African swine fever virus maintenance and transmission dynamics in the sylvatic *Ornithodoros* vector

by

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African swine fever virus maintenance and transmission dynamics in the sylvatic *Ornithodoros* vector

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Declaration

I, Catharina I. Boshoff hereby declare that this thesis which is submitted for the degree of Doctor of Philosophy (Zoology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Date: 27 March 2017

Disclaimer

This thesis consists of a series of chapters that have been prepared as stand-alone papers that are either already published or have been prepared for submission to different scientific journals. Consequently, unavoidable overlaps and/or repetitions may occur between chapters and the reference style and format may differ between chapters.

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Summary

African swine fever (ASF) is a highly contagious and fatal haemorrhagic viral disease of domestic pigs caused by a large, DNA arbovirus with a genome ranging from 170 to 190 kbp in length, the African swine fever virus (ASFV). There is no treatment or vaccine available to combat the disease, and sporadic outbreaks of ASF have been reported from 1928 until present from within South Africa (SA). Control of the disease in SA relies on strict biosecurity measures and the establishment of a control zone that limits movement of pigs and pig products from high-risk areas. The sylvatic cycle, that involves warthogs and *Ornithodoros* soft ticks, plays a crucial role in the maintenance and distribution of ASFV in SA and clarification of key epidemiological factors are needed in order to enhance understanding and to assist with the formulation of more effective disease control strategies. Previous epidemiological surveys were conducted in late 1970's and early 1980's to determine the infection status and distribution of *Ornithodoros* ticks in relation to ASFV. More recently, a reassessment of ticks using more advanced techniques resulted in negative ASF results from a previously positive area, signalling the need for more extensive studies across SA, the results of which will determine the relevance of the current control zone and strategies in place in SA.

A comprehensive survey to confirm the presence of *Ornithodoros* ticks in game parks within the control zone in SA as well as those in neighbouring Swaziland was done to determine the presence of the soft ticks and their ASFV infection status. Phylogenetic analyses based on partial C-terminal *p72* gene sequences, generated for each of the virus-positive ticks revealed the presence of two additional ASF genotypes in SA, whilst characterisation of the 16S rRNA gene sequences of *Ornithodoros* ticks from each of the sampling sites revealed high levels of diversity (4 haplotypes) and confirmed the presence of at least three geographically distinct lineages within SA.

DNA sequences generated through next generation sequencing (NGS) technologies were used to generate full-length viral genomes for two virus isolates, representative of two (genotype I and XIX) of the 23 known genotypes. One of the benefits of whole genome sequence (WGS) generation is the development of improved diagnostic approaches in infectious disease research. Sequencing of full-length viral genomes is however a difficult task due to the presence of contaminating nucleic acids of the host cell, and highly variable terminal ends for which no reference backbone data are available. In this study an enrichment technique was

used to overcome host DNA interference. A long-range PCR approach targeting overlapping viral genome segments 10kbp - 20kbp in length was also evaluated across ten genotypes, with the aim of amplifying viral DNA alone. A drawback of this method is that it is only able to amplify homologous regions of genotypes that are closely related to genotype I against which the primers were designed, and thus the large variable regions at the terminal ends of the ASFV genome cannot be characterised, limiting the viability of this approach.

In an attempt to better understand how ASFV adapts and changes when it cycles between the invertebrate and vertebrate host, a transmission experiment in which naturally infected *Ornithodoros* ticks were used to establish an infection in domestic pigs was conducted. In this transmission experiment, the virus was cycled back from the infected pigs to naïve *Ornithodoros* ticks, thereby emulating the transmission cycle in southern Africa in which sylvatic cycle vectors precipitate infections in domestic pigs. This cyclical vertebrate-invertebrate infection-amplification, which typically involves the warthog vertebrate host under natural settings, assists in the long-term maintenance of the virus in natural tick colonies, ensuring the central role that they play in ASFV transmission in the field. By performing the transmission experiment under laboratory conditions with domestic pigs, the changes and adaptation the virus undergoes when it cycles between its invertebrate host and vertebrate host was investigated for the first time. Results indicate minor difference between the genomes sequenced with no consistency found over the one year time frame investigated.This study gives insight in the understanding and the role that the sylvatic *Ornithodoros* vector plays in African swine fever virus maintenance and transmission dynamics in South Africa.

This study I dedicate to my family

Gerrie, Lana and Wentzel

for their support and inspiration

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Table of Contents

Appendices

List of Figures

List of Tables

Chapter 1

Literature review

1.1 Introduction

The causative agent of African swine fever (ASF) is a large, enveloped icosahedral virus containing a double stranded (ds) DNA of approximately 190 kilo base pairs (kbp). African swine fever virus (ASFV) shares aspects of genome structure and replication strategy with other large dsDNA viruses including *Poxviridae* and *Iridoviridae* (Dixon et al., 2000) but is currently classified as the sole member of the *Asfarviridae* (Asfar, African swine fever and related viruses) family.

ASFV is the only know DNA arbovirus and in sub-Saharan Africa, it is maintained in a sylvatic cycle between wild swine (primarily warthogs) and argasid ticks of the genus *Ornithodoros.* Since most adult warthogs in ASFV enzootic areas are seropositive, it suggests a prolonged existence in wild swine, with an established host-parasite relationship in which infections occur without signs of disease. *Ornithodoros* ticks are persistently infected with ASFV and are thus very important in the maintenance and spread of the virus. ASFV infections in domestic swine range from highly lethal to subclinical infections, depending on the strain involved. In some cases this haemorrhagic disease has case fatality rates approaching 100%. Due to the lack of vaccine and difficulties with eradication following introduction to naïve populations and geographical areas, this disease represents a global threat. As pork seen as a source of affordable protein world-wide, the implications of ASF to the domestic pig industry are substantial, affecting subsistence farming as well as intensive pork production sectors. Currently control is through strict bio-security measures and is reliant on animal quarantine and slaughter in the event of an outbreak.

1.2 Aetiology and genomics

African swine fever is caused by a DNA virus belonging to the *Asfarviridae* family, the sole member of the *Asfivirus* genus (Dixon et al., 2004). The virus is large, with an external diameter of 175 – 215 nm, enveloped and icosahedral, and replicates predominantly in the cytoplasm.

The ASFV genome is a double-stranded linear DNA molecule that ranges in length between isolates from about 170 to 193 kilo base pairs (kbp) (Blasco et al., 1989, Chapman et al., 2008; De Villiers et al., 2010). The ASFV genome contains a conserved, centrally located 125 kbp region in which major insertion-deletion events are rare, but within the left 38 - 47 kbp and right 13 - 16 kbp terminal regions of the genome, large variability occurs (Tabarés et al., 1981, Blasco et al., 1989, Sumption et al., 1990). ASFV encodes between 151 and 167 open reading frames (ORF) and the differences in genome length are largely due to the gain or loss of ORF's encoded within the variable terminal ends of the virus (Dixon et al., 2013).

Initially the genes were named based on *Eco*RI restriction enzyme fragmentation, gene orientations left of right (L and R) and number of amino acids encoded, but the most recent nomenclature is based on the gene family name according to the average number of amino acids in the proteins encoded by each family, the directions of transcription (L or R) and position in that family from the left genome end (Chapman et al., 2008; Dixon et al., 2013). In the left and right variable regions the families include multi-gene family (MGF) 100, 110, 300, 360 and 505/530, with the largest and most variable MGFs being MGF 110 and MFG 360 (Chapman et al., 2008). Most of the variable ORFs are present close to the genome termini, at approximately 30,000 bp from the left genome end and 10,000 bp from the right genome end. The central conserved region consists of 109 unique conserved genes that encode for the structural proteins, proteins involved in virus assembly, enzymes and nucleotide metabolism, DNA replication and repair, and some with as yet unknown functions (Dixon et al., 2013).

1.3 Pathogenesis: ASF virus infection

African swine fever virus infection in domestic swine results in several disease forms, ranging from highly lethal to subclinical, depending on the host as well as the virus strain. In domestic swine the incubation period ranges from 5 – 15 days, with a rise in temperature (41 – 42 °C) and death occurring between 5 – 10 days after onset of fever. In acute cases clinical signs can be observed as early as 48 h post infections (De Tray, 1963; Penrith et al., 2004). Typical signs are cyanosis of the skin, specifically the ears and snout, and haemorrhage of the organs, including lymph nodes, kidneys and heart, with bloody diarrhoea. Extensive necrosis and haemorrhage are important factors leading to death within 1 – 2 days from onset of clinical signs, with a case fatality rate of up to 100% being recorded. Milder forms of the disease can occur with sub-acute cases that last 3 – 4 weeks (Hess, 1981), with low fever, loss of appetite

resulting in loss of body condition and sometimes, but not always, haemorrhage of lymph nodes and tissues. Less acute forms are more difficult to recognize and surviving pigs can remain carriers and potentially spread of the virus. Warthog infection with ASFV is usually asymptomatic, with low levels of virus in tissue and blood of recovered warthogs (Thomson, 1985). In ASFV enzootic areas warthogs probably remain infected for life (Thomson, 1985).

1.4 Epidemiology of African swine fever

ASFV is transmitted and maintained in one of three epidemiological cycles: (i) a domestic pig cycle which occurs without involvement of either the tick vector or wild suids, (ii) an ancient sylvatic cycle involving an association between soft ticks and warthogs or (iii) a soft tick-pig cycle (Penrith et al., 2004). A range of wild suids, including European wild boars (*Sus scrofa*), warthogs (*Phacochoerus africanus*), bushpigs (*Potamachoerus larvatus*), river hogs (*Potamochoerus porcus*), giant forest hogs (*Hylochoerus meinertzhangeni*) as well as domestic pigs (*Sus scrofa*) are susceptible ASFV hosts (Jori and Bastos, 2009). ASFV is presently the only known DNA arbovirus and soft ticks of the *Ornithodoros* genus (Plowright et al., 1994) play an important role in its maintenance and transmission. Depending on the presence of the different susceptible wild suids and vectors, the epidemiology varies between different countries and regions.

1.4.1 Domestic pig-to-pig cycle

Isolates of ASFV cause an acute haemorrhagic fever in domestic pigs which can result in the death of up to 100% of infected animals 8 – 12 days post-infection. Highly virulent strains in acutely infected domestic pigs result in high levels of virus being present in blood and tissues. Onset of viremia is seen from three days post-infection and transmission can occur by direct contact between pigs for up to 30 days post-infection (Wilkinson et al., 1983), with the main route of transmission being the naso-nasal route (Penrith et al., 2004). If blood is shed as may occur during fighting, then it is possible for transmission to occur for up to eight weeks post-infection. In the less virulent strains, pigs recover but can remain persistently infected for up to six months (Wilkinson, 1984). The ASFV persists in tissues of the lymphatic system of recovered pigs for several months and poor disposal of carcasses, or exposure of pigs to swill from persistently infected pigs can result in transmission (Wilkinson, 1989; Figure 1.1).

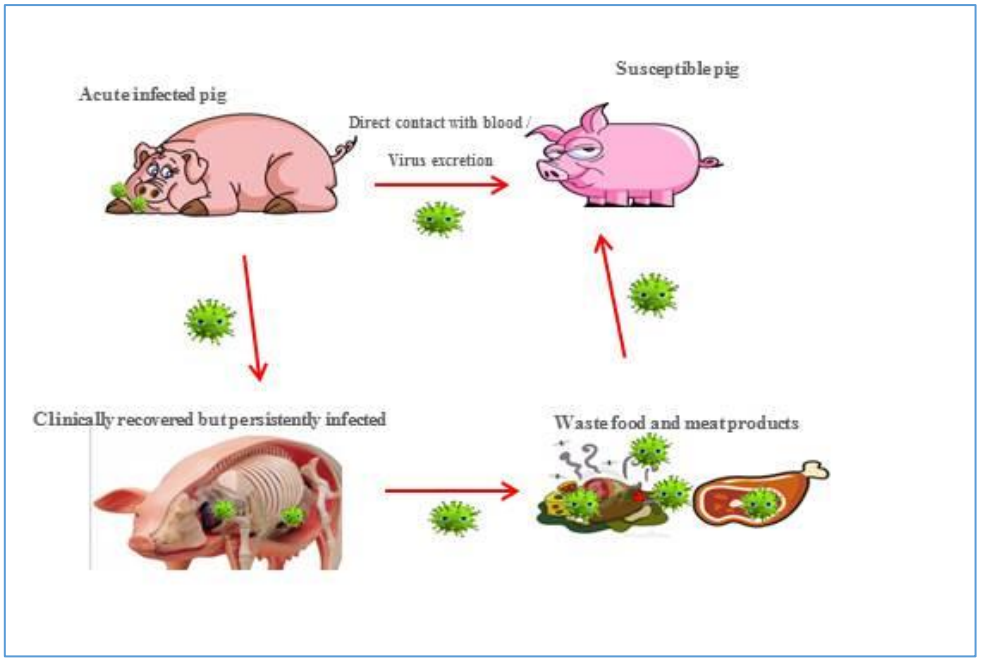

Figure 1.1: Transmission of African swine fever virus in domestic pigs.

1.4.2 Domestic pig-tick cycle

In parts of eastern and Central Africa, the housings that pigs occupy at night provide habitats for *O. porcinus* and these ticks were also reported in human dwellings (Penrith et al., 2004). A domestic cycle between *O. porcinus* and domestic pigs has been demonstrated in Malawi (Haresnape et al., 1988). Such a cycle is also likely to be important in neighbouring countries where suitable conditions exist.

1.4.3 Sylvatic tick-warthog cycle

In southern Africa numerous aspects of the transmission dynamics of ASFV in the sylvatic cycle remain to be clarified. What is acknowledged is that because ASFV is maintained between warthogs and soft ticks (Figure 1.2), the likelihood of elimination of this cycle is very unlikely (Penrith et al., 2013). Infected warthogs show no clinical signs of infection, and levels of virus in tissue are low to undetectable in the blood of adults (Thomson, 1985). These warthogs probably remain infected for life, but horizontal and vertical transmission between warthogs does not occur, maintenance of the virus is dependent on soft ticks of the *Ornithodoros moubata* complex that inhabit warthog burrows (Plowright et al., 1994). During the warthog farrowing season (October – December in South Africa) young warthog piglets become infected when bitten by infected *Ornithodoros* ticks, that reside in the warthog burrows, and develop a viremia lasting two to three weeks (Costard et al., 2013). Naive ticks

feeding on the viremic young warthogs then become infected (Thomson et al., 1980). The virus replicates in the tick mid-gut and spreads to the salivary glands and reproductive organs from where infected ticks secrete virus in both saliva and coxal fluids (Plowright et al., 1969). African swine fever infected *Ornithodoros* ticks remain infected for long periods of time and can thus transmit the virus to susceptible hosts. In the tick population the virus can be transmitted trans-stadially, trans-ovarially as well as sexually (Plowright et al., 1970; Kleiboeker and Scoles, 2001), allowing the virus to persist for long periods of time even in the absence of an infected host. Long-term persistence of ASFV in *Ornithodoros* ticks from Portugal was demonstrated for at least five years (Boinas et al., 2011).

In sub-Saharan Africa, the virus cycle is probably maintained between a cycle of infection of ticks and infection of young warthogs (Thomson et al., 1983). Ticks are also the most likely source of infection for domestic pigs in Africa as direct contact transmission from warthogs to domestic pigs has not been observed (Thomson, 1985). Adult warthogs can act as a carrier for *Ornithodoros* ticks, and can spread infected ticks from burrows to areas where domestic pigs occur, exposing domestic pigs to ASFV *via* the infected ticks. A high potential risk of ASFV infection for domestic pigs exists where ASFV-infected *Ornithodoros* ticks and warthogs occur.

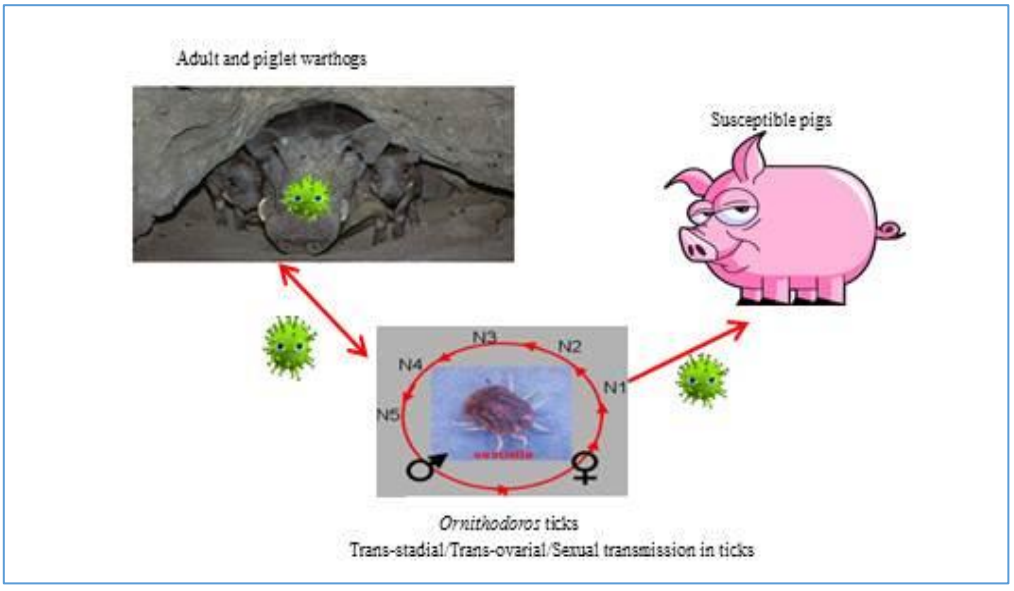

Figure 1:2: Transmission of African swine fever virus in the sylvatic cycle indicating transmission to domestic pigs.

1.5 The *Ornithodoros* tick vector/s of African swine fever

1.5.1 Taxonomy

Ticks are blood-feeding ectoparasites of vertebrates for which main families are distinguished, namely the hard ticks (*Ixodidae*) with 705 species (Guglielmone et al., 2010), the soft ticks (*Argasidae*) with 198 species (Guglielmone et al., 2010; Heath, 2012; Venzal et al., 2012) and the monotypic *Nuttalliellidae* family that is morphologically intermediate. Soft ticks are leathery, they lack a dorsal shield or scutum and their mouthparts are sub-terminally attached and not visible from the dorsal view. Soft ticks feed rapidly, taking in multiple small blood meals, as nymphs and adults, and engorge rapidly within minutes to hours (Soneshine, 1991; Mans and Neitz, 2004). After feeding they drop off and do not stay attached to their hosts.

The genus *Ornithodoros* within the family *Argasidae* consists of approximately 90 species with an almost worldwide distribution (Taylor et al., 2007). *Ornithodoros moubata*, was originally named *Argas moubata* by Murray in 1877 (Leeson, 1952). Murray's specimens originated from Angola (formerly Portuguese West Africa), where Livingstone had noted that such ticks were common in the huts of native inhabitants some twenty years earlier (McFarlane, 1916). A description of the species and a guide to distinguish between *0. moubata* and *0. savignyi* was first published in 1900 (Pocock, 1900).

In East and southern Africa the tick vectors for ASFV belong to the *Ornithodoros moubata* complex of species and in North and West Africa to the *Ornithodoros erraticus* group (Jori et al., 2013). The *Ornithodoros moubata* species complex currently includes four different species of which two: *O. moubata sensu stricto* and *O. porcinus*, are confirmed vectors of ASFV (Jori et al., 2013). Because *O. compactus* and *O. apertus* do not feed on suids they are not considered to play a role in ASFV epidemiology (Walton, 1962). Currently two *Ornithodoros porcinus* subspecies are recognised *viz. O. porcinus domesticus*, associated with human dwellings and *O. porcinus porcinus* associated with warthog burrows (Walton, 1979). Ticks from the *O. porcinus* species complex and *O. erraticus* are both capable of developing a persistent ASFV infection with high virus titres, under experimental conditions (Greig, 1972; Kleiboeker et al., 1998; Basto et al., 2006). The literature pertaining to the taxonomy of the sylvatic-cycle vector of ASFV is inconsistent (Kleiboeker and Scoles, 2001; Bastos et al., 2009), leaving the current taxonomic status of *O. porcinus/moubata* unresolved due to lack of adequate guidelines for classification based on morphological characteristics. A recent molecular phylogenetic study has also called into question the validity of the subspecies status,

and could find no support for *O. moubata* monophyly (Bastos et al., 2009). For the purpose of this literature review, *O. porcinus* will be used when referring to the warthog-associated ticks that transmit ASFV in South Africa. Several species, including, *O savignyi* (Mellor and Wilkinson, 1985), *O. peurtericensis* (Hess et al., 1987), *O. turicata* (Hess et al., 1987) and *O. coriaceus* (Groocock et al., 1980) have been shown to be competent vectors of ASFV under experimental conditions, but none of these have been confirmed as vectors in the field.

1.5.2 Morphology

Ornithodoros porcinus, adults are oval in outline, with four pairs of legs, brown in colour with a leathery, wrinkled body (when unfed) and have no eyes (Figure 1.3). The capitulum which bears the mouth parts is located beneath the anterior margin of the body and is not visible from above. Male and female soft ticks are similar in appearance, with no dorsal plate (scutum) to distinguish the sexes as is the case in hard ticks. Sexual dimorphisms are only visible in adults. The respiratory openings lie on the sides of the body above the third and fourth pair of legs. The sexes can be distinguished by the shape of the genital opening which in males is circular and in females is a transverse split that is wider than it is long (Figure 1.4).

Figure 1.3: External structure and dorsal view of an adult Ornithodoros porcinus tick

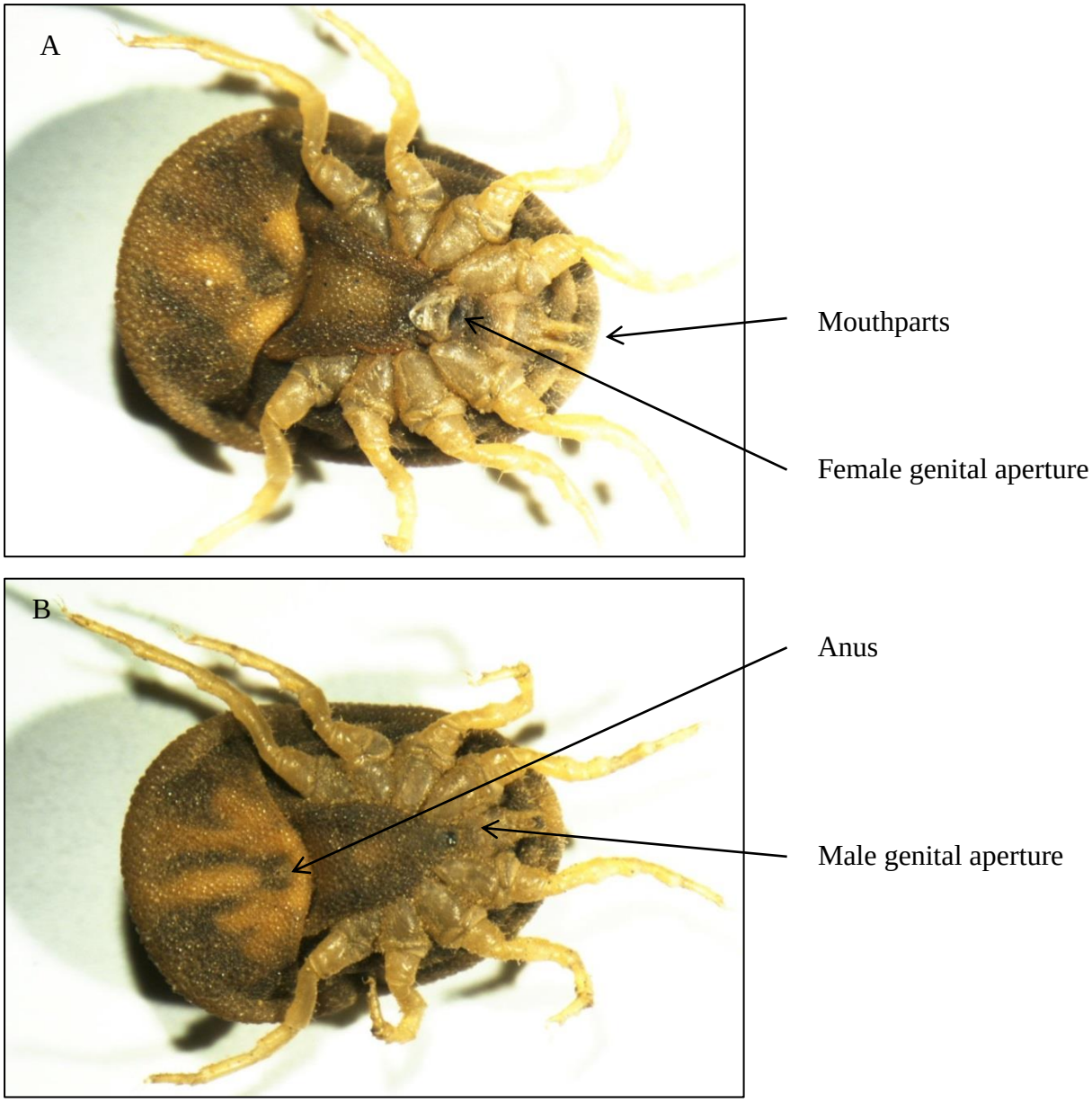

Figure 1.4: External structure and dorsal view of adult *Ornithodoros porcinus* ticks
(A) Female (B) Male

1.5.3 Distribution of ticks

Ornithodoros porcinus are widely distributed in the savanna regions of southern and eastern Africa where warthogs occur. Warthogs still occur in large areas of southern Africa but as the density of human habitation increases, wildlife reserves are becoming the main area of warthog distribution. Locally the nymphal stages, which can be found on warthogs foraging outside their burrows (Horak et al., 1983) are likely to be spread between burrows (Penrith et al., 2004). These ticks have been recorded in Uganda, Kenya, Tanzania, Zambia, Zimbabwe, Malawi,

Mozambique and Namibia in Africa. In South Africa these soft ticks are widespread in the western and northern regions of North West, Limpopo and Mpumalanga Provinces.

1.5.4 Habitats and environmental factors

Soft ticks live apart from their hosts and are most common in the nests and resting places of the animals on which they feed. *Ornithodoros porcinus* ticks associated with the sylvatic cycle live in the sand of warthog burrows or dirt road culverts which warthogs use for shelter at night. Environmental factors such as temperature and rainfall play a major role in the tick's cycle. Warthogs are warm blooded vertebrates and maintain a suitable temperature for the virus, with arthropods being cold-blooded and unable to regulate body temperature. The environmental temperature plays an important role in the activity of the ticks and transmission of the virus. Evidence indicates that *Ornithodoros* ticks are able to survive hot weather well (Butler and Gibbs, 1984), and that an increase in temperature enhances digestion and egg maturation (El Shoura, 1987). In contrast, tick activity is also affected by cool weather, as temperatures below 22 °C reduce tick activity and larval development (El Shoura, 1987).

Most ticks show seasonality in their life cycles. The adult ticks will become more active and feed at the start of the rains. During spring time, which coincides with the start of the rainy season in South Africa (September – November), the eggs are laid, larvae hatch, and the tick larvae feed on the warthogs in the burrows. This coincides with the warthog furrowing season, when young warthogs are confined to the burrows after birth for a period of at least four weeks (Cumming, 2013). The tick nymphs become infected with ASFV and continue to feed on the warthogs confined to the burrow and then also infect the neo-natal warthogs, which in turn act as a source of infection for naïve ticks (Thomson et al., 1983). This however is limited to a very short period of time each year. During winter, regarded as the dry season in the central and northern parts of South Africa (May – July), the tick larvae remain mostly inactive following the feeding during summer and absence of the then older warthogs from the burrows as warthogs weaned at 4 – 5 months and leave the burrows. Factors such as soil temperature, relative humidity, wet and dry seasons can all be climatic conditions that have an impact on the fitness of a region for a tick population (Gray et al., 2009), life-cycle and the ASFV infection cycle of the ticks.

1.5.5 Lifecycle

Ornithodoros porcinus ticks have a hemi-metabolous life cycle, including eggs, larvae, nymphs and adults (male and female). Eggs hatch into larvae that do not feed and moult immediately to the nymphal stage. The first stage or N1 nymphs resemble the adults and have 8 legs, but are smaller and lack the genital pore. They seek and attach to hosts, feed within 15 - 30 minutes and then drop off to moult in the sand, or in cracks of the natural habitat, within 2 – 3 weeks. This is repeated up until the last nymphal moult, after which adult ticks become sexually mature. Mating occurs before as well as after feeding, but rarely if ever on the host itself. Following feeding, oviposition begins and females deposit small egg masses of less than 500 eggs per cycle (Walker et al., 2003). The duration of the life cycle depends on host availability for blood meals. *Ornithodoros porcinus* are fast feeders, engorging up to 5 – 10 times their initial body weight within a few minutes to hours. *Ornithodoros* ticks are able to survive long periods of starvation with lifespans of more than 15 years if they are occasionally fed (Escinas et al., 1999).

1.5.6 Pathogenesis of ASFV in Ornithodoros ticks

ASFV infection of *O. porcinus* ticks represents a well-adapted virus-tick host biological system, but questions still remain on the virus-tick adaptation mechanism. Successful ASFV infection in the ticks and transmission of the virus back to the vertebrate host depends on biological interactions between ticks and the virus (Nuthall, 2009). The midgut of the ticks is the initial site of viral replication and also the site of longest viral persistence (Kleiboeker and Scoles, 2011). Generalization of infection occurs between 15 – 21 days post-infection, indicating that in order for successful ASFV infection that crossing of midgut barrier is crucial. For a tick to be able to transmit ASFV, the virus must overcome a series of obstacles from ingestion until it can be transmitted to another host, the gut wall must be passed, the virus needs to replicate and to reach the secretory glands, mainly the salivary glands and coxal glands. The mechanisms by which the virus reaches the salivary and coxal gland are largely unknown (Nuthall, 2009; Burrage, 2013). Little or no cytopathology has been observed in infected nymphs (Kleiboeker et al., 1998), nor are there significant virus-induced mortalities after infection (Greig, 1972; Kleiboeker et al., 1998), although exceptions to this have been noted (Rennie et al., 2000). ASFV transmission to pigs during tick feeding occurs through saliva secreted by the tick into a feeding lesion and through coxal fluid secreted by the tick during or shortly after feeding. Once *Ornithodoros porcinus* ticks are infected with ASFV they probably remain infected for life (Kleinboeker and Scoles, 2001) indicating the probability that ASFV

infection in its natural *O. porcinus* host represents a well-adapted and possibly coevolved biological system (Kleiboeker et al., 1998). Transovarial and transstadial transmission are likely to be the two most important mechanisms contributing to the maintenance of ASFV infections in tick populations (Kleiboeker and Scoles, 2001). Sexual transmission is unidirectional, occurring between infected males and uninfected females (Plowright et al., 1974).

1.5.7 Sampling of ticks

When investigating the sylvatic cycle and the role that *Ornithodoros* ticks play, standardised tick collection methodologies need to be established that take the characteristics of *Ornithodoros* ticks into consideration. These soft ticks are warthog burrow dwelling, photophobic, rapid feeders, that live underground and do not remain attached to their hosts. In response to these characteristics, three different techniques have been developed to collect soft ticks in the field:

(i) Manual collection, involving removal of ground out of the warthog burrow, using a shovel. The soil content is then spread out in the sun, which due to the photophobic nature and heat-intolerance of the ticks, causes them to move. Each specimen is then collected individually by hand with use of tweezers (Jori et al., 2013).

(ii) Carbon dioxide gas, such as dry ice, acts as a stimulant that mimics vertebrate host breathing and attracts tick species to it, again allowing for individual specimen collection (Jori et al., 2013).

(iii) Vacuum aspiration can be performed by using a petrol blower / vacuum with a pipe modification, whereby the soil content of the burrow is aspirated, and then as with the manual method is spread out and placed in the sun, to observe tick movement (Jori et al., 2013).

The preferred collection method depends on the habitat examined and the environmental conditions of the study area. After collection, ticks should either be kept alive or directly stored in liquid nitrogen to preserve the virus inside the tick and avoid DNA degradation.

1.5.8 Molecular tools for Ornithodoros tick population genetics

Virus replication in ticks has been shown to depend on the geographical origin of tick batches (Plowright et al., 1970; Greig, 1972). It is possible that this previously documented geographical variation is linked to the more recently identified existence of genetically and geographically discrete sub-lineages of soft ticks in Africa (Bastos et al., 2009) and may

explain why replication of a specific virus isolate in *O. moubata* (Diaz et al., 2012) could not be replicated in *O. erraticus* ticks (de Carvalho Ferreira et al., 2014).

Ornithodoros vector taxonomy in ASF literature is fraught with inconsistencies and a preliminary molecular-based assessment of ASFV tick taxonomy (Bastos et al., 2009) has highlighted the need for expanded studies. In the 2009 study, the 16S rRNA gene phylogeny found no support for the two distinct subspecies, nor was there support for *O. moubata* and *O. porcinus* monophyly (Bastos et al., 2009). The one step duplex PCR method described by Bastos et al., (2009) was shown to be best-suited when performing epidemiological studies involving *Ornithodoros* ticks of the sylvatic cycle (Bastos et al., 2009). This method also proved useful for the investigation of the taxonomy of the *Ornithodoros* ticks.

1.5.9 Identification, diagnosis and characterization of ASFV

Diagnosis of ASFV, involves the direct detection of ASFV antigen or DNA, or confirmation of antibodies against ASFV in samples taken from pigs or warthogs. For *Ornithodoros* ticks, virus presence was previously confirmed by virus isolation, either in cell culture or through inoculation into a live pig (Haresnape et al., 1988), but is now reliant on molecular methods to confirm virus genome presence, and guide virus isolation. Laboratory procedures to identify ASF can be divided in virological and serological methods with the various tests recommended by World Organisation for Animal Health (OIE) in its Manual of diagnostic tests and Vaccines for Terrestrial Animal, Chapter 2.8.1 (http://www/oie.int) being updated regularly. Rapid, reliable, specific and sensitive detection of ASFV is very important, to be able to implement control measures that prevent spread of the disease, but also to differentiate ASF from similar clinical diseases such as Classical swine fever, porcine dermatitis and porcine reproductive and respiratory syndrome.

1.6 Virological diagnosis of ASFV

A variety of laboratory methods can be used for the detection of ASFV. Isolation and cultivation of ASFV is a very important component for diagnosis and research of ASFV. Blood, and tissue samples, such as spleen and liver can be used for virus isolation. The test is carried out by inoculating blood or tissue suspensions onto primary porcine bone marrow (PBM) cell cultures. The haemadsorption test (HAD) (Malmquist and Hay, 1960) can aid in confirmation of ASFV, due to the fact that pig erythrocytes adhere to the surface of the pig monocytes infected with ASFV, but not all ASFV isolates have this characteristic. Virus

isolation is however time-consuming and can take up to 6 days to declare a negative results and requires preparation of primary cultures and dedicated biocontainment facilities and personnel with this expertise.

The fluorescent antibody test can be used to detect ASFV antigen in infected tissues and blood smears of suspect pigs (Bool et al., 1969). Microscopic detection of the antigen is done by using ASFV-specific antibody reagents. Viral antigens can also be detected using ELISA, with a number of antigen capture ELISA's available. Different capture approaches are used, such as polyclonal antibodies or a combination of monoclonal and polyclonal antibodies. This is however recommended for acute cases as it is likely that these tests have low sensitivity and are of limited use when dealing with subacute and chronic forms of ASF. A number of commercially produced antigen ELISA kits are currently available, i.e. ELISA INGEZIM K3 (Ingenasa, Spain).

Molecular assays for the detection and diagnosis of ASFV are currently globally used. Many of the current assays amplify sequences of the *p72* gene and can be used to detect ASFV genome in blood, serum or organ samples. Various conventional polymerase chain reaction (PCR) assays that use agarose-gel electrophoresis to visualize products generated by PCR have been described (Agüero et al., 2003; Bastos et al., 2003; Basto et al., 2006). ASFV real-time PCR assays have become more widely used for routine diagnostics with various real-time assays for detection of ASFV having been described (King et al., 2003; McKillen et al., 2010; Tignon et al., 2011). Target sequences are detected by fluorescence signals from target specific primer-probes and have several advantages over conventional PCR's such as increased speed, higher throughput and higher sensitivity and a reduced risk of cross contamination (Oura et al., 2013). PCR enables the diagnosis of ASF to be made within hours of sample receipt. Three diagnostic PCR protocols are prescribed by OIE for the detection of ASFV to be used by approved biocontainment laboratories, two validated conventional PCR procedures and one Taqman PCR protocol (OIE) (Table 1.1)

Table 1.1: OIE prescribed PCR methods for detection of ASFV

PCR Protocol	Primers	Reference
Conventional PCR 1	PPA-1: 5'-AGT TAT GGG AAA CCC GAC CC-3' PPA-2: 5'-CCC TGA ATC GGA GCA TCC T-3'	Agüero et al., 2003
Conventional PCR 2	Primer-1:5'-CTGCT-CATGG-TATCA-ATCTT-ATCGA-3' Primer-2-: 5'GATAC-CACAA-GATC(AG)-GCCGT-3'	King et al., 2003
TaqMan® PCR protocol PCR amplification by TaqMan® assay	TaqMan probe: 5'[6-carboxy-fluorescein (FAM)]-CCACG-GGAGG-AATAC-CAACC-CAGTG-3-[6-carboxy-tetramethyl-rhodamine (TAMRA)]	Fernandez-Pinero et al., 2010

1.6.1 Detection of ASFV in ticks

Previous epidemiological studies used virus isolation in porcine macrophage cultures to test for the presence of ASFV in ticks (Thomson et al., 1983). Virus isolations can be technically difficult due to low viral load in infected ticks as well as small amounts of clinical starting material due to the small size of ticks, making virus isolations not the method of choice. A sensitive nested PCR for *O. erraticus* was developed and confirmed that the PCR approach is valuable for testing *Ornithodoros* ticks for the presence of ASFV (Basto et al., 2006). Due to a higher sensitivity of the PCR approach, which was shown by these authors to be 1.4 times more sensitive than isolation, as well as being a more rapid approach, this method is advantageous for initial screening of large tick populations collected in the field for the presence of ASFV. A duplex PCR method was subsequently reported where simultaneous detection of 16S rRNA of the host mitochondrial genome acts as an internal PCR control (Bastos et al., 2009). Using this approach, integrity of the DNA extract is confirmed for each samples, whilst simultaneously allowing for virus genome confirmation through amplification of the C-terminal end of the *p72* gene (Bastos et al., 2009). These authors also compared the phylogenetic utility of different *p72* gene regions under different epidemiological settings. Their results showed that the central region of the *p72* gene targeted in the nested PCR (Basto et al., 2006), was best suited to resolving genotype I viruses, whereas the longer C-terminal region (Bastos et al., 2003) prescribed by the OIE for genotyping, is best suited for the sylvatic setting.

1.6.2 Serological diagnosis of ASFV

Detection of specific antibodies against the virus act as indicators of previous or current infections if antibodies and viable virus are detected simultaneously. The detection of ASF-specific antibodies is indicative of a prior infection and as antibodies are produced from the

first week of infection and persist for long periods they can be useful to act as appropriate indicators of diagnosis of the disease in sub-clinical cases or in warthogs that show no clinical signs of infections. Highly sensitive and specific serological tests are used to identify high-risk areas and to develop appropriate recommendations for disease prevention. ASF serodiagnosis strategies used in the past included immune-electrophoresis (IEOP) (Pan et al., 1972) and indirect immunofluorescence (IIF) test (Pan et al., 1974). These tests were replaced by the ELISA which is more sensitive, less laborious and more suitable for large scale testing of sera. ELISA is the most widely used technique for the diagnosis of subacute and in apparent ASF carriers (Wardley et al., 1979; Tabarés et al., 1981; Pastor et al., 1990). A disadvantage of the ELISA is however that false positive reactions can occur with field sera. Production of ASF antigen for ELISA was improved to eliminate false positive reactions (Escribano et al., 1989). Although hemadsorption inhibition assay (HAI) can be used to serologically classify ASFV into serogroups (Malogolovkin et al., 2015), serotyping of ASF field isolates is currently not widely used and needs further investigation and standardization. Identification of the most antigenic viral proteins is very important to improve serological tests. The major component of the viral capsid is virus protein (VP) 72, one of the fist viral proteins identified as responsible for the induction of antibodies after a natural infection (Tabarés et al., 1980). VP30 and VP54 were also identified as highly antigenic during infections (Pastor et al., 1989; Gallardo et al., 2006). Antibodies against these three proteins are involved in virus neutralization, attachment inhibition (VP72 and VP54) and internalization (VP30) (Gómez-Peurtas et al., 1996, 1998). Three commercial ELISA tests are currently available with two of these using VP30 antigen as a reagent (SVANOVIR ASFV-AB assay; ID Screen African Swine fever Indirect ELISA kit) (Cubillos et al., 2013) and one using VP72 (INGEZIM PPA COMPAC).

Although laboratory-based serology methods are available, in many developing countries where ASFV is endemic, sophisticated laboratories and equipment are not readily available. Assays that can be used away from dedicated laboratories in the field and that can provide rapid, easily interpreted results are often referred to a pen-side tests. A commercial pen-side test (INGEZIM PPA-CROM antibody) was developed based on immune-chromatography that uses a migration technique by using purified VP72 protein to detect specific antibodies against ASFV in porcine serum samples. The test can be performed without special equipment, directly in the field and results are obtained in 15 minutes.

1.6.3 Molecular characterization

1.6.3.1 Sanger sequencing

Currently genetic typing of ASFV isolates is based on nucleotide sequencing of the *p72* capsid protein gene (Bastos et al., 2003). Molecular characterization of isolates plays an important role in monitoring the distribution and spread of ASFV. It provides insight and understanding into the genetics and interaction of the virus with the different hosts including, pigs, warthogs and *Ornithodoros* ticks. Previously genotyping was achieved by using a restriction fragment length polymorphism (RFLP) approach (Wesley and Tuthill, 1984; Blasco et al., 1989) but this has been replaced by sequencing-based methods (Bastos et al., 2003). Partial sequencing for the gene, B646L, encoding the major capsid protein *p72* has been used to genotype isolates into 23 major genotypes (Bastos et al., 2003; Lubisi et al., 2007; Boshoff et al., 2007; Achenbach et al., 2016), with all 23 genotypes occurring in Africa. Additional strategies to resolve and uncover epidemiological relationships between closely related isolates has been investigated by sequencing of the full-length *p54*-gene (Gallardo et al., 2009) and analysing of tandem repeat sequences identified in the central variable region (Bastos et al., 2004; Phologane et al., 2005; Lubisi et al., 2007). The OIE prescribed approach for typing strains currently includes these three gene targets.

1.6.3.2 Next generation sequencing

Due to the large ASFV genome size, ranging between 170 – 190 kbp, generating complete genome sequences of isolates on a routine basis remains problematic and full-genome sequence data are limited to just 15 ASFV isolates (Yáñez et al., 1995; Chapman et al., 2008; de Villiers et al., 2010; Bishop et al., 2015; Granberg et al., 2016). The 15 complete genome sequences correspond to eight (genotype I, II, III, IV, VII, VIII, IX, X) of the 23 known genotypes identified by partial sequencing of the 3'end of the gene encoding the *p72* protein (Bishop et al., 2015; Granberg et al., 2016). Different approaches have been used to obtain complete ASF genome sequences (Yáñez et al., 1995; Chapman et al., 2008; de Villiers et al., 2010) with next generation sequencing (NGS) approaches largely replacing the shotgun cloning and sequencing methods employed in the past to generate complete ASFV genome sequences (Bishop et al., 2015). Since 2005 different NGS methods, that provide high speed and throughput have been developed and improved upon. This field has undergone a rapid expansion and there are currently multiple approaches available, although five NGS methods currently dominate the market. These approaches have different underlying biochemistries and differ in sequencing protocols and throughput as well as in the length of the sequences that they produce (Table

1.2). Common to all approaches is that numerous sequencing reactions are produced in parallel, leading to the generation of multiple overlapping reads which potentially enable rapid sequencing of an entire genome. However, as all methods produce relatively short reads, the current bottleneck in the process is genome assembly which requires complex alignment algorithms and sophisticated software. Deciding on which platform will be the best depends on the specific experiment and application of the data. For *de novo* assembly of large complex genomes, longer read lengths are a better option, but turnaround time and expenses are also factors to consider.

Sequencing success largely depends on how the template nucleic acid is prepared. Sequencing of full-length viral genomes is a difficult task due to the presence of contaminating nucleic acids of the host cell as viruses are obligate organisms. The viral nucleic acid must be purified and the contaminating host genome nucleic acids need to be removed or reduced in order to ensure that sequences of viral origin predominate in the resulting sequence reads. Several methods have been described to enrich viral nucleic acids from cell cultures or host tissue before extracting genomic DNA/RNA (Barzon et al., 2011) in order to minimize the contamination from the host nucleic acid. Ultracentrifugation and density gradients can be used to concentrate viral genomes, but despite this the resulting virus genome sequences correspond to only a small percentage (less than 1%) of reads (Radford et al., 2012). Other methods based on viral nucleic acid enrichment can be used such as PCR amplification of targeting conserved genome segments (Höper et al., 2011) or whole genome amplification (WGA) by isothermal multiple strand displacement amplification (MDA). The different methods need to be assessed for each project to determine which targeted amplification approach is best for a specific starting material.

After NGS, the reads need to be analysed and multiple software packages have been developed and are commercially available such as CLC Genomics workbench (CLC Bio, Aarhus, Denmark; https://www.qiagenbioinformatics.com) and Geneious (Biomatters Ltd., Auckland; http://www.geneious.com, Kearse et al., 2012). If a reference genome is available, the sequences can be mapped directly to the reference sequence which greatly speeds up assembly. However, when a suitable reference genome is not available, individual sequencing reads must be assembled *de novo* by using available algorithms to find overlapping information between reads, leading to the generation of contigs. The sequence depth and coverage become crucial and potentially limiting factors.

With the advancement of NGS, it should be possible to generate additional complete genome sequences representing all the genotypes. This will enable a much more comprehensive analysis of the relationships between isolates leading to an improved understanding of relationships between isolates and will also permit the establishment of enhanced epidemiological tracing approaches and improved diagnostics, as well as permit investigation of pathogenicity and ultimately guide vaccine strain development and selection.

Table 1.2: Summary of the five major next-generation sequencing platform families (Barzon et al., 2011; Hodkinson and Grice, 2015)

Platform family	Clonal amplification	Chemistry	Separation	Amount of reads per run	Highest average read length	Application
Roche 454*	Emulsion PCR	Pyrosequencing (seq-by-synthesis)	Microbeads and 'picotitre' plate	1 million	700 bp	• De novo genome sequencing • Genotyping • Metagenomics
Illumina HiSeq/MiSeq	Bridge amplification	Reversible dye terminator (seq-by-synthesis)	Glass slide hybridization	4 billion	300 bp	• Genome sequencing • Transcriptomics • Genotyping • Metagenomics
SOLiD	Emulsion PCR	Ligations (seq-by-ligation)	Beads on glass slide	3 billion	75 bp	• Genome resequencing • Genotyping • Quantitative transcriptomics
Ion Torrent	Emulsion PCR	Proton detection (seq-by-synthesis)	Ion spheres and high-density array	50 million	400 bp	• De novo genome sequencing and resequencing • Target sequencing • Genotyping • Metagenomics
PacBio	Linear amplification	Fluorescently labelled dNTP's (seq-by-synthesis)	Captured by DNA polymerase in microcell	47,000	600 bp	

* This platform will no longer be supported by Roche after 2016

1.7 African swine fever and the situation in South Africa

African swine fever was first described in Kenya in 1921 (Montgomery, 1921) followed by the first reports of the disease in South Africa in the mid-1920s and early 1930s (Steyn, 1928; De Kock et al., 1940). In 1926 in the Potgietersrus district in the Northern Transvaal (now Modimolle, in Limpopo Province) outbreaks of a serious disease affecting pigs were reported (Steyn, 1928). This disease was observed in free-living domestic pigs where contact with wild pigs occurred. In 1933 an outbreak of ASF occurred for the first time in the Western Cape Province, in the Wellington district, and the infection was traced back to movement of animals from the Transvaal (now Gauteng Province) (De Kock et al., 1940). Control measures through stamping out resulted in eradication of ASF from the Cape Province (De Kock et al., 1940). In 1934 an outbreak was recorded in the Witwatersrand area, but the origin was unknown (De Kock et al., 1940). For just over 10 years, between 1939 and 1950, ASF was not reported in South Africa but in 1951 outbreaks of the disease occurred in the Northern Transvaal, again affecting Pietersburg (now Pholokwane) and Soutpansberg (Pini and Hurter, 1975). Between 1953 and 1962, 17 outbreaks were confirmed in Northern and Eastern Transvaal. Again for almost 10 years between 1963 and 1973, no cases were reported. By 1970, incidences of the disease had been reported in a large number of southern and East African countries (Penrith et al., 2004). These regions overlap with the distribution and occurrence of warthogs and ticks of the *Ornithodoros moubata* complex. The common / savannah warthog (*Phacochoerus africanus*, formerly denoted *Phacochoerus aethiopicus*) and the bushpig (*Potamochoerus larvatus)* were found to harbour the virus in nature (Steyn, 1932; De Kock et al., 1940; Hammond and De Tray, 1955). It was recognized, very early on, that ASF outbreaks in domestic pigs occurred where warthogs were present and therefore prevention methods were put into place to prevent contact between these two species (Scott, 1965). Between May 1973 and March 1974, 18 outbreaks of ASF were reported from the Letaba, Pietersburg, White River and Thabazimbi district, the origin was again linked to the presence of wild pigs and movement of infected pig products (Pini and Hurter, 1975) (Figure 1.5).

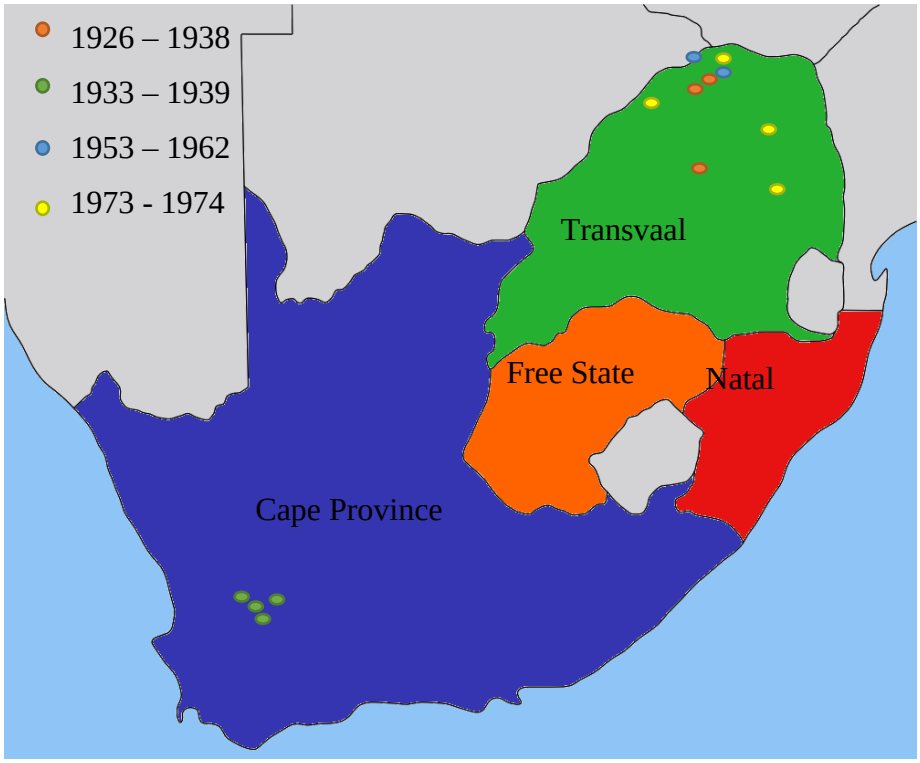

Figure 1.5: Historical map of South Africa indicating the reported cases from 1926 – 1974, according to the prior four-province delineation in South Africa

Prevention and control of the disease relies on strict biosecurity measures, such as movement control as well as prevention of contact between domestic pigs and warthogs, as no vaccine is available. Due to the role of the sylvatic cycle in the maintenance of the disease in South Africa, eradication is considered to be unlikely (Penrith et al., 2013). As only sporadic cases of clinical disease were recorded from time to time in domestic pigs in the control zone between 1973 and 1999 (Boshoff et al., 2007) it appears that the biosecurity measures within the control zone are effective as cases were restricted to this high-risk area of the control zone. A few clinical cases in pigs were reported during 2000 – 2010, but all of these occurred within the control zone area. The first outbreak of ASF outside the control zone was reported in 2012, with Gauteng as well as Mpumalanga Province reporting cases. However, these outbreaks were contained and eradicated without further spread and with limited impact (Penrith, 2013). It is speculated that illegal movement of pigs out of the control zone was the cause of the outbreak (Penrith, 2013). Informal pig movement and long distance trade network practises of emerging small-scale pig farms can pose risks of spread of infection outside the control area. There is a need to enhance surveillance and improvement of bio-security together with educating farmers and implementing rewards as a motivation to prevent ASF outbreaks outside the control zone (Fasina et al., 2015).

1.8 Molecular epidemiology of ASF in South Africa

A retrospective study was done to provide epidemiological data on the ASF genotypes that occurred in South Africa (SA) by using isolates originating from pig outbreaks in South African between 1973 and 1999 (Boshoff et al., 2007). The aim was to understand the origin and spread of the disease in SA. In this study seven of the 23 known genotypes were recovered from SA with five of these being country-specific and two genotypes (genotype III and VII) being shared with neighbouring Botswana (Figure 1.6). Outbreaks caused by genotype XIX viruses occurred in 1987, 1992 and 1996, indicating circulation and dominance of a single genotype in field outbreaks over a 9-year period.

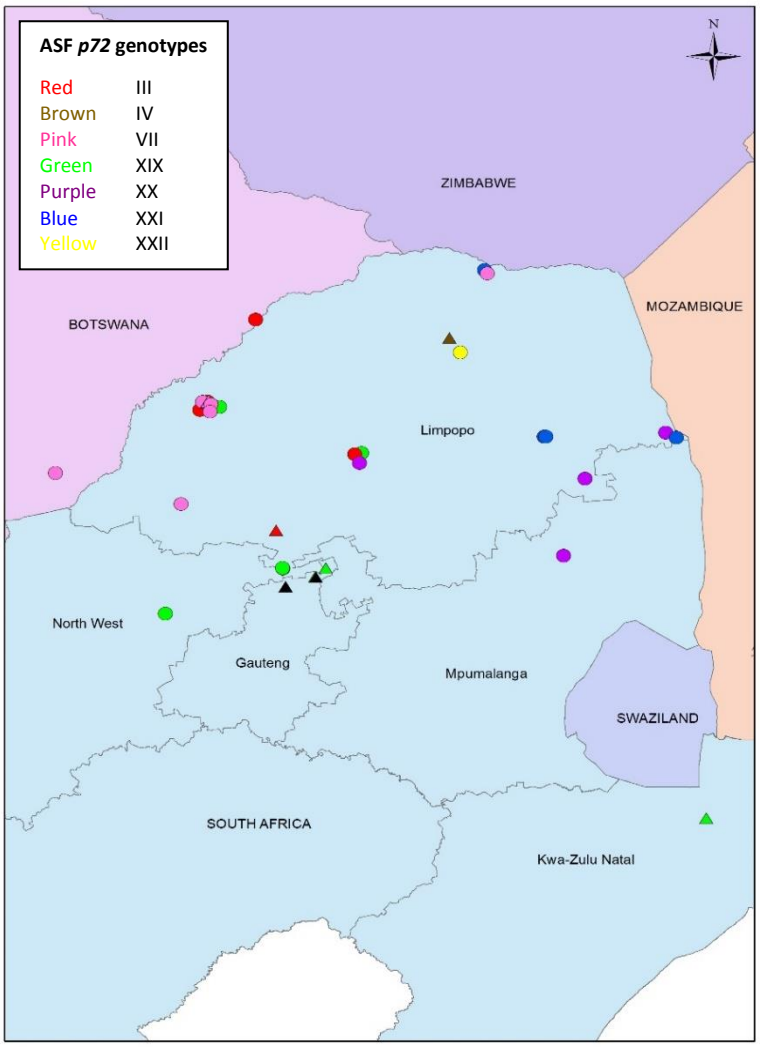

Figure 1.6: Distribution of ASF genotypes based on p72 genotyping of ASFV isolates between 1973 and 2010. Pig origin isolates are indicated by ● and sylvatic cycle hosts (*Ornithodoros* tick/warthog) isolates by ▲ (Boshoff et al., 2007; van Heerden, J., personal communication)

A few clinical cases in pigs were reported from 2000 – 2010, but all within the control zone area. Phylogenetic analysis based on partial *p72* sequences grouped all the cases within the genotypes already established for South Africa (van Heerden, J., personal communication). Genotype XIX identified previously (Boshoff et al., 2007) was again detected in 2005, extending the circulation period of this genotype to 18 years. This genotype was also linked to viruses isolated from *Ornithodoros* ticks in 2003, 2004 and 2005, that were collected during routine survey investigations (unpublished results). This indicated the role of the sylvatic cycle in maintenance of the virus in the field. Identical viruses, isolated from pigs (1987, 1993, 1995, 2001 and 2009) were grouped in Genotype VII, together with viruses of warthog origin sampled in 2003, again confirming the role of the sylvatic cycle in field circulation and maintenance of this genotype in South Africa. All of the above mentioned viruses were isolated from pigs, warthogs and ticks mostly from Limpopo Province except for a few from Mpumalanga Province. From North-West Province the latest virus isolated was in 1992 (pig isolate) and 1978 from Kwa-Zulu Natal (tick isolate). Both areas fall within the current ASF control zone of South Africa.

1.9 Relationships between African swine fever virus, *Ornithodoros* species and warthogs in South Africa

In South Africa the distribution of ASF and the delineation of the ASF control zone coincide with the distribution of warthogs and ASFV-positive *Ornithodoros porcinus* ticks. Large warthog populations where ASFV is present, have high rates (>80%) of ASFV infections, when tested for antibodies against ASF. The highest percentage of serologically positive warthogs are concentrated within the north-eastern part of the country, comprising of parts of Limpopo province, the Kruger National Park (KNP) and adjacent areas in Mpumalanga (80% – 94%), with comparatively lower infection rates being recorded in the north-eastern part of the country, corresponding to North-West province and to the northern part of the Kwa-Zulu Natal Province (2% - 6%), however these estimates are based on historical records (Thomson et al., 1983; Thomson, 1985).

Numerous field studies indicate that the infection rates among *Ornithodoros porcinus* and ASFV infections are relatively low with an average of less than 2% (Plowright et al., 1969; Thomson et al., 1983; Arnot et al., 2009; Boshoff et al., 2014). The last large scale tick study done in 1978 in KNP indicated an ASF infection rate in *Ornithodoro*s ticks of 1.4% based on pooling and virus isolation of the 1026 ticks collected, and the lowest was recorded for Mkuze

Game reserve with an infection rate of 0.06% from the 5018 ticks collected (Thomson et al., 1983). A progressive increase in infection rate was observed with increasing of size of the ticks, with female ticks (8.2%) having a 4-fold greater infection rate than adult male ticks (1.8%) (Thomson et al., 1983; Arnot et al., 2009).

In 2002 a study was done to re-evaluate the status of Mkuze Game reserve (Arnot et al., 2009). Despite screening the ticks with a more sensitive PCR-based approach no evidence of the ASF virus could be found in *Ornithodoros* ticks, suggesting that the virus may have disappeared from the Mkuze Game reserve. This result underscores the value of regular surveillance and the need for a large scale comprehensive tick surveys across the ASF control zone in South Africa, and in bordering regions and countries, as well as serological surveys of wild and domestic suids.

1.10 Treatment and prevention: Control of disease in South Africa

Currently there is no vaccine available for ASF and the disease is controlled by animal slaugher and bio-security measures. A few experimental vaccines have been developed, but an effective vaccine has been elusive up to date (Coggins, 1974; Forman et al., 1982; Mebus, 1988). Protection against homologous and heterologous virulent inoculums of ASFV may be acquired, but in the majority of cases there is a lack of heterologous protection that is an impediment to the development of a vaccine (Kleiboeker, 2002). Pigs surviving viral infection develop homologous protective immunity but generally show no resistance to heterologous virus challenges (Hamdy and Dardiri, 1984).

In South Africa a control zone and control measures were put in place in 1935, preventing movement of pigs as well as pig products in areas where wild pigs and *Ornithodoros moubata* ticks are found. The African Swine fever control area, proclaimed in 1935 (De Kock et al., 1940), is in the north-eastern part of the country where the virus is maintained in the sylvatic cycle and includes parts of Mpumalanga-, Limpopo-, North-West- and Kwa-Zulu Natal provinces. The control zone remains unchanged since its inception, and regular monitoring of the control line is recommended (Magadla et al., 2016). The area was based on a geographical distribution survey of warthogs as well as occurrence of outbreaks, but the inclusion of Kwa-Zulu Natal in the control zone is not clear as no history of outbreaks are known in the province, although it is possible that it may have been modified to include this area based on the presence of serologically positive warthogs and virus-positive ticks reported based on surveys conducted

in the late 1970s (Thomson et al., 1983). Control measures to prevent spread to domestic pigs include double fencing around piggeries situated within the control zone, and strict movement control in and out of the ASF zone area to prevent spread.

A stamping out policy exists to address outbreaks of ASF outside the control zone in South Africa. During the 2012 outbreak in Gauteng, 500 pigs were quarantined on infected farms. The owners received goodwill payments and the pigs were humanely slaughtered and carcasses were destroyed. After disposal the facilities were disinfected and remained empty for 60 days before restocking was allowed. This lead to effective containment of the outbreak, and was aimed at reducing possible spread and minimising the duration of the crisis (www.pigprogress.net access 10 November 2015).

1.11 Justification of the study

African swine fever in SA has been well controlled for over 60 years by applying the legislation of an ASF control zone. However, *Ornithodoros* ticks involved in the sylvatic ASF cycle was reported near Gauteng, an area that falls outside of the control zone, and ASF viral genomic material was detected by PCR (Penrith and Vosloo, 2009). This report was followed by an outbreak south of the ASF control area in 2012, raising the question as to whether the ASF control area is still relevant today. The last in-depth epidemiological studies, based on distributions of *Ornithodoros* ticks, as well as ASFV infection of these ticks, were done in the late '70's and early '80s (Thomson et al., 1983) leaving an almost 30 year gap between these surveys and those conducted early in the 21st century (Arnot et al., 2009; Jacquier et al., unpublished). In South Africa it is noticeable that the sylvatic cycle including warthogs and *Ornithodoros* ticks plays a very important role in the transmission and maintenance of ASFV. In the absence of a vaccine it is particularly important determine factors impacting the epidemiological role of the sylvatic cycle vector, which has been previously identified as a crucial role player in the distribution of the disease. Main research priorities were identified in this review as being crucial and the ultimate goal of this study was to understand African swine fever virus maintenance and transmission dynamics in the sylvatic *Ornithodoros* vector.

1.12 Key research questions

The key research questions addressed in the present study include:

Chapter 2 – The role of *Ornithodoros* ticks in maintaining African swine fever virus in South Africa

Key research Aim:

A1: To ascertain the ASFV status of diverse tick populations from game parks across the ASF control zone of South Africa with the aim of more fully understanding the role of the sylvatic invertebrate host in the epidemiology of the disease and the current distributional limits of control area

Chapter 3 - First molecular assessment of the African swine fever virus status of *Ornithodoros* ticks from Swaziland

Key research Aim:

A1: The ASFV status of Swaziland is currently unknown, despite the presence of both invertebrate and wild suid species associated with the sylvatic cycle, and it being located within a geographical continuum between southern Mpumulanga and northern Kwa-Zulu Natal, which fall within the South African ASF control zone. This initial survey was undertaken to determine the distribution of the sylvatic cycle *Ornithodoros* ticks and the possible presence of the virus in this invertebrate reservoir in Swaziland.

Chapter 4 – Phylogeography of the *Ornithodoros* vector of African swine fever in South Africa with specific emphasis on the Kruger National Park

Key research Aim:

A1: The aim was to assess geographical variation within tick populations within the ASF control area of South Africa by means of a mitochondrial gene sequencing approach.

Chapter 5 – Evaluation of two enrichment strategies for genetic characterisation of the full-length genomes of ten African swine fever virus genotypes

Key research Aim:

A1: The development of a novel approach, using long-range PCR and deep sequencing, to improve on whole genome sequencing of ASF viruses, allowing for comparisons with available reference sequences.

Chapter 6 – Experimental transmission of African swine fever between the sylvatic *Ornithodoros* tick vector and domestic pigs: Transmission dynamics and genomic variation

Key research Aims:

A1: The aim of this study was to reconstruct the sylvatic cycle transmission by cycling the virus to a vertebrate host (the domestic pig) and back to naïve uninfected ticks, under experimental conditions, with emphasis on clinical observations.

A2: Genomic evolution of the ASFV during tick-domestic pig-tick cycling, under experimental conditions.

Chapter 2

The role of *Ornithodoros* ticks in maintaining African swine fever virus in South Africa

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Abstract

African swine fever (ASF) virus is maintained in a sylvatic cycle of infection between ticks of the *Ornithodoros moubata* complex and warthogs (*Phacochoerus africanus*). In South Africa this cycle plays an important role in the epidemiology of the disease, and the distribution of virus-infected ticks is key to delineating an effective ASF control zone. As large-scale assessment of virus presence in tick populations was last conducted in the late 1970s / early 1980, this study aimed to assess the current ASF-infection status of ticks by sampling specimens from warthog burrows in nine game parks inside the ASF control zone in South Africa. The DNA extracts prepared from individual ticks were assessed using a duplex PCR that amplifies tick and virus genomes, with the former serving as an internal control. In eight of the nine sampling areas infested with ticks, two parks tested positive for ASF virus (ASFV). Phylogenetic comparison of the partial C-terminal *p72* viral gene target was used to determine the ASFV genotypes in these tick populations in South Africa. Results indicate the presence of two additional genotypes (XXIV and XXV) specific to South Africa, as well as one transboundary genotype (II) that had not been described from South Africa before. This indicated the important role that *Ornithodoros* ticks play in the transmission of ASF with specific reference to Kruger National Park and surroundings in South Africa. The absence of ASF from North-West province highlight the need for more intensive investigations and confirmations to determine if the ASF control zone in SA are still relevant or need to be extended or condensed in certain areas.

2.1 Introduction

In South Africa between 1920 and 1940 several outbreaks of a virulent disease in pigs occurred. Initially described in Potgietersrus (now Mokopane, Limpopo Province) in 1926 it spread to the Cape Province in the early 1930's due to pig movement from the northern parts of the country, and continued until 1939 (De Kock et al., 1940). Uncertainty existed regarding whether the causative agent was the same virus responsible for Classical Swine fever, or the virus described by Montgomery in 1921 as causing 'East African' swine fever. It was however clear that an association between warthogs and the disease in the north-eastern part of South Africa existed. Based on sylvatic cycle involvement, as well as epidemiological and immunological differences from classical swine fever, it was accepted to be the 'East African' virus (Penrith et al., 2004). A swine fever control area was put in place in South Africa in 1935 (De Kock et al., 1940). The disease was confined to the African continent until middle of the 20th century and then spread to Europe, South America and Caribbean (Costart et al., 2013). The viruses adapted and adjusted to survive in domestic pigs.

The incursion of this virus into Europe was crucial to identify the role of soft tick vectors in the epidemiology of the disease on that continent. Research indicated that *Ornithodoros erraticus* that live in pig sties played a role in maintenance and transmission of this disease to pigs (Boinas et al., 2011). Based on these findings the role of *Ornithodoros moubata* a soft tick that lives in warthog burrows in Africa was investigated. Plowright and co-workers confirmed in 1969 that these eyeless, soft-shelled ticks were indeed involved in the maintenance and transmission of the virus to warthogs and domestic pigs (Plowright et al., 1969).

In South Africa the sylvatic cycle plays a very important role in the distribution of the disease and this cycle involves a relationship between the common warthog, *Phacochoerus africanus,* which are considered the original vertebrate hosts of ASF, and ticks of the *Ornithodoros moubata* complex. Warthogs are considered a very important vertebrate host of ASFV in South Africa, due to their wide distribution, persistent infection and asymptomatic carrier status of the disease (Thomson et al., 1980). The detection of specific antibodies against ASFV in the warthogs can be indicators of historic infections, or current infections, if presence of antibodies coincides with the presence of ASFV in *Ornithodoros* ticks in areas where the sylvatic cycle occurs (Katale et al., 2012).

In Africa, *O. moubata* ticks are a source for ASFV for both domestic and wild pigs (Costard et al., 2013). Infected ticks are able to maintain the virus for long periods of time and transmission of the virus to susceptible hosts occurs *via* blood meals. Persistent infections of ASFV in these ticks occur due to transstadial, transovarial and uni-directional sexual transmission of the virus in the tick host (Plowright et al., 1970, 1974; Wardeley et al., 1983; Kleiboeker and Scoles, 2001). *Ornithodoros moubata* ticks are widely distributed in southern Africa and are considered important for the persistence of ASF. Studies on ticks of the *Ornithodoros moubata* complex and the warthog's role in the disease, in South Africa was investigated through large-scale sampling conducted in the 1970's and 80's (Thomson et al., 1983; Thomson, 1985), based on studies described in East Africa (Plowright et al., 1969).

As ASFV infection fails to induce protective neutralizing antibodies and a vaccine is not available; control relies on slaughter and stamping out of infected pigs when outbreaks occur, as well as strict bio-security measures, such as prevention of contact between domestic pigs and free-living hosts to limit outbreaks (Penrith et al., 2004). The ASF control zone in South Africa was established based on the distribution of infected warthogs and *Ornithodoros* ticks and includes most of Limpopo Province as well as part of North-West Province, Mpumalanga and Northern-KwaZulu-Natal (Figure 2.1) (Thomson et al., 1983). This control approach seems to be effective in South Africa, as only sporadic ASF cases have been reported in the control zone. The ASF control zone relevancy was recently investigated (Magadla et al., 2016) and was found to be well-positioned with the possible exception of Limpopo Province as a single, small outbreak occurred just outside the zone in this province in 1996 (Penrith and Vosloo, 2009). These sporadic outbreaks were probably caused by contact between inadequately enclosed pigs and warthogs that resulted in infected ticks biting the pigs (Penrith and Vosloo 2009). To ensure that the current disease control strategies in place are adequate and effective, regular intensive epidemiological investigations need to be conducted inside the control zone and regular monitoring of the control line is necessary. An accurate understanding of the epidemiology of ASF in South Africa is an essential element for effective control of this disease.

Molecular epidemiological approaches have proved to be useful in investigations of ASF epidemiology patterns. To date, 23 genotypes have been identified (Bastos et al., 2003; Lubisi et al., 2005; Boshoff et al., 2007; Achenbach et al., 2016) to assist in the investigation of outbreaks and to provide epidemiological insight in the distribution of the disease and virus.

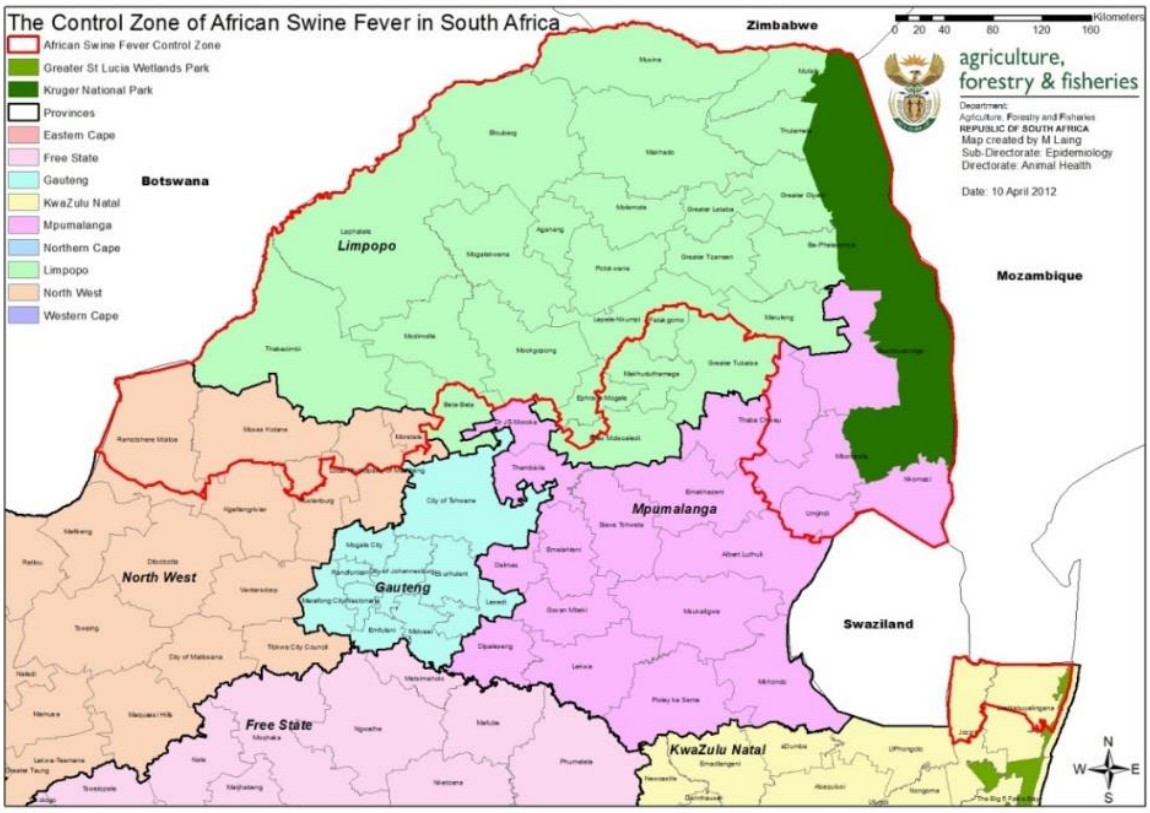


Figure 2.1: Map indicating the ASF control zone (red-line) in South Africa

The current approach is to partially sequence the gene B646L encoding the major virus protein 72 (VP72) with primers that target the C-terminal end of the gene (Bastos et al., 2003). Following this approach, the epidemiological complexity of ASF has been illustrated, ranging from a single genotype affecting an entire region and multiple countries, such as genotype I in West Africa (Lubisi et al., 2005), and as many as seven genotypes being recorded in a single country, viz. South Africa (Boshoff et al., 2007). The southern African region, is particularly genotype rich, with 14 of the 23 known genotypes occuring here (Bastos et al., 2003; Lubisi et al., 2005; Boshoff et al., 2007). This genotype and genetic diversity are linked to the presence of the sylvatic cycle and unrestricted cross border movements of role players in the transmission cycles. Genotypes can be country-specific but can also be spread across neighbouring countries through animal / animal product movement (Boshoff et al., 2007) having an impact on disease control and precipitating outbreaks.

In South Africa a thorough investigation on *Ornithodoros* ticks and the relationship with ASF and warthogs was done in the 1970's and 1980's (Thomson et al., 1983; Thomson, 1985). Mkuze Game Reserve in northern KwaZulu-Natal was included in this study and results

indicated the presence of ASFV infected ticks and warthogs, *albeit* both at very low levels compared to other infected wildlife areas (Thomson et al., 1983). Three decades later, this reserve was re-visited, and despite an increase in burrow infestation, warthog densities as well as the availability of more sensitive screening methods, no evidence of ASFV could be found in soft ticks (Arnot et al., 2009). This study highlighted the need to re-evaluate the entire ASFV control zone status in South Africa through conducting ASFV surveillance in *Ornithodoros* ticks, the sylvatic host that plays a central role in the ASF epidemiology.

A good correlation between presence of antibodies in warthogs and the occurrence of *Ornithodoros* was previously found in the Kruger National Park (Thomson et al., 1983). The ELISA test is most widely used for diagnosis of subacute and inapparent ASF carriers (Wardley et al., 1979; Pastor et al., 1990) and is considered to be the most sensitive and suitable method. However, one of the disadvantages of the ELISA is that false positive results can occur with field sera from wild species for which the test is not validated and thus confirmation with a second serological test is required (Cubillos et al., 2013). A rapid chromatographic strip test, that's reliable, accurate, sensitive and provide results within 15 minutes can be used as an initial indications followed by confirmation with the ELISA test results. The pen-side test is robust and simple to perform, making it ideal to perform field tests. Serological tests can be utilized to improve the understanding of the disease and assist to identify high risk areas.

Continuation of surveillance is important to understand the epidemiology and risks associated with ASF. With a three decade lapse since the last intensive *Ornithodoros* tick survey (Thomson et al., 1983) and the availability of genotyping methods that allow for identification of virus relationships, the current study was undertaken to better understand the role of the tick vector in ASF epidemiology. This was achieved by sampling sylvatic cycle hosts in game parks and nature reserves throughout the ASF control zone in South Africa. Although efforts focussed primarily on the detection and characterisation of ASF viruses in vector populations, the presence of antibodies in some populations of the natural vertebrate hosts was also assessed, with the aim of obtaining new insights into the epidemiology of the disease in South Africa.

2.2 Materials and Methods

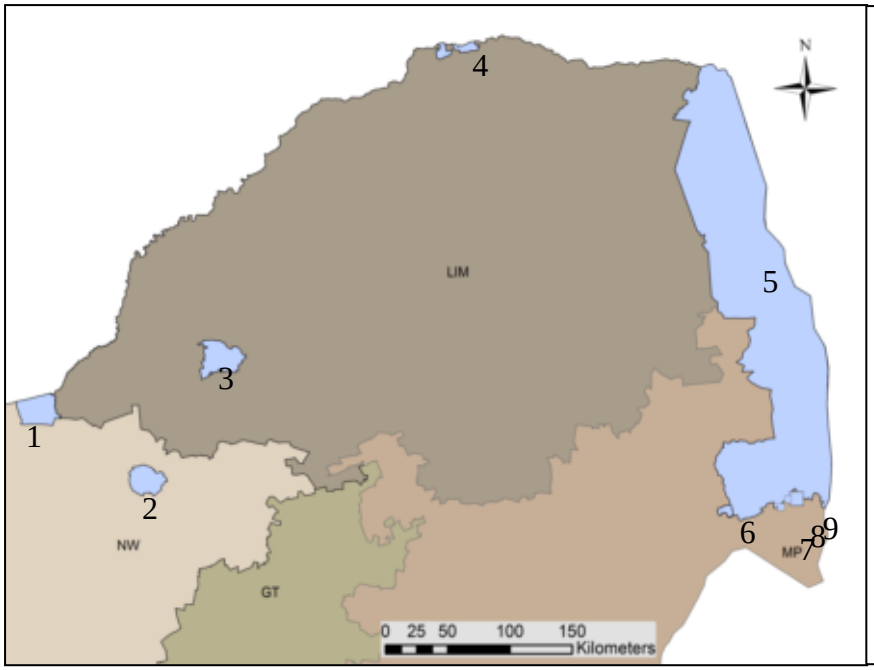
2.2.1 Study design and tick collection

National Parks (NP), Nature Reserves (NR) as well as Private Game Reserves (PGR) situated inside the ASF control zone were selected for tick sampling (Table 2.1). These parks are located in the three north-eastern provinces of South Africa and include Mpumalanga, North-West and Limpopo Provinces (Figure 2.2).

Table 2.1: Parks and reserves selected for sampling

Park/reserve name	Province	Co-ordinates
Kruger National Park	Mpumalanga	24.0114°S 31.4853°E
Kwa Madwala Private Game Reserve	Mpumalanga	25.4932°S 31.7269°E
Madikwe Nature Reserve	North-West	24.8167°S 26.2167°E
Marekele National Park	Limpopo	24.4138°S 27.6098°E
Marloth Park, Private Game Reserve	Mpumalanga	25.3477°S 31.7577°E
Mapungubwe National Park	Limpopo	22.2220°S 29.3472°E
Methethomusha Nature Reserve	Mpumalanga	25.4542°S 31.2721°E
Mjejane Private Game Reserve	Mpumalanga	25.3802°S 31.7274°E
Pilanesberg Nature Reserve	North-West	25.2611°S 27.1008°E

To ensure consistency in sampling, a single team, consisting of a team leader, a field assistant and a field ranger at each specific park, did the sampling throughout all the parks and nature reserves. Estimated collection days per park varied depending on the size of a park, with multiple collections being carried out in Kruger National Park, due to its large size. Storm water pipes on dirt and tar roads, and anthills near roads, which can both serve as overnight warthog shelters, as well as warthog burrows in the field were inspected for tick presence (Figure 2.3). The warthog burrows / shelters were selected to ensure broad representation across all areas of the parks. In KNP the sampling areas were spread from north to south across the rainfall gradient. The main focus areas for sampling were on the gravel roads. The main road (H1) was avoided as several samples had been collected along this road in a previous study (Jacquier et al., in prep). The exact GPS position of each burrow was documented and each burrow was photographed.



1. Madikwe Nature Reserve
2. Pilanesberg Nature Reserve
3. Marekele National Park
4. Mapungubwe National Park
5. Kruger National Park
6. Methethomusha Nature Reserve
7. Kwa Madwala Private Game Reserve
8. Mjejane Private Game Reserve
9. Marloth Park, Private Game Reserve

Figure 2.2: Map indicating the national parks, nature reserves and game reserves included in the study. GT: Gauteng Province; NW: North-West Province; LIM: Limpopo Province; MP: Mpumulanga Province

Figure 2.3: Culverts and burrows investigated for the presence of *Ornithodoros* ticks.

Ticks were collected according to a modified manual collection method (Jori et al., 2013). Briefly, soil was collected from each burrow and spread in a thin layer across a large, black plastic sheet placed in the sun. As soft ticks are photophobic and do not tolerate warm temperatures well, this encourages *Ornithodoros* tick movement and facilitates tick detection and collection. A minimum of 30 minutes per burrow was allowed, to ensure that tick movement was elicited and that all observable ticks were collected. Ticks were placed in labelled and sealed containers according to necessary bio-security regulations and transported

to Onderstepoort Veterinary Institute, Transboundary Animal Disease Programme (OVI-TADP) laboratory for analysis, with the necessary permits. Individual ticks were temporarily incapacitated by refrigeration, photographed and sorted microscopically into the various nymphal stages. Adult ticks were sorted on the basis of gender, and each tick was placed in an individual, labelled tube.

2.2.2 DNA extraction

Individual ticks were weighed and placed in a 1.5 ml Eppendorf tube and frozen for 10 min at -86 °C prior to the addition of the required volume of phosphate-buffered saline (PBS, pH 7.4) to achieve a final W/V of 10%. Each tick was ground individually with a pestle and mortar and the resulting homogenate was stored. Pools were made using 100 µl of three individual tick homogenates, thus DNA was extracted from 300 µl of the resulting homogenate pool using a modified silica-guanidium-thiocyanate method (Boom et al., 1990) for initial screening of all the ticks (Jacquier et al. in prep). The DNA extracted from each tick was eluted in a final volume of 50 µl with Tris-EDTA buffer (pH 7.4). Upon obtaining a positive ASFV *p72* PCR result from a pooled tick homogenate DNA extract, 100 µl of each of the individual ticks from that positive pool were subjected to DNA extractions using the High Pure PCR template preparation kit (Roche) to determine which of the three individual ticks were positive for ASFV. The final elution volume of each extract was 100 µl.

2.2.3 Genomic amplification

A duplex-PCR incorporating a tick mitochondrial 16S rRNA gene target as internal control together with primers that target the *p72* gene of the ASFV genome was performed (Bastos et al., 2009). By incorporating primers that target the *p72* gene of the ASF virus and primers that target a conserved region of the mitochondrial 16S rRNA gene of *Ornithodoros* ticks, virus genome presence and nucleic acid integrity could, respectively, be assessed. Genomic amplification was performed in a 50 µl volume in the presence of 0.2 mM dNTP, 0.5 µM of each primer, 1 U thermostable Go Taq G2 DNA polymerase (Promega Corp., Madison, WI) and 3 µl DNA extract. Thermal cycling conditions prescribed for C-terminal *p72* gene amplification (Bastos et al., 2003) were used. PCR products were evaluated by a 1.5% agarose gel electrophoresis against a 100-bp ladder (Promega Corp., Madison, WI) with amplification of a 478 bp band confirming virus presence and amplification of the 313 bp host gene target verifying DNA integrity.

2.2.4 Purification and nucleotide sequencing of PCR products

Bands of the expected size for *p72* were excised from the gel and purified. Two independent amplification and sequencing reactions were performed for positive samples with both PCR primers in separate reactions, in order to generate a partial *p72* gene sequence contig. The purified products were submitted for nucleotide sequencing to Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa).

2.2.5 Sequence comparison and phylogenetic analysis

The nucleotide sequences obtained were aligned and compared with at least one virus representative of each of the 23 (I – XXIII) known *p72* genotypes (Table 2.2) described to date (Bastos et al., 2003; Lubisi et al., 2005; Boshoff et al., 2007; Achenbach et al., 2016) resulting in a final dataset comprising 39 taxa, 405 nucleotides (nt) in length. Neighbor-joining (NJ) and minimum evolution (ME) trees were constructed in MEGA 5.2 (Tamura et al., 2011) with 10,000 bootstrap replications being performed to assess nodal support.

2.2.6 Virus isolation

The 10% (W/V) prepared for each individual tick identified as ASFV-positive by *p72* PCR, was inoculated with antibiotics and fetal bovine serum into primary swine macrophage cultures prepared in 96 well plates, with slight modification (Malmquist and Hay, 1960). The cells were examined daily for cytopathogenic effect or haemadsorption over a period of five days, and were blind passaged for at least two passages, if negative in a previous isolation attempt.

2.2.7 Serology

Fifty warthog (*Phacochoerus africanus*) sera, including juvenile, young adults, and adult, males and females (Figure 2.4) (Appendix A) originating from the Kruger National Park (KNP) were obtained from Veterinary Wildlife Services: KNP BioBank, SANParks. These were retrospective samples collected between 2009 – 2015 from animals immobilized for management and research purposes. Nine male adult sera were also obtained from Mauricedale, a privately owned game farm in the Marloth Park area in 2014, during animal hunting practices for farm management purposes.

Table 2.2: Summary of the ASF viruses used for the construction of the phylogenetic trees based on partial p72 gene sequences

Isolate	Country of origin	Host species	Sampling year	*p72* gene Genbank accession number	*p72* genotype	Reference
NAM/1/80	Namibia	Warthog	1980	AF504881	I	Bastos et al., 2003
MOZ/60-98	Mozambique, Tete	Pig	1998	AY274455	II	Bastos et al., 2004
RSA/5/95	South Africa, Ellisras	Pig	1995	DQ250124	III	Boshoff et al., 2007
RSA/1/99/W	South Africa	Warthog	1999	AF449477	IV	Bastos et al., 2003
MOZ/1960	Mozambique	Pig	1960	AF270708	V	Bastos et al., 2004
MOZ/94/1	Mozambique	Pig	1994	AF270711	VI	Bastos et al., 2003
SPEC/260	South Africa, Thabazimbi	Pig	1993	DQ250121	VII	Boshoff et al., 2007
MOZ-/A98	Mozambique	Pig	1998	AY274452	VIII	Bastos et al., 2004
UGA/1/95	Uganda	Pig	1995	AF449475	IX	Bastos et al., 2003
UGA/3/95	Uganda	Pig	1995	AF449476	X	Bastos et al., 2003
KAB/62	Zambia	Tick	1983	AY351522	XI	Lubisi et al., 2005
MFUE6/1	Zambia	Tick	1982	AY351561	XII	Lubisi et al., 2005
SUM/1411	Zambia	Tick	1983	AY351542	XIII	Lubisi et al., 2005
NYA/12	Zambia	Tick	1986	AY351555	XIV	Lubisi et al., 2005
TAN/1/01	Tanzania	Pig	2001	AY494552	XV	Lubisi et al., 2005
TAN/2003/1	Tanzania	Pig	2003	AY494550	XVI	Lubisi et al., 2005
ZIM/92/1	Zimbabwe	Pig	1992	DQ250119	XVII	Boshoff et al., 2007
NAM/1/95	Namibia	Pig	1995	DQ250122	XVIII	Boshoff et al., 2007
RSA/3/96	South Africa, Pienaarsriver	Pig	1996	DQ250127	XIX	Boshoff et al., 2007
RSA/1/95	South Africa, Hoedspruit	Pig	1995	DQ250123	XX	Boshoff et al., 2007
SPEC/53	South Africa, Letaba	Pig	1985	DQ250111	XXI	Boshoff et al., 2007
RSA/1/96	South Africa, Gravelotte	Pig	1996	DQ250125	XXI	Boshoff et al., 2007
SPEC/245	South Africa, Louis Trichardt	Pig	1992	DQ250117	XXII	Boshoff et al., 2007
ETH/04	Ethiopia. Gondar	Pig	2014	KT795355	XXIII	Achenbach et al., 2016
SAT/20/1	South Africa, KNP	Tick	2013			This study
SAT/21/2	South Africa, KNP	Tick	2013			This study
SAT/22/5	South Africa, KNP	Tick	2013			This study
LB/17/7	South Africa, KNP	Tick	2013			This study
LB/21/57	South Africa, KNP	Tick	2013			This study
LB/53/61	South Africa, KNP	Tick	2013			This study
LB/58/5	South Africa, KNP	Tick	2013			This study
LB/68/28	South Africa, KNP	Tick	2013			This study
SK/2/4	South Africa, KNP	Tick	2013			This study
SK/8/1	South Africa, KNP	Tick	2013			This study
N/71/66	South Africa, KNP	Tick	2013			This study
N/84/6	South Africa, KNP	Tick	2013			This study
N/84/21	South Africa, KNP	Tick	2013			This study
N/85/85	South Africa, KNP	Tick	2013			This study
N/85/98	South Africa, KNP	Tick	2013			This study
Nkom/6/5	South Africa, Marloth Park	Tick	2012			This study

Blocking enzyme-linked immunosorbent assay (ELISA) (Ingezim PPA CROM antibody detection Ref: 11.PPA.K41) as well as immuno-chromatographic assay (Ingezim PPA Compac Ref: 11.PPA.K3) for detection of antibodies against VP72 of ASFV in porcine sera was carried out according to the manufacturer's (Ingenasa) specifications. The ELISA sera were diluted 1:1 as prescribed in the manufacturer before testing and all samples were tested in duplicate. The test was considered valid when the test optical density (OD) of the negative control was at least four times higher than the OD of the positive control. Sera were considered positive for ASFV antibodies if the OD was lower than the positive cut off.

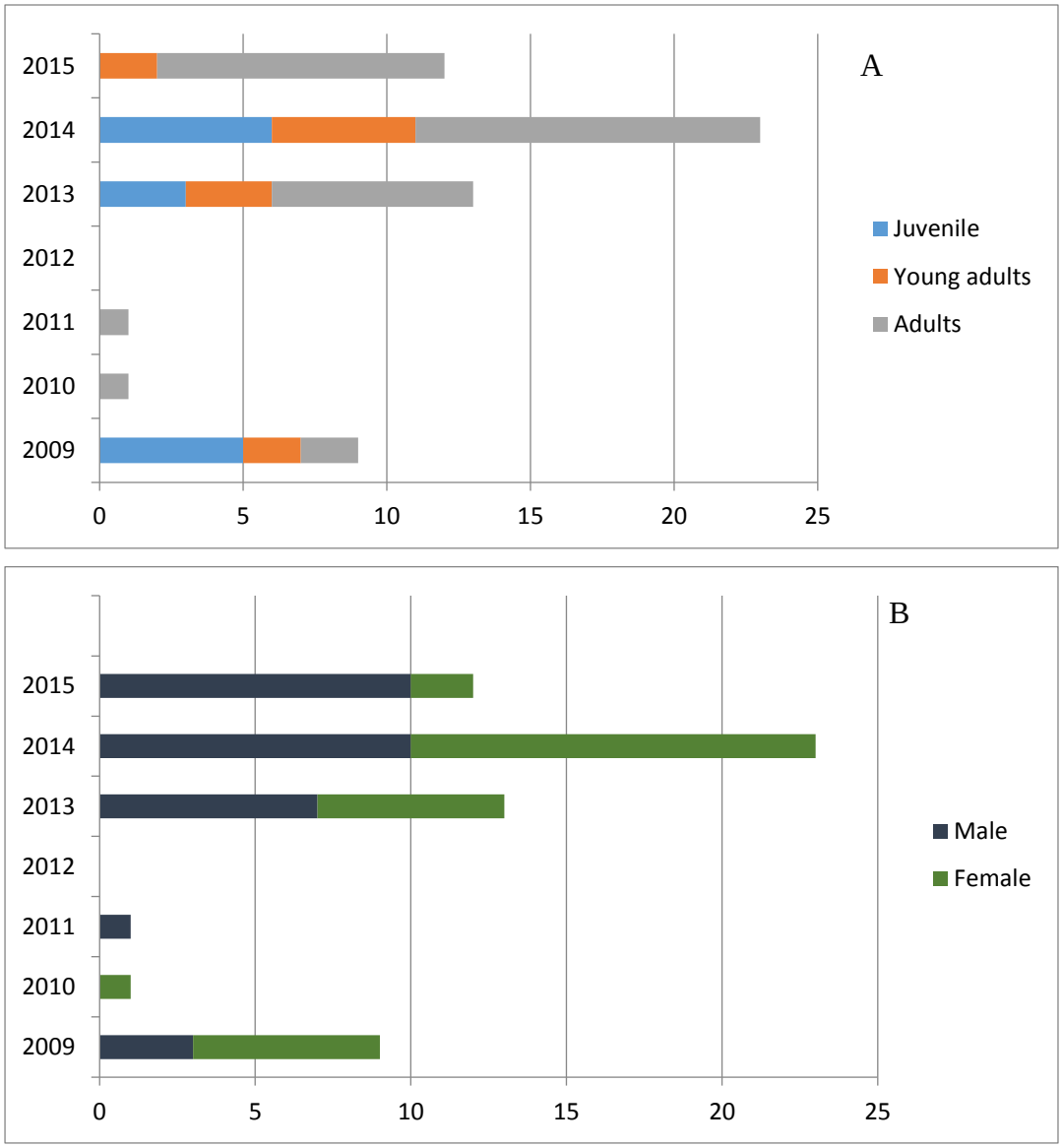


Figure 2.4: Charts indicating the representation of warthog samples tested, grouped according to age class (A) and sex (B)

All the sera were tested with the immuno-chromatographic assay. Ten microliters of each serum sample was micro-pipetted onto the test device and after a minute diluted with running buffer (provided with the kit). A maximum time of 10 minutes was allowed before reading the results. A test was considered valid when a blue line appeared in the control area. If a line appeared in the control area as well as in the result window, the serum sample was considered positive for ASFV antibodies (Figure 2.5).

Figure 2.5: Immuno-chromatographic assay, indicating a negative result above (only one line, the control (C) appears positive) and a positive result below (two lines, the control (C) and the test sample (T), appear positive).

2.3 Results

2.3.1 Burrow infestation rates

Nine national parks, nature reserves and private game reserves were investigated for the presence of *Ornithodoros* ticks. In total 268 burrows were examined (Appendix B) and ticks were found in 125 burrows (Figure 2.6), corresponding to an overall infestation rate of 46.6%. In eight of the sampling areas ticks were found, with Pilanesberg NR the exception where no ticks were present. The infestation rates in the eight positive sampling areas were compared (Table 2.3) and large differences were detected, ranging from a 5.2% tick infestation rate in Madikwe NR up to 63.2% in KNP.

Table 2.3: Results from different sample areas and the burrow infestation rates

National Park / Nature reserve / Private game reserve	Burrows infested by ticks / Burrows examined	Infestation rate
Kruger National Park	110 / 174	63.2%
Kwa Madwala Private Game Reserve	2 / 4	50.0%
Madikwe Nature Reserve	1 / 19	5.2%
Marekele National Park	5 / 22	22.7%
Marloth Park, Private Game Reserve	1 / 3	33.3%
Mapungubwe National Park	2 / 8	25.0%
Methethomusha Nature Reserve	3 / 10	30.0%
Mjejane Private Game Reserve	1 / 3	33.3%
Pilanesberg Nature Reserve	0 / 25	0%
TOTAL	125/268	46.6%

2.3.2 Tick numbers and ratios

A total amount of 2616 ticks were collected in the eight tick infested sampling areas (Appendix B). In seven sampling areas the tick numbers were low ranging from six in Kwa-Madwala PGR to 171 in Methethomusha NR, with the highest number of ticks (2320) being collected in KNP (Table 2.4). Of the 2616 ticks collected in all the parks, 327 were N1 (12.5%); 842 were N2 (32.2%); 611 were N3 (23.3%); and 222 were N4 (8.5%) resulting in a total of 2002 (76.5%) nymphs. Of the 614 (23.5%) adults, 279 (10.7%) were adult females and 335 (12.8%) were males (Figure 2.7).

2.3.3 Screening for ASFV genome presence

All 2616 ticks were screened for the presence of ASFV using the sylvatic tick duplex PCR (Bastos et al., 2009). Six out of the eight tick-infested parks tested negative for ASFV (Table 2.4). Of the 125 ticks infested burrows evaluated, 15 tested positive for ASFV, with positive amplifications for the ASFV *p72* gene being confirmed in Marloth Park as well as in KNP. In total 35 out of the 2616 ticks tested positive for ASFV, corresponding to an overall molecular prevalence of 1.34%. In Marloth Park, seven out of the 14 ticks tested positive, and in KNP 28 out of 2320 were positive, corresponding to individual park infection rates of 50% and 1.3%, respectively. For the KNP tick population, ASFV nymphal stage positivity ranged from 0.5% for N3 to 2.3% for N2. For the adult ticks, ASFV positivity was higher in males (1.2%) than in females (0.6%; Table 2.5).

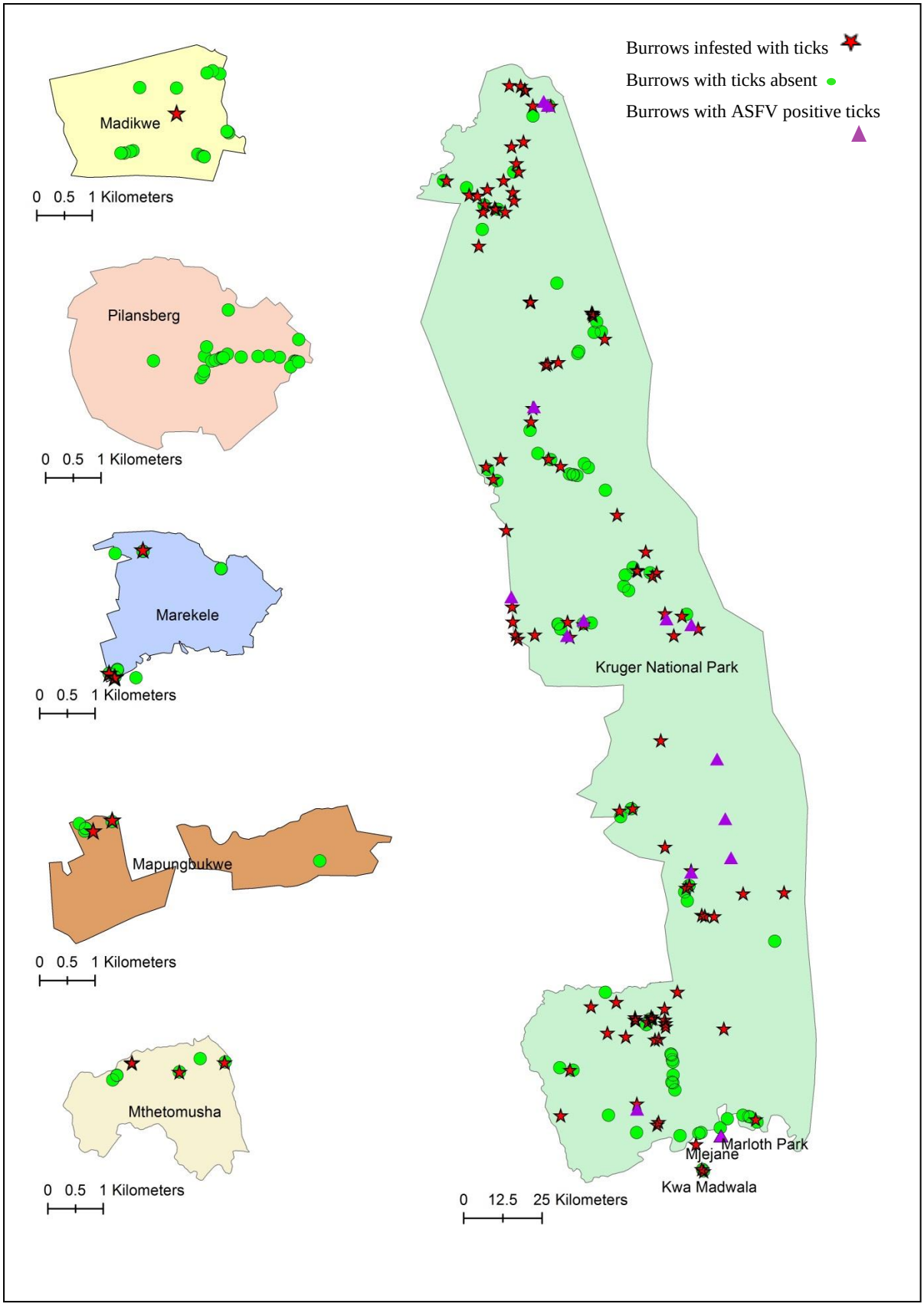


Figure 2.6: Map of the different parks, indicating localities of burrows investigated, burrows with and without ticks and burrows with ASFV positive ticks.

Table 2.4: Different sample areas indicating the number of ticks and infection rates

National Park / Nature reserve / Private game reserve	Date sampled	No of ticks ASFV positive / No of ticks examined	Infection rate	No of ASFV positive burrows / No of tick infested burrows
Kruger National Park	July and August 2013	28 / 2320	1.3%	14 / 110
Kwa Madwala	April 2013	0 / 6	0%	0 / 2
Madikwe Nature Reserve	May 2013	0 / 10	0%	0 / 1
Marekele National Park	May 2013	0 / 35	0%	0 / 5
Marloth Park	April 2013	7 / 14	50.0%	1 / 1
Mapungubwe National Park	June 2013	0 / 47	0%	0 / 2
Methethomusha	November 2013	0 / 171	0%	0 / 3
Mjejane	April 2013	0 / 13	0%	0 / 1
Pilanesberg	May 2013	0 / 0	0%	0 / 0
TOTAL		35/2616	1.3%	15/125

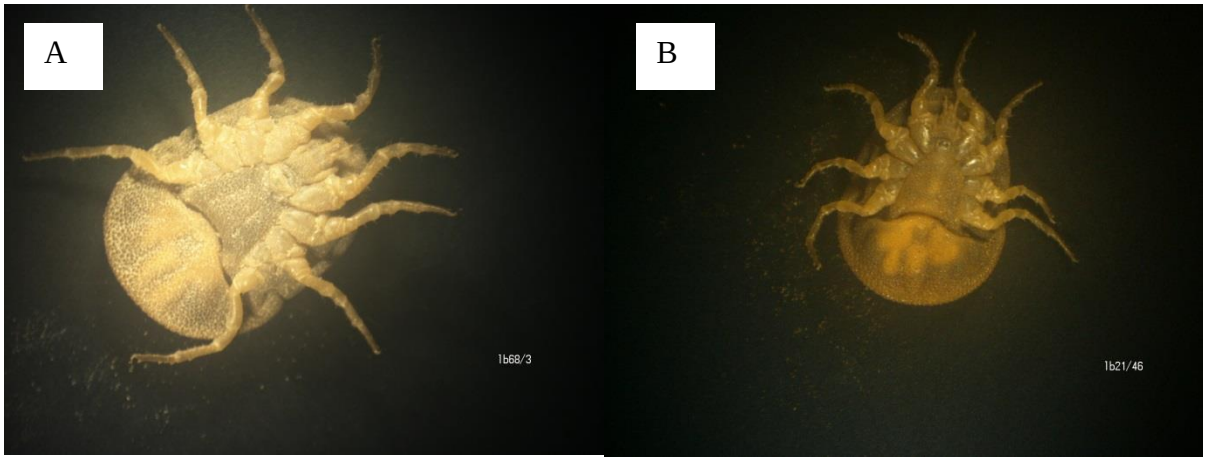


Figure 2.7: a) Female tick b) Male tick

2.3.4 P72 gene phylogeny

The final *p72* dataset comprised out of 40 taxa, and contained one representative haplotype for each of the 16 haplotypes identified from the 35 ASFV positive ticks sequenced. An AT bias (57.1%) was observed with the individual base frequencies for A, C, G and T being 0.277, 0.240, 0.180 and 0.294 respectively. Of the 405 nucleotide sites included in the analyses, 62 were variable and 36 were parsimony informative. On amino acid level, 11 of the 135 codon sites were variable and five of these variable sites were parsimony informative.

Comparable topologies were obtained with all methods of phylogenetic inference. The 40 viruses clustered within 25 discrete lineages, representative of distinct 25 genotypes. Of these, genotypes I – XXIII correspond to those identified previously (Bastos et al., 2003; Lubisi et al., 2005; Boshoff et al., 2007; Achenbach et al., 2016), whilst *p72* genotypes XXIV and XXV are reported here for the first time and therefore regarded as novel (Figure 2.8). The novel genotypes appear to be associated with a sylvatic cycle as these viruses are all from the ticks collected from the warthog burrows during this study. Seven genotypes were previously described in South Africa (Boshoff et al., 2007) with five specific to South Africa (IV; XIX; XX; XXI; XXII) and two displaying a transboundary distribution, being shared between South Africa and neighbouring Botswana (III; VII). Genotype II initially identified in Mozambique, Zambia and Madagascar (Lubisi et al., 2005) was found in this study for the first time to be present in South Africa, in the sylvatic tick host, indicating either movement of argasid tick vectors across boundaries, or due to limited sampling in the past, that it was missed before.

With the three additional genotypes identified in South Africa, the total number of genotypes occurring in this country increased from seven to ten genotypes. Genotypes XX and XXI previously detected in domestic pigs and warthogs, were shown to be present in ticks for the first time, and thus far appear to be restricted to South Africa. The three distinct evolutionary lineages previously identified (Boshoff et al., 2007), were again recovered in this study (Figure 2.8), with lineage A representing mostly Southern Africa viruses, lineage B comprised of seven genotypes (78% bootstrap support) mostly from East Africa and lineage C comprising out of two genotypes (100% bootstrap support) exclusively from East Africa.

2.3.5 Virus isolation

All PCR positive ticks were individually subjected to virus isolations on PBMC. No viruses could be isolated, despite blind passage performed twice. These negative results were probably due to the fact that the majority of ASFV positive ticks (mention how many of the total) were nymphal stage ticks, and thus due to their very small size there was a limited amount of starting material.

Table 2.5: Infection with ASFV in different sex and size classes of ticks collected from different parks in the ASF control zone of South Africa

Locality	Adult Females		Adult Males		N1		N2		N3		N4	
	No Tested	PCR positive (%)	No Tested	PCR positive (%)	No Tested	PCR positive (%)	No Tested	PCR positive (%)	No Tested	PCR positive (%)	No Tested	PCR positive (%)
Kruger National Park	311	2 (0.6)	260	3 (1.2)	252	2 (0.8)	710	16 (2.3)	584	3 (0.5)	203	2 (0.98)
Kwa Madwala Private Game Reserve	-	-	-	-	-	-	2	-	1	-	3	-
Madikwe Nature Reserve	-	-	-	-	2	-	8	-	-	-	-	-
Marekele National Park	-	-	2	-	16	-	11	-	6	-	-	-
Marloth Park, Private Game Reserve	1	1 (100)	3	1 (33.3)	-	-	2	2 (100)	4	1 (25)	4	2 (50)
Mapungubwe National Park	17	-	4	-	-	-	10	-	7	-	9	-
Methethomusha Nature Reserve	4	-	9	-	57	-	96	-	5	-	-	-
Mjejane Private Game Reserve	2	-	1	-	-	-	3	-	4	-	3	-
Pilansberg National Park	-	-	-	-	-	-	-	-	-	-	-	-
Total	335	3 (0.89)	279	4 (1.43)	327	2 (0.61)	842	18 (2.13)	611	4 (0.65)	222	4 (1.8)

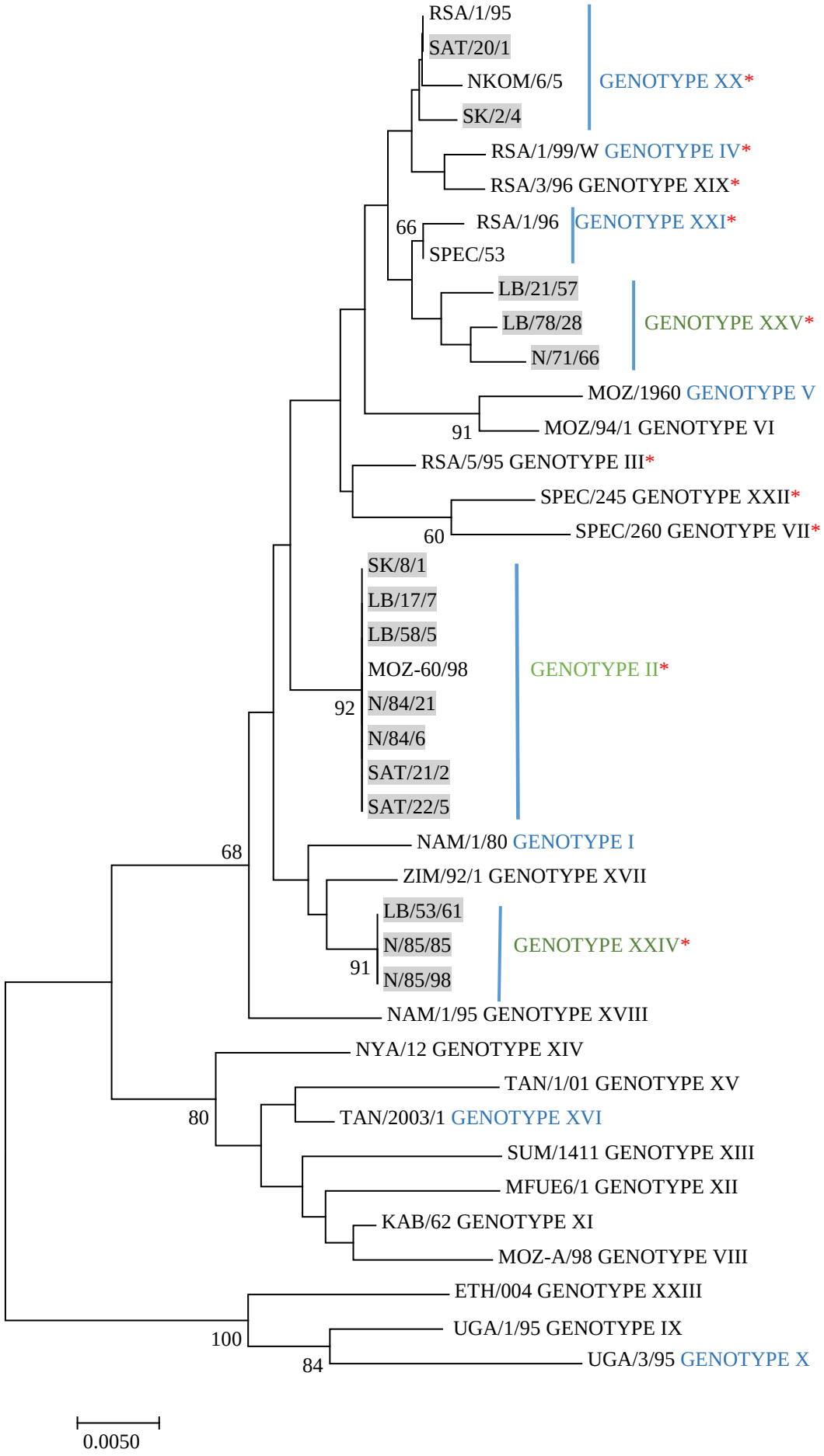


Figure 2.8: Neighbor-joining tree depicting ASFV relationships of the 23 (I-XXIII) genotypes, recovered from previous studies, and two new southern African genotypes, recovered in this study, designated XXIV and XXV. Bootstrap values > 60% obtained from 10,000 data replications are indicated next to each node. Viruses characterised in this study have a grey background, with genotypes from South Africa being indicated by a indicated by *

2.3.6 Antibody to ASF virus in warthog sera

Fifty nine warthog sera, originating from Skukuza, KNP (n=50) as well as Mauricedale, Malelane (n=9) that is in the same vicinity as Marloth Park, were tested. Both sampling areas were identified as being ASF PCR positive based on *Ornithodoros* tick screening. Both serological methods namely, ELISA (Figure 2.9) as well as the rapid pen-side immuno-chromatographic assay, tested positive for all 59 sera tested and there was therefore a 100% correlation between the conventional lab-based ELISA and rapid pen-side test.

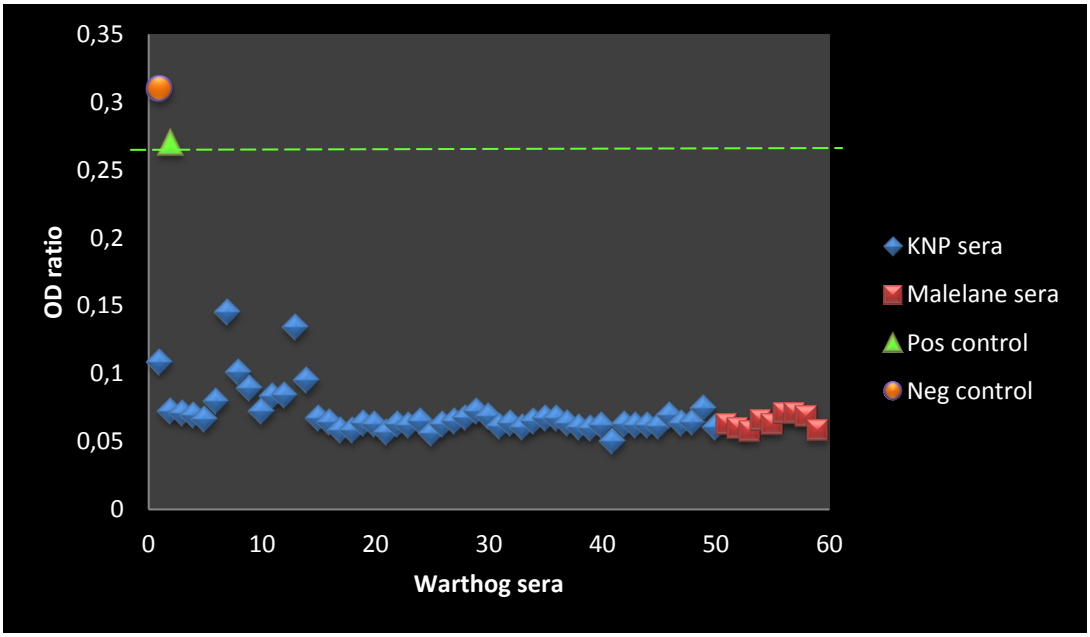


Figure 2.9: Detection of ASFV positive and negative warthog serum samples using the Ingezim ELISA. Results are expressed as ratio ODs where each dot corresponds to duplicate analyses of individual samples. Sera with OD lower than the positive cut off (shown by dotted line), indicate samples positive for ASFV antibodies, and sera samples with OD higher than negative cut off indicate samples negative for ASFV antibodies

2.4 Discussion

The aims of the current study were firstly to determine the prevalence of *Ornithodoros* ticks in various game parks and nature reserves in South Africa and secondly to investigate ASFV infections in these ticks. Thomson and co-workers (1983) reported that ticks were absent in North West Province (previously known as North-West Transvaal) south of latitude 25° S. This correlates to Pilanesberg nature reserve (25.2611° S, 27.1008° E) where ticks were also shown to be absent in the 2013 sampling effort conducted in the course of this study. This is the only reserve investigated that is located in North-West Province, south of latitude 25° S, and the only reserve negative for the presence of ticks. Results from KNP indicate an increase

in burrow infestations from 55.6% (10/18) in 1983 to 63.2% (110/174) in 2013, but this can be due to the much larger sample size in the amount of burrows investigated (18 *vs* 174). In North-West Province, north of latitude 25°S, ticks were found in 44.4% of burrows (Thomson et al., 1983) *vs* 5.2% and 22.7% respectively in Madikwe NR and Marekele NP which both fall within the same geographical area of North-West Province, and which were sampled in 2013. The exact warthog numbers are not known in any of the investigated parks, therefore correlations between warthog numbers and tick numbers could not be made.

The higher number of nymphs collected is consistent with tick populations sampled in the 1970's by Thomson and co-workers (1983) reported 13% adults, and 87% nymphs, compared to 23% adults and 77% nymphs in our 2013 sampling. Differences were observed with the ASFV infection rates, but this can depend on the season and month of the year when sampling took place. Infection rates in warthogs and ticks can vary due to seasonal effects (Thomson et al., 1983) as it is known that warthogs are infective during the warthog furrowing season (from October to January in Southern Africa), which corresponds to the wet season in the northern and eastern parts of South Africa. More recently, Jacquier an co-workers (in preparation) detected seasonal variation in tick infection rates in the Kruger National Park, however, the differences were not significant.

Previously a nine-fold higher infection rate was observed in adult ticks (Arnot et al., 2009 based on results of Thomson, 1985, whereas the overall infection rates for adults *vs* nymphs was lower in this study, viz. 0.7% in adults and 1.3% in nymphs, corresponding to an almost two-fold higher infection rate in nymphs. The overall low ASFV infection rate in ticks from KNP described by Thomson *et al*. in 1983 (1.4%) correlates with the 1.3% infection rate of the ticks collected from KNP during 2013, despite the differences in methods used to detect ASFV. Thomson and co-workers (1983) used tick pools for virus isolations and in this study, PCR was used, an approach found to be 1.4 times more sensitive than ASFV isolations (Arnot et al., 2009). Positive ticks were found in Marloth Park, which is situated on the southern boundary of KNP, and separated from it by the Olifants River. Animal movement and distribution of diseases are therefore possible during the dry season. In four parks, Kwa- Madwala PGR, Mapungubwe NR, Methethomusha NR and Mjejane PGR, ten or less burrows were investigated. Although tick infestation rates ranged from 25% to 50%, ASFV was absent. This is however inconclusive due to the low number of burrows investigated. Due to difficult

terrains, smaller sampling areas and fewer roads in these parks, sampling was more challenging.

The *p72* sequencing approach, used with success to identify country-specific genotypes previously (Lubisi et al., 2005; Boshoff et al., 2007) was used to investigate the relationships of the viruses obtained during this study from *Ornithodoros* ticks. The presence of two more novel genotypes confined to South Africa was revealed by molecular phylogeny, and in addition a known genotype (genotype II), already described for Mozambique (Bastos et al., 2004) was also described for the first time in South Africa, resulting in 10 genotypes in South Africa (Figure 2.10). Genotype XX and XXI are genotypes previously linked to outbreaks in domestic pigs, and warthogs, that are now linked directly to the sylvatic invertebrate host. The novel genotypes (XXIV and XXV) are currently restricted to *Ornithodoros* ticks in the Kruger National Park, making this park an important source of ASF strains, and highlighting the importance of physical separations of pigs and wild suids in this area and the strict implementation of control measures in adjacent communities.

Antibodies to ASFV in warthogs were present in KNP and Marloth Park. For both the localities examined, 100% of the sera were positive in the ELISA and in the immuno-chromatographic assay. This is higher than the 85% reported in 1983 in the KNP (Thomson et al., 1983). Both of these areas correspond to the two areas out of the eight tick-infested parks, where positive ASFV ticks were found. The detection of ASF-specific antibodies is indicative of previous infection, and confirms the presence of the virus in the warthog population. This was only a preliminary investigation and warthog sera of all nine areas investigated in this study need to be tested.

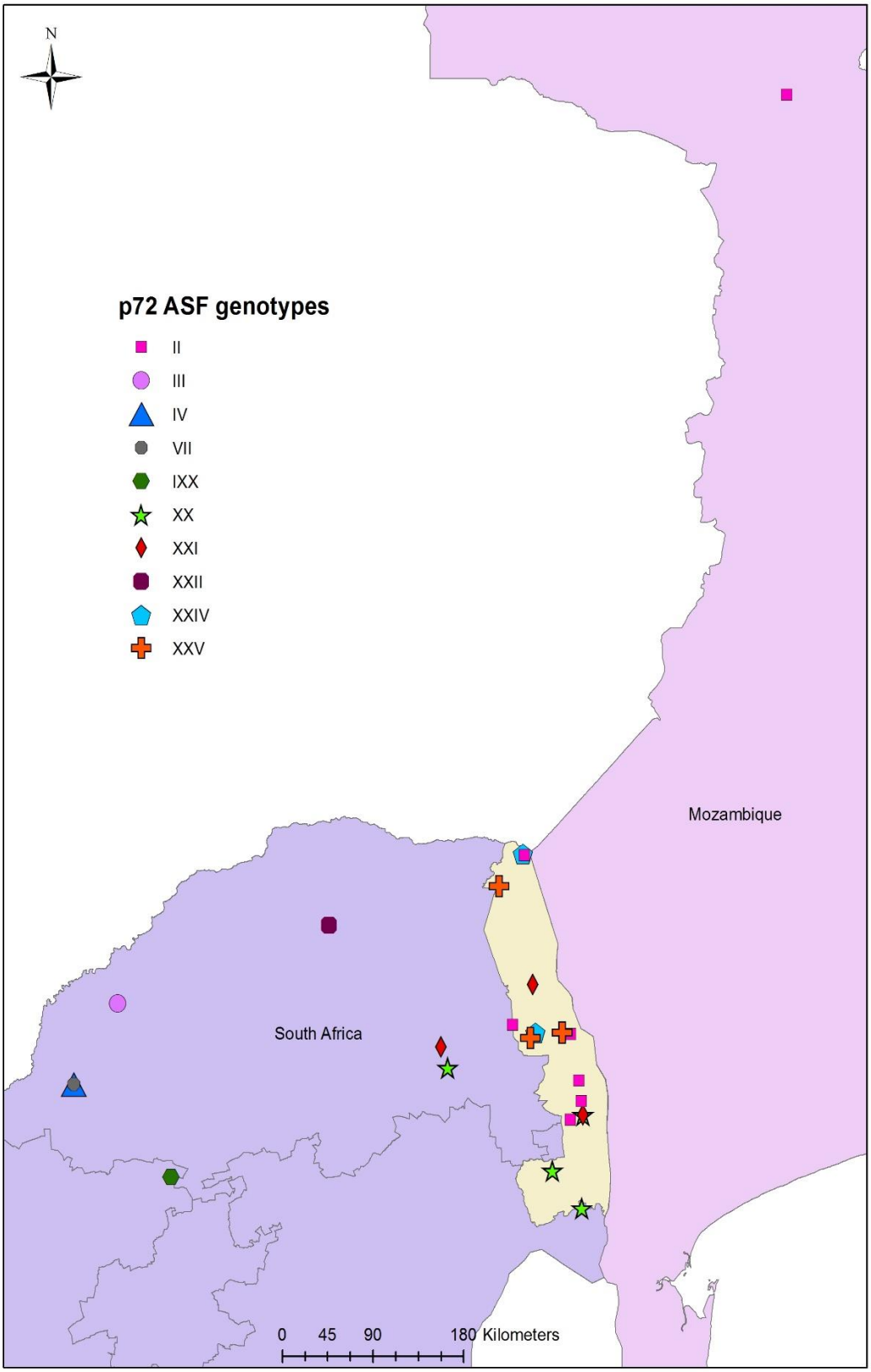


Figure 2.10**: Geographical distribution of the African swine fever genotypes identified in South Africa by *p72* genotyping.**

2.5 Conclusion and future work

African swine fever is a controlled disease of economic importance and has major effects on pig farming inside the control area. As it seems that *Ornithodoros* ticks are absent from Pilanesberg NR but present in Madikwe NR (although no ASF virus could be detected), with both NRs located in North-West Province, included in the control zone. Based on the current results, it is debatable whether these parks should continue to be included or whether inclusion within the ASF control area should be restricted to the northern part of North-West Province. This highlights a need for more intensive investigations for the presence of *Ornithodoros* ticks as well as serological surveys on warthogs in both parks. This study also highlights the need and the value of regular surveys in countries where a sylvatic and a tick-domestic cycle occur in order to ensure that adequate and effective control measures are in place to limit the threat, and to ensure that control is not unnecessarily being applied to disease-free areas.

African swine fever positive ticks found outside the southern border of Kruger National Park, in the region between Kruger National Park and Swaziland is important and should be more intensively investigated. This is because the southern-most distributional limit of ASFV-positive ticks is unclear since the recent report of the apparent disappearance of ASFV Mkuze GR (MGR) in northern Kwa-Zulu Natal Province (Arnot et al., 2009). The latter suggests that the continued inclusion of MGR, may not be necessary. However, before changes to the current control zone can be made it is important to assess the area south of KNP continuously through to, and inclusive of Mkuze. The inclusion of Swaziland, which is currently assigned ASF negative status, is crucial as it falls directly within the geographical continuum between KNP and MGR.

Acknowledgments

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Chapter 3

First molecular assessment of the African swine fever virus status of *Ornithodoros* ticks from Swaziland

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Abstract

African swine fever (ASF) is an economically significant haemorrhagic disease of domestic pigs, caused by African swine fever virus (ASFV), a DNA arbovirus. Argasid ticks of the genus *Ornithodoros*, which are widely distributed throughout southern Africa, play a primary role in virus maintenance and spread, within the endemic sylvatic cycle. The ASF status of Swaziland is not known, but this land-locked country is surrounded by ASF-positive countries, has a growing pig industry and sylvatic cycle hosts present within its borders. In this first assessment of ASF status, warthog burrows in seven nature reserves and game management areas in Swaziland were investigated for tick and virus presence. Tick infestation rates of between 33.3% and 88.8% were recovered for the four *Ornithodoros* infested parks. A total of 562 ticks were screened for virus genome presence using a duplex PCR that targets the C-terminal end of the *p72* gene of ASFV, and confirms DNA integrity through amplification of the 16S rRNA tick host gene. All samples were negative for virus genome presence and positive for the tick genome target. Nucleotide sequencing of the latter confirmed that *Ornithodoros* ticks from Swaziland are identical to those from the Kruger National Park in South Africa, across the gene region characterised. Whilst this first evaluation of ASF presence in Swaziland indicates that the virus does not appear to be present in the key virus vector, the presence of sylvatic cycle hosts, together with the country's proximity to ASF-affected countries calls for expanded investigations and regular monitoring of the ASF status of Swaziland.

Parts of the results presented in this chapter have been published in the *Onderstepoort Veterinary Journal*, Vol 81, Article 846: doi:10.4102/ojvr.v81i1.846 (2014)

3.1 Introduction

African swine fever (ASF) is an infectious viral haemorrhagic disease of domestic pigs. It is caused by the African swine fever virus (ASFV), the only known DNA arbovirus and sole member of the Asfarviridae family (King et al., 2012). Warthog-associated argasid ticks of the genus *Ornithodoros*, which are widely distributed throughout Southern Africa, play a primary role in maintenance and spread of the virus within the endemic sylvatic cycle, and virtually all regions in which the sylvatic hosts co-occur are ASF positive (Jori and Bastos, 2009). The sylvatic cycle of ASFV established between wild suids and ticks allows for the circulation and perpetual maintenance of the virus. Currently, no vaccines are available to control the disease and prevention relies on regulated control zones such as the one in South Africa (Figure 3.1) as well as on strict bio-security measures that prevent contact between domestic pigs and *Ornithodoros* ticks (Penrith, Thomson and Bastos, 2004). Swaziland is situated between the KwaZulu-Natal and Mpumalanga provinces of South Africa to the south and north, respectively; it shares its eastern border with Mozambique (Figure 3.1).

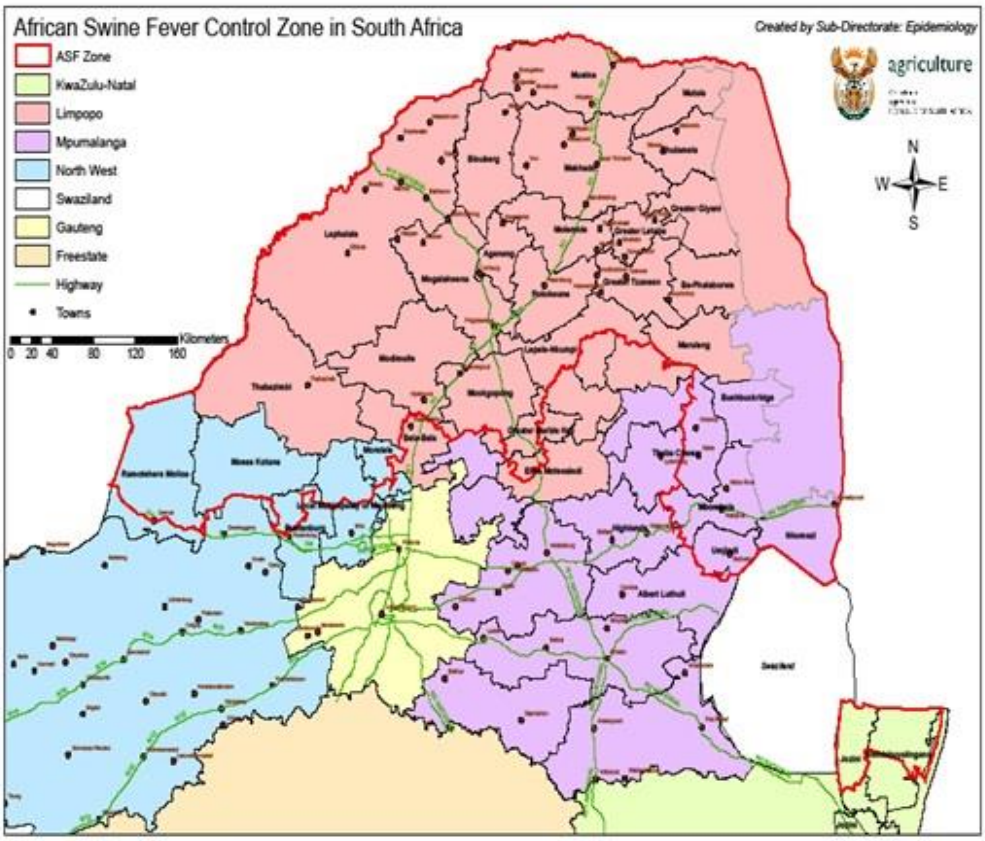

Figure 3.1: Map indicating Swaziland surrounded by African swine fever control zone of South Africa (indicated in red).

An important objective of livestock development in Swaziland is to increase animal production to meet the growing demand for animal protein. Swaziland has an estimated population of +/- 30,000 domestic pigs in 2009, and the number is increasing (Swaziland BEFS, Country brief, FAO). These are in commercial setups, but a significant number are in the rural area where bio-safety measures are not observed. The consumption of pork and pork products in the country is on the increase, with 8,6 tons of pork imported in 2010 (equivalent to 122,694 pigs). These products are mainly imported from South Africa, as such the pig population in the country is set to increase as the consumption and the market increases. In Swaziland piggery production is as yet not well established but is one of the industries that is rapidly growing. Recent increases in small-scale pig farming, particularly in communities in close proximity to game reserves containing sylvatic cycle hosts, signalled the need for assessment of ASF status as the disease is an important, but as yet unevaluated constraint for pig production.

Swaziland has game reserves with warthogs and bush pigs along the borders with South Africa and Mozambique, two countries in which ASF has been recorded. African swine fever is arguably the most serious constraint to pig production wherever it occurs (Penrith, 2009). The presence of warthogs, and possibly the virus vector, make ASF a possible threat to pig production in Swaziland. It is therefore important to fully assess and understand the risks. No prior surveys have been conducted in Swaziland, with the ASF status currently unknown and assumed negative, despite the presence of both invertebrate and wild suid species associated with the sylvatic cycle and the encirclement of this land-locked country by ASF-positive regions. As such, Swaziland is currently considered to be an area that is under constant threat of incursion, but if found to be ASF-positive, can potentially also serve as an uncontrolled reservoir of infection. This initial survey was therefore undertaken to determine the distribution of sylvatic cycle *Ornithodoros* ticks and possible presence of the virus in this invertebrate reservoir. The results of the study are crucial for formulation of appropriate disease control strategies for this and neighbouring countries, and complement ongoing efforts by the Swaziland government and the Food and Agriculture Organisation (FAO), with support of the European Union (EU), to reverse the country's declining agricultural productivity and to increase pig production (FAO, 2011).

3.2 Materials and Methods

3.2.1 Swaziland as study area

Swaziland is the smallest country in Africa, and is bordered in the North, West and South by South Africa and by Mozambique in the East. It has a land area of just over 17,000 square kilometres, and are divided into four regions of Hhohho, Manzini, Lubombo and Shiselweni. These regions are distinct from the four geographical zones (Highveld, Middleveld, Lowveld and Lubombo plateau) which are based on altitude and vegetation, and run from west to east, varying in altitude from 1800 to 400 metres above sea level. Along the eastern border with Mozambique, the Lubombo mountain ridge has an altitude of around 600 m. Along the north-western border, the rainforest Highveld region, which has an average altitude of 1,200 m, lies on the edge of the escarpment. The Middleveld, has an average altitude of 700 m above sea level with the Lowveld at around 250 metres and presents a typical African bush country of thorn trees and grasslands (Figure 3.2). Swaziland's climate ranges from moderate to subtropical. The weather depends on the region of the country, with the Lowland areas being subtropical, with temperatures reaching up to 42 °C during peak summer, whereas at higher altitudes in the Highveld regions temperatures in summer average 20 °C and the weather is cloudy and cool. Rain falls mostly during the summer months, with the winter months of June - August being dry. Annual rainfall is highest on the Highveld in the West, with between 1,000 and 2,000 mm being recorded, with the Lowveld in the East recording between 500 and 900 mm per annum (Atlapedia; Embassy of the Kingdom of Swaziland).

3.2.2 Study design

In Swaziland there are national, private and community-owned protected areas including national parks, nature reserves, wildlife sanctuaries and game reserves. Access was granted for sampling in seven nature reserves and game parks, including parks located near the borders with South Africa and Mozambique. These parks form part of the Songimvelo-Malolotja Transfrontier conservation area (TFCA) and the Lubombo Conservancy, which incorporate South Africa and Mozambique, respectively. As warthog censuses have not been performed, exact numbers of these sylvatic vertebrate host species are not known for the seven parks included in this study.

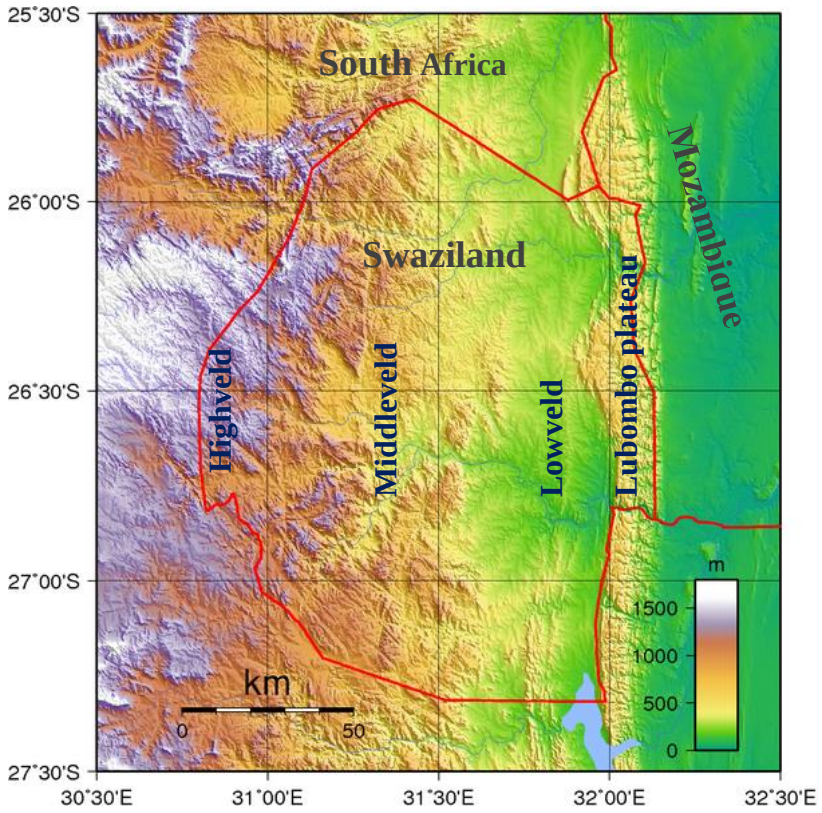

Figure 3.2: Topographical map of Swaziland, indicating the four geographical zones (http://upload.wikimedia.org/wikipedia/commons/0/04/Swaziland Topography.png)

In order to ensure consistency between sampling opportunities, a single team, consisting of a sampling team leader and field assistant did the collection under the field guidance of a knowledgeable ranger at each park. Estimated collection days per park were determined based on the size of a park, with a maximum of 5 days and a minimum of 2 days being allocated for, Hlane (22,000 ha) and Mbuluzi (3,000 ha), respectively. Storm water pipes on dirt and tar roads, and anthills near roads which can both serve as overnight warthog shelters, as well as warthog burrows in the field were inspected for tick presence.

3.2.3 Tick collection

Seven nature reserves and game parks in Swaziland, located near the borders with South Africa and Mozambique were selected for this study. These game reserves are both privately and publicly owned. Burrows presumed to be used by warthogs present in each of the parks were investigated, in total 58 burrows were investigated (Appendix C). Ticks were collected according to a modified manual collection method (Jori et al., 2013; Chapter 1). Briefly, sand was collected from each burrow and spread in a thin layer across a large, black plastic sheet, placed in the sun. As soft ticks are photophobic and do not tolerate warm temperatures, this

encourages *Ornithodoros* tick movement and facilitates tick detection and collection. A minimum time span of 30 minutes per burrow was allowed, to ensure that tick movement was elicited and that all visible ticks were collected. Ticks were hand-picked, labelled and transported to Onderstepoort Veterinary Institute, Transboundary Animal Disease Programme (OVI-TADP) laboratory for analysis, in sealed containers, under the necessary permits. Individual ticks were temporarily incapacitated by refrigeration, photographed and sorted microscopically into the various nymphal stages. Adult ticks were sorted on the basis of gender, and each tick was placed in an individual, labelled tube.

3.2.4 DNA extraction and genomic amplification

Individual ticks were weighed and placed into a 1.5 ml Eppendorf tube and frozen for 10 min at -86 °C prior to the addition of phosphate-buffered saline (PBS). Each tick was ground with a pestle and DNA was extracted from 100 µl of the resulting 10% W/V homogenate using a modified silica-guanidium thiocynate method (Boom et al., 1990). The DNA was eluted in 50 µl of Tris-EDTA buffer (pH 7.4). A duplex-PCR incorporating a tick mitochondrial 16S gene target as internal control was performed when screening for ASFV genome presence (Bastos et al., 2009). By incorporating primers that target the *p72* gene of the ASF virus genome and primers that target a conserved region of the mitochondrial 16S rRNA gene of *Ornithodoros* ticks, virus genome presence and nucleic acid integrity could respectively be determined. Genomic amplification was performed in a 50 µl volume in the presence of 0.2 mM dNTP, 0.5 µM of each primer, 1U thermostable Go Taq G2 DNA polymerase (Promega Corp., Madison, WI) and 3 µl DNA extract. Thermal cycling conditions prescribed for C-terminal *p72* gene amplification (Bastos et al., 2003) were used. PCR products were evaluated by 1.5% agarose gel electrophoresis against a 100 bp ladder (Promega Corp., Madison, WI) with amplification of a 478 bp band confirming virus presence and amplification of the 313 bp host gene target verifying DNA integrity. Positive and negative controls were included to address the possibility of false negative and false positive results, respectively.

3.2.5 Purification and nucleotide sequencing of PCR products

Host genome bands of the expected ~313 bp size were excised from the gel and purified by using a Zymoclean Gel DNA recovery kit (Zymo research), and submitted to a commercial company for Sanger cycle sequencing. Nucleotide sequences were generated for the tick mitochondrial 16S rRNA gene target (Inqaba Biotechnologies) of a geographically representative subset of tick samples and complemented with homologous data from a prior

study (Bastos et al., 2009) and aligned using the ClustalW programme embedded in Mega 5 (Tamura et al., 2011). The best-fit model of sequence evolution for the final dataset, 267 nt in length and comprising 19 taxa, was selected. A phenetic tree was inferred using the neighbour-joining algorithm and p-distance model of sequence evolution. Gaps were deleted in a pairwise manner and nodal support was calculated based on 10,000 bootstrap replications.

3.3 Results and Discussion

Ornithodoros ticks were found to be present in four of the seven game reserves sampled (Table 3.1; Appendix C). Ticks were only observed in parks situated in the eastern region of the country and were absent in the parks in the mountainous western region. Swaziland has a bowl-like terrain, being predominantly mountainous with forest escarpment in the north-western side, flattened throughout the centre of the country and becoming mountainous in the north-east along the Mozambique border (Figure 3.2). The game reserves positive for *Ornithodoros* tick presence all fell within the eastern savannah area and were associated with lower altitude localities (Figure 3.3). Tick infestation rates varied from 33.3% to 88.8% between the four tick-infested parks (Table 3.1). All ticks tested by duplex PCR were positive for the host 16S rRNA gene, but negative for ASFV, suggesting that despite the presence of sylvatic cycle hosts, the virus appears to be absent from *Orntihodoros* ticks in the game reserves evaluated.

A total of 562 ticks were collected, of which 113 were male, 105 were female and the remaining 344 were nymphs. The slight male bias in adult ticks collected in Swaziland is consistent with previous studies (Thomson et al., 1983; Arnot et al., 2009), but the adult to nymph ratio of 39:61% was nearly three-fold higher than the average adult to nymph ratio of 13:87% from a previous tick survey conducted in South Africa (Thomson et al., 1983). This adult bias is important, as there is an on average nine-fold higher infection rate in adults versus nymphs (Arnot et al., 2009) calculated based on results of the Thomson et al., 1983 study. This, together with the 1.4-fold higher sensitivity of PCR versus isolation, mitigates some of the concerns regarding the adequacy of the Swaziland sample size and underscores the advantages of performing adult-tick-biased surveys (Arnot et al., 2009).

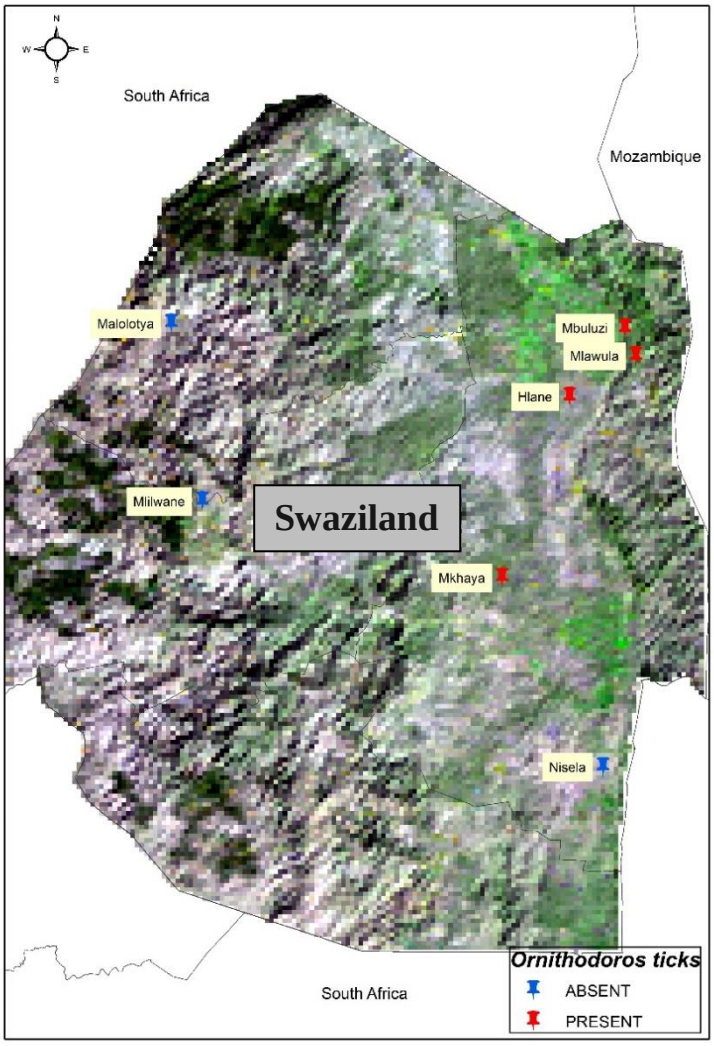

Figure 3.3: Map indicating the topography of Swaziland, the game reserves sampled in this study and *Ornithodoros* tick presence or absence

Despite intensive searching, and re-visiting of some of the parks, warthog burrows proved difficult to locate, particularly in the western half of the country, which is mountainous and densely vegetated. These sampling difficulties and the high altitudes likely underlie the comparatively lower number of burrows sampled in Mlilwane and Nisela, and the relatively low overall tick numbers from western Swaziland. Similar difficulties were encountered in Malawi, where similarly large differences in occurrence of *Ornithodoros* ticks were observed, leading to the speculation that altitude may be an important factor influencing tick distribution (Haresnape and Mamu, 1986).

Table 3.1: Game parks and *Ornithodoros* tick infestation rates of warthog burrows sampled in Swaziland

Game Reserve / National Parks	Latitude; Longitude co-ordinates for the game reserve's		Region in Swaziland	Approximate size of the game park	Altitude range of park (a.s.l)	Date sampled	No. of burrows with ticks / No. burrows examined	Total number of ticks
Malolotya	-26.146946°	31.137904°	North-Western	18,000 ha	1830m - 640m	November 2012	0/11	0
Mlilwane	-26.477807°	31.195466°	Western	4,600 ha	1450m - 670m	March 2013	0/4	0
Nisela	-26.972308°	31.941367°	Southern	4,000 ha	230m - 140m	April 2013	0/4	0
Hlane	-26.283136°	31.879031°	North-Eastern	22,000 ha	570m - 150m	March 2013	3/9	20
Mkhaya	-26.619919°	31.753700°	South-Eastern	10,000 ha	310m - 225m	May 2013	4/8	41
Mbuluzi	-26.156048°	31.982346°	North-Eastern	3,000 ha	460m -150m	April 2013	8/13	232
Mlawula	-26.207428°	32.002820°	North-Eastern	16,500 ha	470m -150m	November 2013	8/9	269

a.s.l.: above sea level

Sequencing of the host mitochondrial gene of ticks sampled from three infested reserves was used to confirm the *Ornithodoros porcinus* species designation and to evaluate relatedness of the ticks from Swaziland to reference data from southern and East Africa. Bastos et al., (2009) identified three geographically distinct *O. porcinus* clades in southern Africa, with South African samples falling in the south-east lineage. The gene phylogeny (Figure 3.4) confirmed that the sampled soft ticks from Hlane, Mlawula and Mbuluzi game reserves were identical to each other and all clustered within the geographically distinct *O. porcinus* south-east lineage (Bastos et al., 2009). The ticks from Swaziland had 100% and 99.63% pairwise sequence identities to ticks from two South African localities, namely the Kruger National Park (KNP) and Mkuzi game reserve (MGR), respectively, across the gene region sequenced.

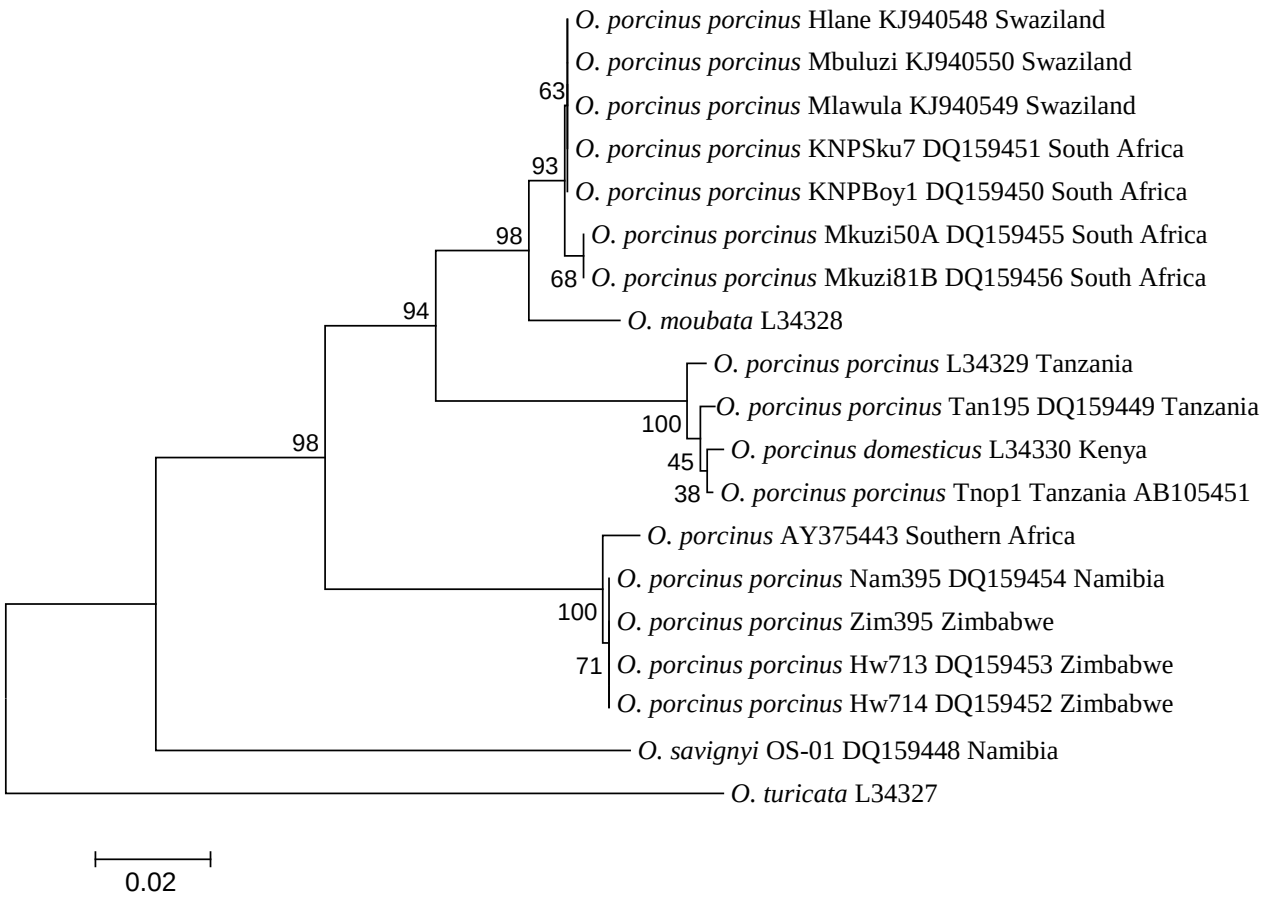

Figure 3.4: Neighbor joining tree depicting 16S rRNA gene relationships of Ornithodoros ticks sampled from three Swaziland wildlife reserves. Bootstrap support values >55 are shown next to the relevant nodes and sequences generated in this study are indicated in bold. For each of the reference sequences, the species / genus, voucher number, genbank accession number, and finally the country of origin is provided (if available).

Swaziland is surrounded by countries and regions where ASF has previously been reported. In Mozambique, bordering Swaziland to the east, disease control is challenging and the disease is known to be endemic in the country (Penrith et al., 2007). In South Africa, the disease is endemic in Mpumalanga province, which borders Swaziland to the north. In this and other provinces within in ASF control zone of South Africa, measures that prevent contact between pigs and sylvatic hosts of ASFV are implemented with success to prevent spread of the disease. In a study performed in MGR, in KwaZulu-Natal Province, which borders Swaziland to the south, it was shown that the virus was not present in any of the ticks sampled, signalling a possible change in the ASF status of this game reserve since the last survey in the late 1970s (Arnot et al., 2009). Together, the negative results from Swaziland and Mkuze, and apparent absence of the virus from both regions calls for additional, more extensive studies of the ASF status of both. These efforts will assist in delineating, with greater confidence, the southernmost distributional range of ASFV-positive ticks in South Africa.

Swaziland has an estimated population of 42,000 domestic pigs, but as the country is presumed to be ASF negative, there are no bio-security measures in place to prevent the spread of the disease. As ASF is an economically important disease with implications for all burgeoning pig industries across Sub-Saharan Africa (Penrith et al., 2013), the apparent absence of the virus indicated by this preliminary PCR-based tick assessment suggests that Swaziland should continue to focus efforts on minimising incursions in order to retain this status, particularly as other studies have shown that control measures implemented to limit ASF, also have a positive impact on control of other pig diseases (Fasina et al., 2015).

3.4 Conclusion and future research

Fifty-eight burrows were examined for the presence of *Ornithodoros* ticks from seven Nature reserves in Swaziland. A total of 562 ticks were tested for the presence of ASFV. The relatively low number of warthog burrows and ticks was likely due to topography and to the relatively low numbers of warthogs in the parks, as no warthogs were observed by the sampling team. The hypothesis that ASFV may be absent from sylvatic cycle hosts in Swaziland needs to be more fully investigated. It is proposed that parks in adjacent countries forming part of the trans-frontier parks be sampled to confirm the presence or absence of the ticks and the virus. The preliminary results highlight the need for additional research, including serological surveys of warthogs in all the parks and of domestic pigs across the country, and testing of these wild suids and domestic pigs for prior exposure to *Ornithodoros* ticks with an ELISA capable of

detecting OpTSGP1 argasid salivary protein antibodies (Díaz-Martín et al., 2011) in order to clarify all epidemiological aspects that impact ASF status in Swaziland.

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Chapter 4

Phylogeography of the *Ornithodoros* vector of African swine fever in South Africa with emphasis on the Kruger National Park

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Abstract

Warthog-associated soft ticks of the *Ornithodoros moubata* species complex play a central role in the epidemiology of African swine fever (ASF). Whereas ASF virus (ASFV) maintenance and transmission has been intensively studied through experimental infection of *Ornithodoros* ticks, the diversity and distribution of natural populations throughout the sylvatic cycle range in sub-Saharan Africa is understudied, and key taxonomic uncertainties remain. In this study we address the paucity of data for the southern African region by genetically characterising the 16S rRNA gene of *Ornithodoros* ticks sampled from eight game parks within the ASF control area of South Africa, and from neighbouring Mozambique and Swaziland. When complemented with homologous data from previously characterised populations in Zimbabwe, Namibia, Tanzania and Uganda, the analyses confirm the presence of four geographically discrete warthog-associated *Ornithodoros* clades, of which three occur in South Africa. In addition to the high levels of intra-lineage variation within the southern African region, finer scale analyses of ticks from the Kruger National Park in South Africa confirm the presence of four topotypical haplotypes suggestive of historical barriers to gene flow in the largest game park within the ASF control zone of South Africa.

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4.1 Introduction

African swine fever (ASF) is a highly contagious viral haemorrhagic fever of domestic swine, for which neither vaccine nor treatment options are currently available (Bastos et al., 2014). The ASF virus (ASFV), the only known DNA arbovirus, is maintained in an ancient sylvatic cycle in which the warthog burrow-dwelling, eyeless argasid tick plays an important role in maintenance and spread of the disease (Kleiboeker and Scoles, 2001). The sylvatic cycle involving the common warthog (*Phacochoerus africanus*) and *Ornithodoros* ticks is present throughout Central, East and southern Africa where the two species co-exist (Jori and Bastos, 2009). Warthogs live in burrows that are often infested with thousands of *Ornithodoros* ticks (Plowright et al., 1969) and these infected ticks are the link between ancient sylvatic cycle (involving wild suids) and domestic pigs.

The genus *Ornithodoros* consists of approximately 90 species of which 10 are of veterinary importance (Taylor et al., 2007). Confusion currently exists regarding the taxonomic status of *Ornithodoros* tick species that vectors ASFV (Kleiboeker and Scoles, 2001; Bastos et al., 2009). Walton (1962) initially assigned four species to the *O. moubata* species complex based on morphological and biological differences. In addition to *O. moubata sensu stricto*, *O. porcinus*, a species whose existence and interaction with wild suids has been closely linked to the ASF sylvatic cycle was defined. Two additional species, *O. compactus* and *O. apertus*, which are not associated with suids were also included in the species complex. Murray described *Ornithodoros moubata* in 1877 as a new domestic species characterised by high resistance to the effects of desiccation, although the description was insufficient and the type specimen was lost (Walton, 1964). *Ornithodoros porcinus* was the second species defined, for which it was noted that it was only able to withstand half the levels of desiccation of *O. moubata*. Based on consistent differences two subspecies were defined, *viz*. *O. porcinus porcinus* for the wild form and *O. porcinus domesticus* for the domestic form (Walton 1964). *Ornithodoros porcinus porcinus* are associated with warthog burrows and *Ornithodoros porcinus domesticus* with domestic pigs and human dwellings.

Reports before 1979 referred to *O. porcinus porcinus* ticks interchangeably as *O. moubata porcinus* or *O. moubata*. The lack of adequate guidelines based on morphological characteristics and the high biodiversity of *Argasidae* has made *Ornithodoros* spp taxonomy in literature very confusing (Estrada-Peña et al., 2010). A recent molecular investigation of *Ornithodoros* from East and southern Africa did not support the recognition of two *O. porcinus* subspecies. Instead, three geographically distinct *O. porcinus* clades were recovered and the

single sequence representative of *O. moubata* clustered within one of the three geographically separated O. *porcinus clades*, rather than on its own (Bastos et al., 2009). Further taxonomic investigations are therefore needed to confirm and resolve the taxonomic status of the ASF vector. As the presence of discrete geographical clades is undisputed, based on limited regional sampling we conducted a more in depth investigation into the diversity of *Ornithodoros porcinus* tick populations in South Africa, with emphasis on game parks within the ASF control zone. The Kruger National Park (KNP), one of the biggest national parks (19,485 km²) in the country, resides within the ASF control zone and has a high density and diversity of wildlife. KNP, is zonated into 35 landscapes, based on specific geomorphology, climate, soil and vegetation patterns together with the associated fauna (Gertenbach, 1983). As this landscape, diversity is likely to reflect genetically, a phylogeographic approach was employed in an attempt to unravel patterns of inter- or intra-specific genetic polymorphism in relation to landscape / geography. To achieve this we genetically characterised *Ornithodoros* ticks sampled across a ~400 km longitudinal gradient in KNP, to determine whether the broad regional geographical structuring observed previously (Bastos et al., 2009) may also reflect at a finer geographical scale. Characterisation of warthog-associated *Ornithodoros* ticks will be valuable for assessing vector diversity in relation to virus diversity. This is important as ASF virus diversity is extensive in South Africa (Boshoff et al., 2007), yet the underlying reasons for this, particularly with regard to *Ornithodoros* vector diversity are presently poorly understood.

4.2 Materials and Methods

4.2.1 Tick collection and genomic amplification

For Kruger National Park, ticks from 60 burrows were selected for the phylogeographical component of the study (Table 4.1). In addition, ticks from 30 burrows sampled at eight discrete localities in South Africa were randomly selected for genetic characterisation (Table 4.2). Individual ticks were placed for 10 minutes at -86 °C and a 10% W/V homogenate was prepared using phosphate buffered saline (PBS) from which DNA was extraction with the High Pure PCR Template preparation kit (Roche). DNA was eluted in final volume of 100 µl and stored at -20 °C until further use. A ~313 bp fragment of the mitochondrial 16S rRNA gene was amplified using previously described primers and thermal cycling conditions (Bastos et al., 2009). The resulting amplicon was excised from the agarose gel, purified by means of the Zymoclean Gel DNA recovery kit (Zymo research) and submitted to Inqaba Biotec (Pretoria, South Africa) for Sanger cycle sequencing.

Table 4.1: Kruger National Park Samples used in the phylogeography dataset

Sample name	Geographical origin	Co-ordinates	Sample name	Geographical origin	Co-ordinates
LB#1	Letaba	-23.78284, 31.57583	N#71	Punda Maria rest camp	-22.70177, 31.03859
LB#3	Mooiplaas	-23.61781, 31.46317	N#75	Punda / Pafuri road	-22.71844, 31.16704
LB#16	Balule Road	-23.94409, 31.69526	N#76	Punda / Pafuri road	-22.69447, 31.16392
LB#17	Olifants tar road	-23.93275, 31.67583	N#79	Punda / Pafuri road	-22.54986, 31.19420
LB#18	Letaba	-23.90728, 31.64878	N#81	Pafuri	-22.44685, 31.22085
LB#19	Letaba	-23.96260, 31.62573	N#82	Pafuri	-22.44750, 31.27241
LB#21	Letaba	-23.91671, 31.60474	SK#3	Skukuza	-25.06289, 31.51494
LB#27	Letaba	-23.77817, 31.52148	SK#6	Doispane Road	-25.01183, 31.46029
LB#32	Macene	-23.35110, 31.21626	SK#8	Hamiltons Camp	-24.64116, 31.67512
LB#53	Letaba	-23.92063, 31.36652	SK#10	Hamiltons Camp	-24.67878, 31.67000
LB#55	Letaba	-23.92387, 31.32066	SK#14	Tshokwane	-24.56789, 31.60045
LB#56	Letaba	-23.92336, 31.16417	SK#17	Tshokwane	-24.46488, 31.47007
LB#57	Letaba	-23.88122, 31.16175	SK#19	Orpen	-24.45824, 31.50768
LB#58	Letaba	-23.85385, 31.1596	SK#23	Skukuza	-25.08228, 31.60199
LB#59	Letaba	-23.66209, 31.14477	SK#25	Malelane road	-25.11972, 31.57143
LB#62	Phalaborwa	-23.96185, 31.17144	SK#27	Skukuza	-25.05919, 31.56544
LB#64	Phalaborwa	-23.00880, 31.21368	SK#28	Sabie river	-24.98302, 31.63565
LB#65	Phalaborwa	-23.00784, 31.21551	TS#22	Tshokwane	-24.76281, 31.70591
LB#66	Phalaborwa	-23.96705, 31.32773	TS#23	Tshokwane	-24.76551, 31.71431
LB#68	Phalaborwa	-23.96391, 31.31929	TS#24	Tshokwane	-24.76667, 31.74136
LB#69	Phalaborwa	-23.94491, 31.30057	SAT#22	Satara	-24.48784, 31.77322
LB#71	Mahlangeni	-23.51586, 31.10824	SAT#21	Satara	-24.31726, 31.74919
LB#74	Mahlangeni	-23.45892, 31.12859	SAT#20	Satara	-24.60006, 31.78946
N#78	Shingwedzi	-22.61200, 31.17377	ORP#20	Satara	-24.26346, 31.58797
N#3	Shingwedzi river	-23.18112, 31.29427	S#64	Mathekenyane	-25.06927, 31.55083
N#4	Shingwedzi river	-23.18808, 31.25854	S#66	Nwathimi road	-25.08812, 31.76917
N#61	Shingwedzi	-23.04846, 31.39451	S#71	Crocodile Bridge	-25.33644, 31.30100
N#63	Shingwedzi	-23.04230, 31.39235	S#75	Numbi gate	-25.20661, 31.32769
N#66	Punda Maria gate	-22.75121, 31.14143	S#78	Jock Safari	-25.31950, 31.52011
N#69	Punda Maria gate	-22.74048, 31.11165	S#79	Mlambane	-25.36310, 31.57744

Table 4.2: Samples used in the 16S rRNA dataset

Sample name	Geographical origin	GenBank	Reference
O. moubata	NK[1]	L34328	Black and Piesman
O. porcinus domesticus	Kenya	L34330	Black and Piesman
O. porcinus	Southern Africa	AY375443	Scoles (2004)
O. porcinus domesticus	Madagascar	HM588699	Ravaomanana et al.,
Mkuze50A	Mkuze, SA[2]	DQ159455	Bastos et al., (2009)
Mkuze81B	Mkuze, SA	DQ159456	Bastos et al., (2009)
KNPBoy1	Boyela, KNP[3], SA	DQ159450	Bastos et al., (2009)
KNPSku7	Skukuza, KNP, SA	DQ159451	Bastos et al., (2009)
Zim395	Zimbabwe	DQ159454	Bastos et al., (2009)
Hw713	Hwange NP[4], Zimbabwe	DQ159453	Bastos et al., (2009)
Hw714	Hwange NP, Zimbabwe	DQ159452	Bastos et al., (2009)
Nam395	Namibia	DQ159454	Bastos et al., (2009)
O. moubata Tn1.	Tanzania	AB057541	Fukunaga et al.,
Tan195	Serengeti, Tanzania	DQ159449	Bastos et al., (2009)
Tnop1	Tanzania	AB105451	Mitani et al., (2004)
O. porcinus porcinus	Shiyanga, Tanzania	L34329	Black and Piesman
O. savignyi	Namibia	DQ159448	Bastos et al., (2009)
Hlane	Hlane NP, Swaziland	KJ940548	Boshoff et al., (2014)
Mbuluzi	Muluzi NP, Swaziland	KJ940549	Boshoff et al., (2014)
Mlawula	Mlawula NP, Swaziland	KJ940550	Boshoff et al., (2014)
Mkhaya	Mkhaya NP, Swaziland		This study
Mthethomusha 10	Mthethomusha Game		This study
KNP LB18	Letaba, KNP, SA		This study
KNP LB27	Letaba River road, KNP,		This study
KNP LB57	Letaba, KNP, SA		This study
KNP LB62	Phalaborwa, KNP, SA		This study
KNP LB74	Mahlangeni, KNP, SA		This study
KNP N61	Shingwedzi, KNP, SA		This study
KNP N71	Punda, KNP, SA		This study
KNP N79	Pafuri, KNP, SA		This study
KNP S71	Pretoriuskop, KNP, SA		This study
KNP S78	Malelane, KNP SA		This study
KNP SAT21	Sweni Road, KNP, SA		This study
KNP SK6	Paul Kruger Gate, KNP,		This study
Nkomazi 6	Marloth Park, Nkomazi,		This study
Nkomazi 10	Mjejane, Nkomazi, SA		This study
Marekele 1	Marekele NP, SA		This study
Marekele 8	Marekele NP, SA		This study
Mapungubwe 6	Mapungubwe NP, SA		This study
Mapungubwe 4	Mapungubwe NP, SA		This study
Alldays	Alldays, SA		This study
RSA/2010/01	Loodswaai, SA		This study
Madkiwe 13	Madikwe Game Reserve,		This study
Mongena11	Dinokeng Game Reserve,		This study
Kwalata NR 08/1429	Dinokeng Game Reserve,		This study
Melkfontein	Dinokeng Game Reserve,		This study
Kromdraai 06/1429	Dinokeng Game Reserve,		This study
Leeuwfontein NR	Dinokeng Game Reserve,		This study
Gorongosa NP A001-1	Gorongosa NP,		Unpublished
Gorongosa NP 12	Gorongosa NP,		Unpublished

[1]NK not known; [2]SA South Africa; [3]KNP Kruger National Park; [4]NP National Park

4.2.2 Phylogenetic and haplotype network analyses of the 16S rRNA

Partial 16S rRNA nucleotide sequences generated in this study were aligned with homologous *Ornithodoros moubata* and *Ornithodoros porcinus* sequences in the GenBank database, resulting in a final 279 nt long dataset comprising of 50 taxa. *Ornithodoros savignyi*, which was previously shown to be sister to the *O. moubata* complex (Bastos et al., 2009), was included for outgroup purposes. Gaps were coded for maximum likelihood analyses conducted with PhyML (Guindon et al., 2010) and treated as a fifth character state for parsimony analyses in PAUP (Swofford, 2003). When constructing a pairwise p-distance matrix, gaps were deleted in a pairwise manner at positions with less than 95% site coverage (Table 4.3). Haplotype network analyses were constructed using median-joining networks (Bandelt et al., 1999) with default settings in PopART (Leigh and Bryant, 2015).

Table 4.3: Estimates of Mean Evolutionary Divergence over Sequence Pairs between and within clades

South-East	0.0122				Within clades
North-East	0.0761	0.0047			Between clades
South-Central	0.0454	0.0784	0.0012		
South-West	0.0856	0.1192	0.0962	0.0000	
	South-East	North-East	South-Central	South West	

4.3 Results

4.3.1 16S rRNA tick gene phylogeny

The 50 taxon 16S rRNA dataset comprising of 279 nucleotide sites contained 70 variable sites, of which 6 corresponded to indels inserted for alignment purposes and 47 to parsimony informative sites (Appendix D). The dataset displayed AT bias with base frequencies of T, A, G and C, being 0.39, 0.34, 0.16 and 0.10 respectively. As a previous study comparing Maximum parsimony (MP), Neighbor-joining (NJ), Maximum Likelihood (ML) and Bayesian interference (BI) phylogenetic reconstruction methods with an homologous 16S rRNA dataset recovered topologically similar trees (Bastos et al., 2009), the analyses in this study were limited to ML and BI. Both methods resulted in topologically similar trees when applied to the 16S rRNA dataset generated for this study. Results confirmed that specimens sampled in this study cluster according to geographic origin in four distinct clades (denoted i – iv in Figure 4.1A), with three of these occurring in South Africa (SA). Thus, in addition to the three regionally distinct clades incorporating diverse African countries (Bastos et al., 2009), a fourth southern African clade was recovered. On the basis of the previous clade naming our results

confirmed the presence of a (i) "south western" clade incorporating *O. porcinus* from Namibia, Zimbabwe as well as the North-West and Gauteng Provinces of SA; (ii) a "north eastern" clade including ticks from Tanzania, Kenya and Madagascar, (iii) a "south eastern" clade including ticks from South Africa (Mkuze, Northern KwaZulu-Natal province, Kruger National Park, Mpumalanga and eastern Limpopo), Swaziland and Mozambique, and a newly defined "south central" clade comprising of ticks from north-western Limpopo Province. Thus, it is clear that three of the four geographically discrete clades occur within the borders of South Africa.

Of interest is that clade (iii) comprises out of a number of geographically distinct lineages, and contains a discrete sub-lineage in which ticks from Mozambique cluster (Figure 4.1). This clade also contains ticks from the Kruger National Park for which four distinct sub-lineages were recovered, all of which cluster within clade (iii). In addition, *Ornithodoros moubata* sequence (L34328) clusters within the "south eastern" clade (iii), again calling into question whether *O. moubata* is indeed a monophyletic species. Similarly, in the "north eastern" clade (ii) *O. porcinus porcinus* (L34329), *O porcinus domesticus* (L34330) and *O. moubata* (AB057541) cluster together.

Average number of pairwise differences are shown in Table 4.3. Between clusters pairwise differences were highest between the north eastern (ii) and south western (i) clades, while the south eastern clade shows highest level of within lineage diversity, likely due to higher sample size and characterisation of multiple sub-lineages and haplotypes, particularly within the Kruger National Park.

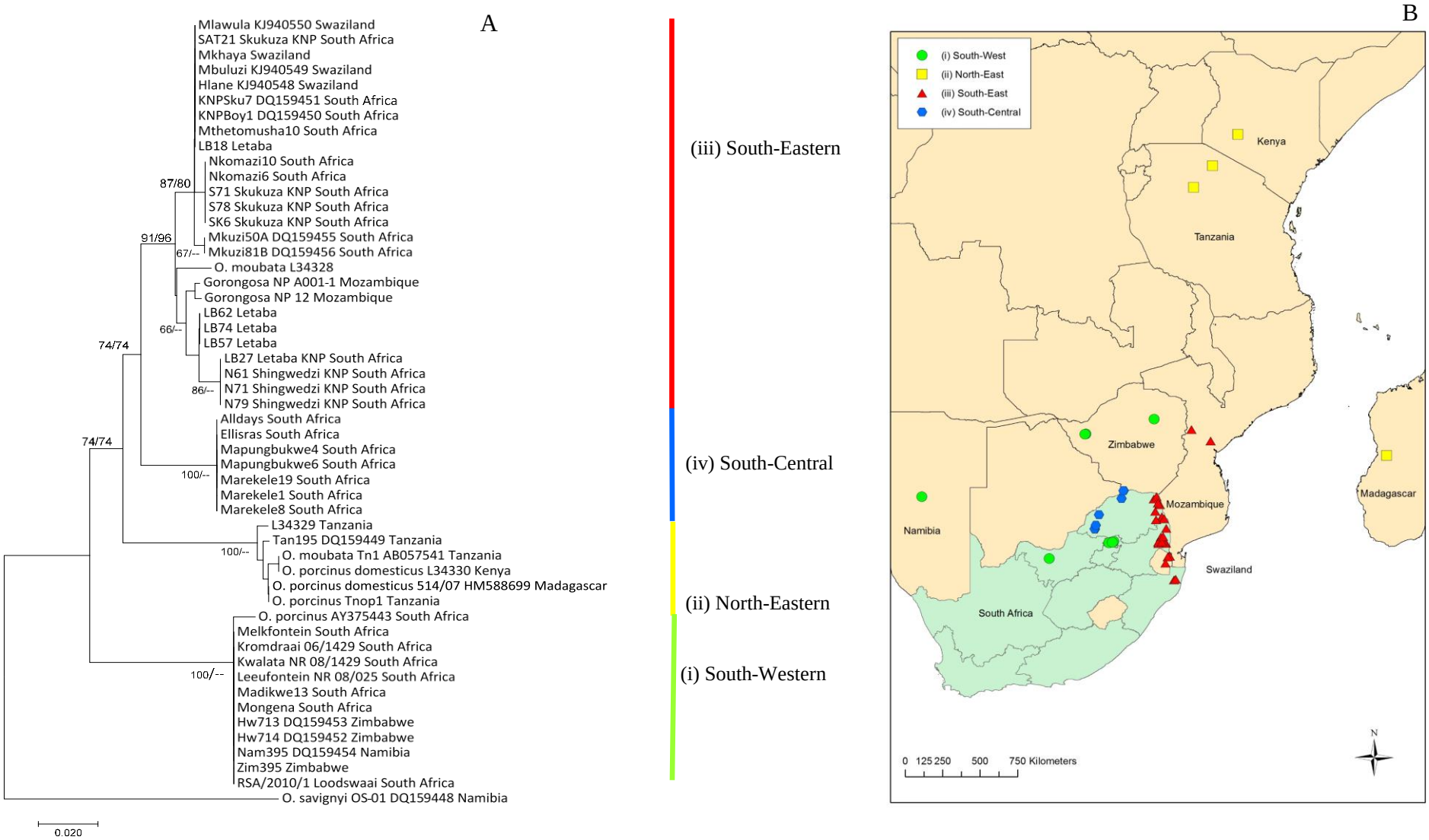

Figure 4.1: (A) Neighbor joining tree of the 16S rRNA tick host gene, indicating the four regional clades (i – iv) identified. Percentage bootstrap support values >60% are those obtained following 10,000 replications for NJ and Maximum likelihood (ML) estimates of posterior probability are expressed as percentages. Values are indicated as NJ/ML next to the relevant nodes. **(B) Map depicting the origin of the tick samples used in this study**, indicating the identified clade distribution; i South-west clade (●); ii North-east clade (■); South-east clade (▲); south-central clade (')

4.3.2 Phylogeography of KNP data set

The 16S rRNA data set of *Ornithodoros porcinus* ticks sampled from KNP used for haplotype network analyses incorporated 60 specimens. The data generated for ticks, randomly selected from infested warthog burrows geographically distributed across the park confirmed the presence of four unique haplotypes (Op1-4, Figure 4.2B) and low levels of nucleotide diversity (π = 10.8791). The phylogenetic analyses were consistent with the haplotype network construction, recovering the same four KNP clusters. The largest haplotype, Op2, comprised of 31 individuals sampled from diverse localities from southern KNP up to northern KNP and encompassing approximately 70% of the longitudinal span of this game park. In contrast, haplotype Op1 was the most geographically restricted haplotype being limited to southern KNP below the Sabie river (Figure 4.2C). No discernible association was observed between the distribution of the four haplotypes and the 35 zonated landscapes (Figure 4.2).

4.4 Discussion

Warthog-associated *Ornithodoros* soft ticks have a distribution encompassing large parts of South Africa, including the Limpopo, Mpumalanga, and North-West Provinces and the distribution of ticks is linked to the distribution of warthogs in South Africa. Our phylogenetic analyses of the 16S rRNA gene of ticks representing the different provinces and game parks, recovered three distinct geographical *O. porcinus* clades in South Africa, with representatives clustering within the previously described South-East and South-West clades (Bastos et al., 2009), and within a newly identified south-central clade. The recovery of an additional evolutionary lineage and the presence of three of the four clades in South and southern Africa underscores the high levels of variation in both the vector and the virus in this sub-region.

Two *O. porcinus* subspecies, *Ornithodoros porcinus domesticus* (associated with human dwellings) and *Ornithodoros porcinus porcinus* (a warthog burrow-dwelling tick) were described based on morphological and biological differences (Walton 1962, 1964) with *O. porcinus porcinus* being considered the reservoir of ASF. The supplementary sample inclusive of 16S rRNA gene phylogeny from this study, support the findings of Bastos et al. (2009) for the recognition of three geographically distinct *O. porcinus* clades, with no support for the recognition of two distinct subspecies within *O. porcinus.*

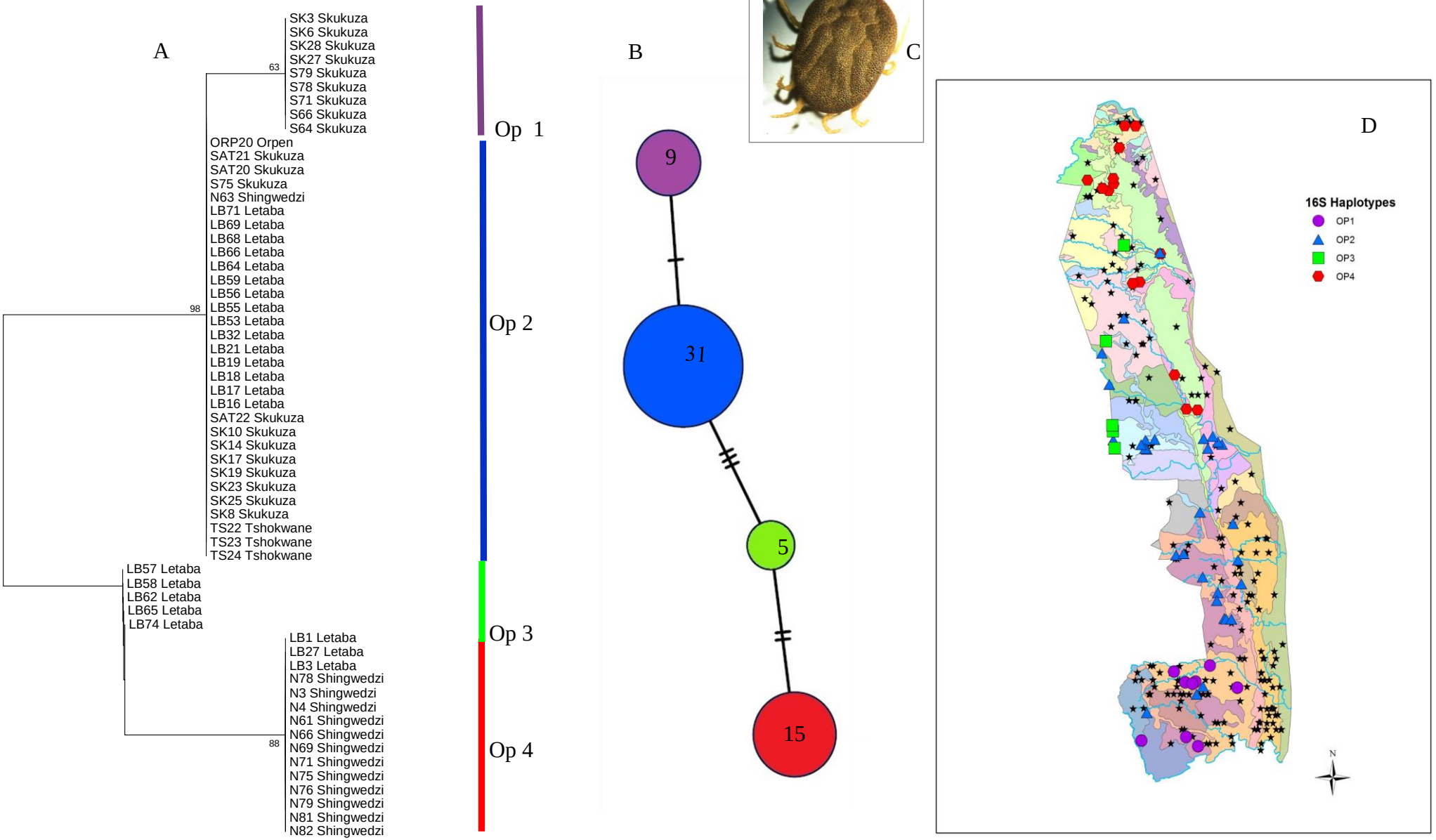

Figure 4.2: Results of phylogenetic and phylogeographic analysis of *Ornithodoros porcinus* originating from Kruger National Park (A) Neighbor-joining tree of the 16S rRNA tick host gene, indicating the four geographically distinct haplotypes (OP1-4). (B) Median-joining network of *Ornithodoros porcinus* 16S haplotypes. Colours denote the major clades as identified with size proportional to their frequencies. Hash marks indicate mutational differences among haplotypes. (C) *Ornithodoros porcinus* (D) Geographical location of the sampled populations and their haplotype composition, indicating the 35 geographical zones together with warthog distribution (*)

At the game park level, evidence was found for four geographically discrete 16S rRNA haplotypes in the Kruger National Park. Clade Op1 – Op4 formed a monophyletic group ranging from most northern to most southern regions of KNP. It is clear from the map that the geographical range of these haplotypes overlap in areas. Haplotype Op2 is most widespread and occurs across more than 70% of the longitudinal sampling grid in KNP. The geographic distribution of three haplotypes (Op 2, 3, 4) does not coincide with current positions of the major river systems. For instance, Op2 is found on both sides of Sabie and Olifants Rivers, which are major perennial rivers in KNP. However, Op1 was only found south of the Sabie River, indicating that for this haplotype that there is some degree of geographical clustering / restriction. No specific zonal correlations could be found to explain either the discrete or the overlapping distribution of the tick haplotypes in this game park.

Kruger National Park, within the nearly 20,000 km^2, was divided into significant landscape zones for the purpose of conservation management and functionality of the ecosystem based on climate, geology, soils, vegetation and animal life (Gertenbach, 1983). Warthog distribution patterns and their association with habitat types is directly linked to tick distribution. Warthogs do not exhibit territorial behaviour and did not appear to favour any habitat type (Chirima et al., 2012). Different groups of warthogs have extensive overlap of home ranges with warthogs sharing resting, feeding, drinking, and wallowing sites. Warthogs occurred over most of the KNP as indicated by the warthog census data from 2011, although as with other mammals were more densely distributed in the southern part of Kruger than the drier northern part (Figure 4.2). Occasionally warthogs shift their home ranges in response to seasonal water shortages but it was found that they were restricted more narrowly to the vicinity of rivers in the northern half of KNP around 1960 (Pienaar, 1963) than was evident after 1980 (Chirima et al., 2012). Warthogs showed a slight shrinkage of their distribution and became less common in mopane-shrubveld and mountain bushveld zones in the northern part of KNP, and more concentrated populations in the southern half of the KNP (Chirima et al., 2012), indicting a shift in movement of warthogs to the south. This recently observed shift may explain the observed overlap in three of the 16S rRNA haplotypes in the central to northern region of the park.

Virus replication in ticks has been shown too influenced by the geographical origin of tick batches (Plowright et al., 1969), yet comprehensive assessment of host tick diversity is lacking. The recovery of three major evolutionary clades in South Africa and four haplotypes in a single game park, the Kruger National Park, is thus of relevance and may underlie the previously

reported differential virus replication, ultimately impacting spread of ASFV. The results further suggest historical barriers to gene flow between northern and southern Kruger National Park, and that tick variation is largely underestimated at local and regional scales. In conclusion, this study represents the first investigation of the population dynamics of *Ornithodoros* ticks in South Africa. Further investigations into the distribution patterns of the different clades are needed in order to understand historical and contemporary drivers the geographical ranges of the ticks and how this influences spread of ASF virus in South and southern Africa.

Acknowledgments

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Chapter 5

Evaluation of two enrichment strategies for genetic characterisation of the full-length genomes of ten African swine fever virus genotypes

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Abstract

Nucleic acid sequencing is used in diagnostic and research virology laboratories to genetically characterise disease-causing agents. The development of second generation sequencing technologies has resulted in the rapid production of large volumes of sequence data at relatively low cost, thereby substantially increasing the quantity of data available for phylogenetic, evolutionary and pathogenicity studies. Despite technological advances, assembling longer sequences, such as that of entire genomes, has not been straightforward. Recent advances in next generations sequencing (NGS) technologies allow rapid sequencing of virus genomes and different NGS platforms are available for sequencing of large genomes. Viral nucleic acid enrichments plays an important role in protocol design as viral preparations are usually contaminated with host DNA, which needs to either be eliminated or reduced. Challenges remain with choosing a suitable platform, enrichment of nucleic acid and analysis of large amounts of data generated to ultimately ensure sequencing success. Long range PCR and polymerase rolling circle amplification (RCA) were evaluated as nucleic acid enrichments / amplification methods, to identify the best approach to achieve comprehensive representation of viral sequences. To determine suitable methods to use for genome sequencing of African swine fever virus (ASFV), a genotype I field strain (Zaire) as well as genotype XIX tick strain (Alldays) from South Africa was selected to compare the two different enrichment protocols. Furthermore the long range PCR approach was evaluated across ten genotypes to test the suitability of this enrichment method across different genotypes. The results thus indicate that RCA is the superior enrichment approach for ASFV genome characterisation using NGS.

5.1 Introduction

During the past two decades, methods to generate nucleotide sequence data have vastly improved. In the 1970's a primer extension sequencing method based on the dideoxynucleotide chain-termination method was developed by Fred Sanger (referred to as Sanger sequencing) (Sanger et al., 1977), and this approach still forms the basis of sequencing methods in use today. During the late 1990's several newer methods were developed to improve the throughput by using fluorescent dyes and capillary separation methods. These methods, which produced longer sequence reads made it possible to assemble 10 kb of genomic DNA with ease. A different approach to sequencing was established at the start of the 21st century, which saw a shift to parallel sequencing of shorter reads and which we refer to as Next Generation Sequencing (NGS). This high-throughput sequencing capacity makes it possible to generate larger genome sequences (≥ 10 kb) rapidly and at a low cost (Radford et al., 2012). Nucleic acid sequencing is now used on an almost daily basis in research and diagnostic virology laboratories, as the data generated can be used to compare viral strains and identify genetic variants that can be used to evaluate evolution and pathogenicity and for fine scale molecular epidemiological studies.

The starting material for Sanger and NGS is double-stranded DNA (dsDNA). In the Sanger method the workflow starts with nucleic acid extractions, followed by PCR and/or cloning, following which a dsDNA fragment of defined size is characterised. As some viruses have RNA genomes, a cDNA synthesis step is added additionally prior to PCR. In NGS, template DNA is extracted / prepared and used for library preparation. The purity and concentration of the DNA is of utmost importance to ensure good results. The quality of input material strongly influences the success of downstream NGS results as well. Due to the fact that viruses are obligate intracellular organisms and propagation occurs in cell-cultures, viral preparations are often contaminated by host nucleic acid. In clinical samples, the ratio of viral genome copies to host genome copies is very low (Depledge et al., 2012). Direct sequencing from clinical samples that contain a mixture of viral and host nucleic acid can lead to difficulties in mapping of the viral genome due to overrepresentation of host DNA sequenced. Purification is therefore of utmost importance to eliminate or reduce contamination with host nucleic acid. Current methods for preparation of DNA of sufficiently high quantity and quality for NGS of ASFV virus genomes are hampered by the imbalance between host and target DNA. In the process of NGS of ASFV genomes, it is important that prior to sequencing, viral enrichment methods such as polymerase chain reaction (PCR) amplification (Höper et al., 2011) or rolling circle

amplification (RCA) are considered (Radford et al., 2012) to ensure adequate amounts of viral DNA sequences.

Traditional PCR reactions have a product length limit, with most thermostable polymerases having generally only allowing for amplification fragments up to 5 kb in size (Barnes, 1992). Modification of the polymerase used during traditional PCR led to development of long range PCRs increasing the size of the amplicons from 3-5 kb to over 30 kb, and making genomic sequencing specific targets, enriched by PCR, a real possibility. These improvements have made it possible to use PCR for genomic mapping and sequencing. The strategy involves the design of primers across conserved regions of the genome, amplifying approximately 10 kb amplicons that span across the entire genome, and that have terminal overlaps to facilitate the generation of contigs. Long range PCR methods not only generate sufficient quantities of the target viral nucleic acids but also eliminate host genome contaminants. This method however is primer dependent and relies on the availability of complete genome sequence from public domain databases to be able to design primers across conserved regions of the aligned sequences that will allow for amplification of diverse isolates.

Alternative methods that do not require prior sequence knowledge of the virus can also be used for random amplification of the viral genome. Nucleic acid amplification can be performed with a polymerase rolling circle amplification (RCA). This method utilizes the bacteriophage phi29 DNA polymerase enzyme to exponentially amplify DNA *in vitro* approximately 10,000-fold (Lizardi et al., 1998). This is due to the ability of phi29 DNA polymerase to incorporate more than 70,000 nucleotides during a single binding event.

African swine fever virus is a large DNA virus, ranging from 170 – 190 kbp in length, depending on the isolate (Dixon et al., 2013). This length variation is primarily due to insertions and deletions in the terminal regions of the genome, referred to as the left variable region (LVR) and right variable region (RVR; De Villiers et al., 2010). Due to the large size of ASFV and the variation that occurs in the terminal regions, sequencing of the complete genome across genotypes is challenging. At present, 15 complete ASFV genome sequences are available, representing seven (de Villiers et al., 2010; Bishop et al., 2015; Granberg et al., 2016) out of the 23 genotypes identified to date, based on partial *p72* gene sequencing (Bastos et al., 2004; Lubisi et al., 2005; Boshoff et al., 2007; Achtenberg et al., 2016) (Table 5.1).

Table 5.1: Table indicating current available full-length ASFV genome sequences

Isolate and Genbank accession number	Country	Host	Genotype	Reference
BA71V U18466	Spain	-	I	Yanez et al., 1995
Benin 97/1 AM712239	Benin	Domestic pig	I	Chapman et al., 2008
E75* FN557520	Spain	Domestic pig	I	De Villiers et al., 2010
OURT88/3 AM12240	Portugal	Tick	I	Boinas et al., 2004
Georgia 2007/1* FR682468	Georgia	Domestic pig	II	Chapman et al., 2011
Kenya 1950 AY261360	Kenya	Domestic pig	IX	Zsak et al., 2005
Ken05/Tk1** KM111294	Kenya	Tick	X	Bishop et al., 2015
Ken06.Bus** KM111295	Kenya	Domestic pig	IX	Bishop et al., 2015
Malawi AY61361	Malawi	Tick	VIII	Haresnape et al., 1988
Mkuzi 1979 AY261362	Zululand	Tick	VII	Zsak et al., 2005
Pretoriuskop AY261363	South Africa	Tick	I	Zsak et al., 2005
Tengani 62 AY261364	Malawi	Domestic Pig	I	Pan, 1992
Warmbaths AY261365	South Africa	Tick	I	Zsak et al., 2005
Warthog AY261366	Namibia	Warthog	IV	Zsak et al., 2005
47/Ss/08** KX354450	Sardinia	Domestic pig	I	Granberg et al., 2016

* Sequenced by 454 Life Sciences pyrosequencing platform

** Sequenced by Illumina platform

The first complete ASFV genome sequence was generated from a vero cell culture adapted strain (BA71V) using conventional Sanger sequencing of plasmid clones prepared using a shotgun approach (Yáñez et al., 1995). Subsequently, two more genotype I whole genome sequences of a tick isolate from Portugal (OURT88/3) and a domestic pig virus from Benin (Benin97/1) were reported (Chapman et al. 2008). The first ASFV genome determined by using next generation pyrosequencing was a virulent ASF Spanish isolate (E75), which like BA71V was also a genotype I virus. The sequence was generated by a commercial company with a 454 Life Sciences sequencer (de Villiers et al., 2010). Recently two viruses representative of genotypes IX (Ken06.Bus) and X (Ken05/Tk1) from Kenya (Bishop et al., 2015), as well as a genotype I isolate (47/Ss/08) from Sardinia (Granberg et al., 2016) were sequenced using an Illumina platform (Table 5.1). With the advances made in next generations sequencing technologies, which permit larger amount of data to be generated at a faster throughput rate, the opportunity to sequence complete genomes of ASFV isolates across all genotypes is nearing realisation. The generation of genome data for genotypes presently not represented in public databases will ensure that data are available to perform comparative genomic analyses. These data will enhance our understanding of evolutionary relationships between isolates, contribute

to knowledge on the origin and spread of viruses as well provide insights into complex host-pathogen interactions at the genetic level between. The aim of this study was therefore to investigate two different approaches to prepare target viral template for NGS and secondly to compare different sequencing platforms for their suitability for generating complete genomes across different ASFV genotypes.

5.2 Materials and Methods

5.2.1 Virus isolates

Ten virus isolates representing nine genotypes, including genotype I, four genotypes described from South Africa (III, VII, XIX, XXI, XXII) and four genotypes from central and eastern Africa (XV, IX, XII, II), were selected for this study (Table 5.2). The virus isolates were randomly selected from viruses available at the Onderstepoort Veterinary Institute - Transboundary Animal Disease Programme (OVI-TADP). All isolates were cultured on Primary Pig Bone Marrow (PPBM) cultures. A volume of 200 µl was used to perform DNA extractions using the High Pure PCR template preparation kit (Roche) according to the manufacturer's instructions. Extracted DNA was stored at -20 °C until further use.

Table 5.2: Virus isolates included in this study

Isolate name	Country	Year	Host	Genotype
Zaire	Zaire	1977	Pig	I
RSA/2007/1	South Africa	2007	Pig	III
SPEC 260	South Africa	1993	Pig	VII
SPEC 251	South Africa	1992	Pig	XXII
TAN/2008/01	Tanzania	2008	Pig	XV
UGA/2003/1	Uganda	2003	Pig	IX
MZI/92/1	Malawi	1992	Pig	XII
Alldays	South Africa	2014	Tick	XIX
MAL/01/2011	Malawi	2011	Pig	II
RSA/2008/01	South Africa	2008	Pig	XXI

5.2.2 Nucleic acid enrichment methods: Long range polymerase chain reaction

5.2.2.1 Primer Design

A long range PCR protocol was received from Ingenasa, Spain, containing primer sequences designed to amplify Genotype I viruses (Immunology and Applied Genetics, SA (INGENASA), Spain, Table 5.2). Additional primers were designed based on alignment of full length ASFV genomes of five genotypes (I-FN55720; III-AY261363, IV-AY261366, VIII-AY261361, X-AY261360) available from Genbank using CLC main workbench 6 (CLC bio, Qiagen) (OVI-TADP primers; Table 5.2). All primer sequences were referenced against E75

(Genbank FN557520), a genotype I isolate from Spain (Figure 5.1) when estimating the expected target fragment sizes. The strategy for designing of primers was to generate amplicons ranging from 10 – 20 kbp in size, covering the entire ASFV genome, with small terminal overlaps of between 200 and 500 bp (Figure 5.1) between fragments. Different primer combinations were initially assessed in the laboratory, by performing long range PCR's, for amplification across different genotypes to determine the most suitable primer pair combinations (data not shown). From the total of 58 primers (Ingenasa and this study) (Appendix E), 40 primers, resulting in 20 primer combinations (Table 5.2), were selected to test across all 10 genotypes.

5.2.2.2 Reaction mixture and PCR conditions

KOD Extreme Hot start (Novagen) DNA polymerase was used which is an optimized PCR system for amplification of long DNA templates. The amplification reactions were done according to the reaction mixture and cycling conditions of the manufacturer. Thermal cycling condition consisted of an initial denaturation at 94 °C for 2 min, followed by 40 cycles of amplification (denaturation 98 °C, for 10 s; annealing at primer-pair annealing temperature (dictated by the lowest primer Tm) °C for 30 s; extension at 68 °C for 1 min/kbp) and a final extension step at 65 °C for 10 min. The products were separated on a 0.8% agarose gel containing ethidium bromide. Amplicons of the expected size were purified using the Zymoclean™ Large Fragment DNA Recovery Kit (Zymo Research Corporation). All purified long range PCR products were submitted for Sanger sequencing with the relevant forward primer to Inqaba Biotech (Pretoria, South Africa), to confirm amplification of the ASFV genome.

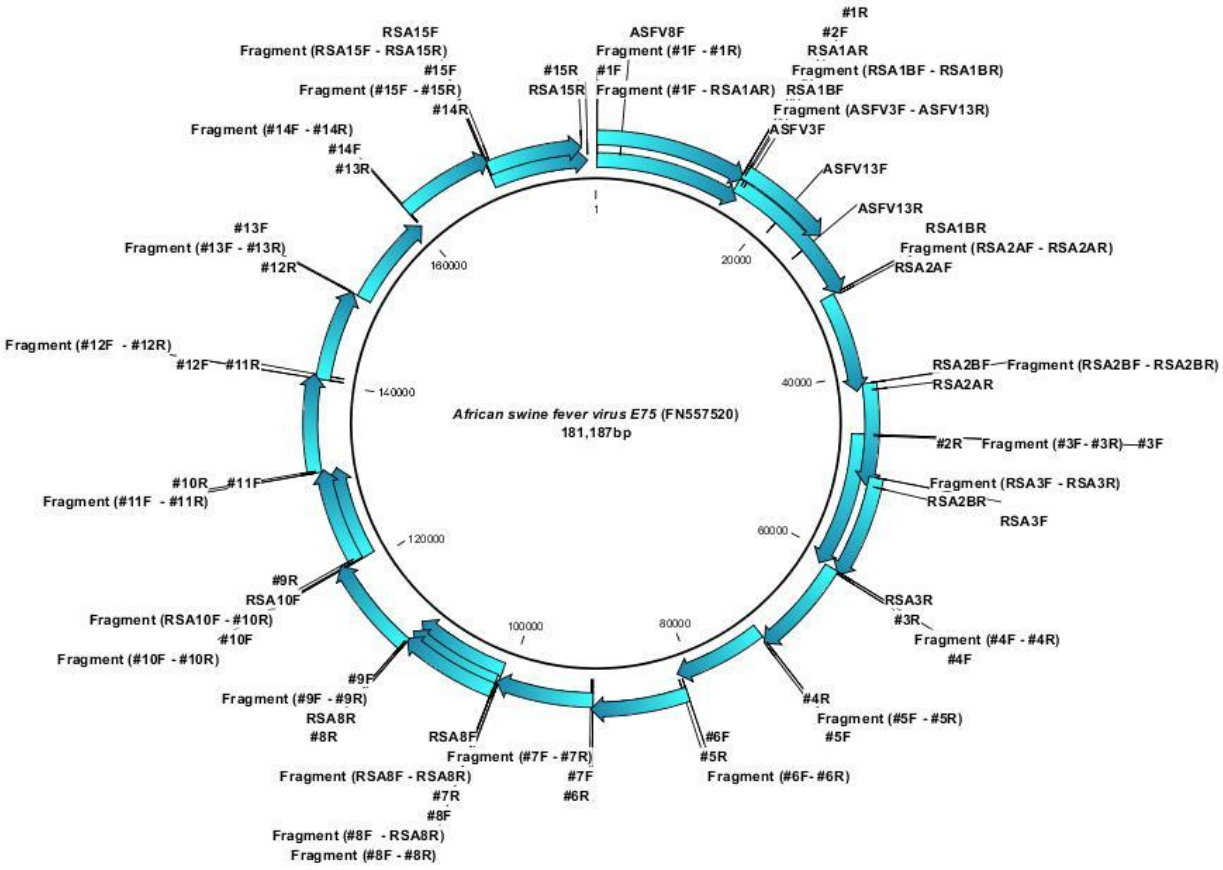

Figure 5.1: Diagram indicating the long range PCR strategy used to amplify the complete genome sequence of ASFV (that for presentation purposes the genome is being represented as being circular). Primer positions in relation to E75 are indicated and fragments of between 10 and 20 kb generated by PCR are indicated in blue arrows.

5.2.2.3 NGS for long range PCR amplicons

To test the suitability of long range PCR amplification approach as an enrichment method prior to NGS, the PCR products generated for the genotype I Zaire isolate, were submitted for MiSeq® (Illumina®) sequencing (OVI-Biotechnology platform).

For the remaining 9 isolates only long range PCR amplicons of primer combinations 9, 10, 11, 12, 15, 16, 17 and 18 (Table 5.2 and 5.3), were selected for downstream processing to test the suitability to obtain large fragments across genotypes. DNA concentrations were determined with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and the different long range PCR amplicons for each individual isolate were combined at equal quantity ratios prior to library preparation, to ensure equal representation of all the long range amplicons. NGS reactions were performed at OVI-Biotechnology platform using the MiSeq® platform.

5.2.2.4 Nucleic acid amplification methods: Rolling circle amplification

To test the suitability of RCA approach for NGS, the DNA extracted from the Alldays (genotype XIX) and Zaire (genotype I) isolates were used for the polymerase rolling circle amplification according to the manufacture's protocol (Illustra GenomiPhi V2 DNA Amplification Kit). A total of 1µl of DNA was mixed with 9 µl of Genomiphi sample buffer, provided with the kit and denatured at 95 °C for 3 min, followed by cooling on ice. After the addition of 9 µl reaction buffer and 1 µl Genomiphi enzyme mix, the mixture was incubated at 30 °C for 2 hours and thereafter inactivated at 70 °C for 10 minutes. RCA amplifications for both isolates were submitted for MiSeq sequencing at OVI-Biotechnology platform.

5.2.3 Bioinformatics

The sequencing data analysis, including quality control and mapping, was performed with CLC genomics workbench version 7 (CLC bio, Qiagen) and Geneious R9 (Biomatters). Reads shorter than 100 bp was discarded and reads were trimmed with low quality bases from 3′ end of the read using threshold value of quality score of $p > 0.05$. The reads were mapped against E75 (Genbank FN557520) as the reference sequence with a length fraction of 0.5 and a similarity fraction of 0.8 with CLC bio, (Qiagen) and Geneious R9 (Biomatters) for comparison purposes. For each isolate, a consensus sequence was extracted (CLC Genomics workbench) from the mapped reads and genome alignment was performed with progressive Mauve alignment (Darling et al., 2004) with Geneious R9. *De novo* assembly was performed on un-mapped reads with resulting contigs compared with published sequences in GenBankTM using the BLAST system (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, Database Resources of the National Centre for Biotechnology Information) to determine the origin of the un-mapped sequences obtained.

5.3 Results

5.3.1 Amplification of the viral genome by long range PCR

Ten virus isolates, representing ten genotypes, were selected to evaluate the long range PCR protocol. Various primer combinations were initially tested, with in 20 primer combinations ultimately being used across all 10 virus isolates selected for characterisation. The amplicons ranged in size from 9 kb – 15 kb (Table 5.3). Variation in amplification success was experienced, ranging from low to high intensity bands as well as non-specific bands (Figure 5.2). None of ten virus isolates amplified across all 20 primer combinations. The amplification

success varied from 6/20 (30% UGA/2003/1 – genotype IX) to 19/20 (95% Zaire – genotype I) across the different primer combinations evaluated (Table 5.4).

Table 5.3: Primer combinations tested on 10 ASF virus isolates in this study

Combination number	Primer combination		Fragment length in bp
	Forward primer	Reverse primer	
1	#1F	#1R	16,061
2	ASFV3F	ASFV13R	9,815
3	RSA1BF	RSA1BR	15,805
4	ASFV13F	ASFV14R	14,478
5	RSA2AF	RSA2AR	10,892
6	RSA2BF	RSA2BR	10,742
7	RSA3F	RSA3R	10,583
8	#3F	#3R	14,897
9	#4F	#4R	10,578
10	#5F	#5R	10,102
11	#6F	#6R	9,962
12	#7F	#7R	10,321
13	#8F	RSA8R	10,086
14	#9F	#9R	10,021
15	RSA10F	#10R	10,210
16	#11F	#11R	10,084
17	#12F	#12R	9,681
18	#13F	#13R	10,254
19	#14F	#14R	9,867
20	RSA15F	RSA15R	10,707

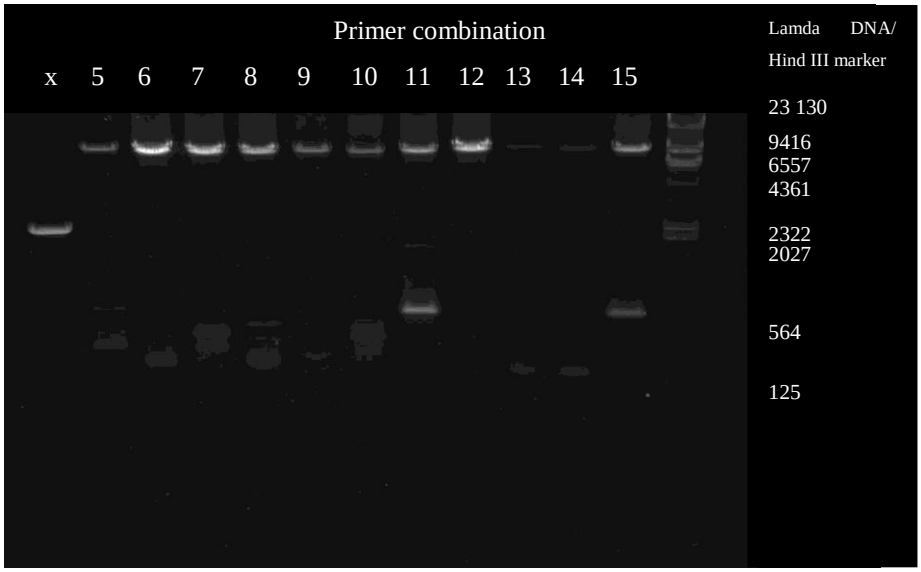

Figure 5.2: Gel electrophoresis of PCR products from the long range PCR amplification of the genotype I Zaire strain. Variation in intensities of amplicons were observed as well as non-specific amplifications (x=primer pair not used in final testing)

Table 5.4: Overview of the PCR results obtained across the 20 primer pairs tested on the 10 virus isolates included in this study.

Primer Combination / Virus Isolate	1	2	3	4	5	6	7	8	9*	10*	11*	12*	13	14	15*	16*	17*	18*	19	20
Zaire#	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RSA/2007/1	-	-	-	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
SPEC 260	-	-	-	-	-	+	-	-	+	+	+	+	+	-	+	+	+	+	-	-
SPEC 251	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	+	-	-
TAN/2008/01	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	-	-
UGA/2003/1	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	-	+	-	-
MZI/92/1	-	-	-	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-	-	-
Alldays	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
MAL/01/2011	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
RSA/2008/01	-	-	+	-	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-

* PCR amplicons used for NGS sequencing
# Zaire all the positive amplicons were submitted for NGS sequencing

5.3.2 Sequencing long range PCR amplicons on MiSeq

For Zaire all the positive PCR amplicons were used for sequencing. Primer pairs 9 – 12 and 15 – 18 (indicated with * in Table 5.4), successfully amplified between 78.8% (7/9) and 100% (9/9) of the remaining nine virus isolates. These fragments were purified, combined and sequenced in a single run on a per-isolate basis. On average, each sample had 3.5 million QC passed reads (range: 1.8 – 5.8). With CLC genomics used as bioinformatics analysis software an average of 34.66% (range: 27.49% – 54.27%) of the reads could be mapped against a reference genome FN557520 (genotype I virus isolate E75), used as a known ASFV genome and 18.46% (range: 6.19% - 26.19%) with Geneious 9.0 (Table 5.5). Mapping performed with Geneious 9.0 resulted in lower percentage reads mapped against the reference strain. Variation in sequencing read depth and coverage was observed for the different isolates (Figure 5.3). Comparison of contigs resulting from *de novo* assembly of the unmapped reads with previously published sequences in GenBankTM using the BLAST system (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) showed the highest identity (up to 98%) with African swine fever viruses. Only a small fraction of the total (<5%) appeared to be attributable to environmental / downstream contaminations.

Table 5.5: Summary statistics of NGS sequencing of long range primers pairs 9 – 12 and 15-18

Virus isolate	Total reads	Reads Mapped		Number of bases mapped
		CLC genomics	Geneious 9.0	
Zaire[#]	7 716 798	1 594 518 (20.66%)	2 021 801 (26.19%)	479 949 918
RSA/2007/1	2 809 718	1 143 997 (40.72%)	499 848 (17.79%)	343 199 100
SPEC 260	1 895 274	899 856 (47.48%)	411 463 (21.71%)	296 956 800
SPEC 251	5 831 454	1 603 349 (27.49%)	361 550 (6.19%)	481 394 716
TAN/2008/01	4 552 440	1 543 958 (33.91%)	967 393 (21.24%)	463 187 400
UGA/2003/1	3 759 625	1 102 763 (29.33%)	860 578 (22.88%)	330 828 900
MZI/92/1	2 840 422	1 265 302 (44.55%)	485 712 (17.09%)	379 590 600
Alldays	3 851 212	1 318 445 (34.23%)	712 474 (18.49%)	395 709 644
MAL/01/2011	4 103 262	2 226 934 (54.27%)	997 092 (14.03%)	668 502 756
RSA/2008/01	2 402 252	913 851 (38.04%)	454 025 (18.89%)	24 155 300

[#] Zaire all the positive PCR amplicons were submitted for NGS sequencing

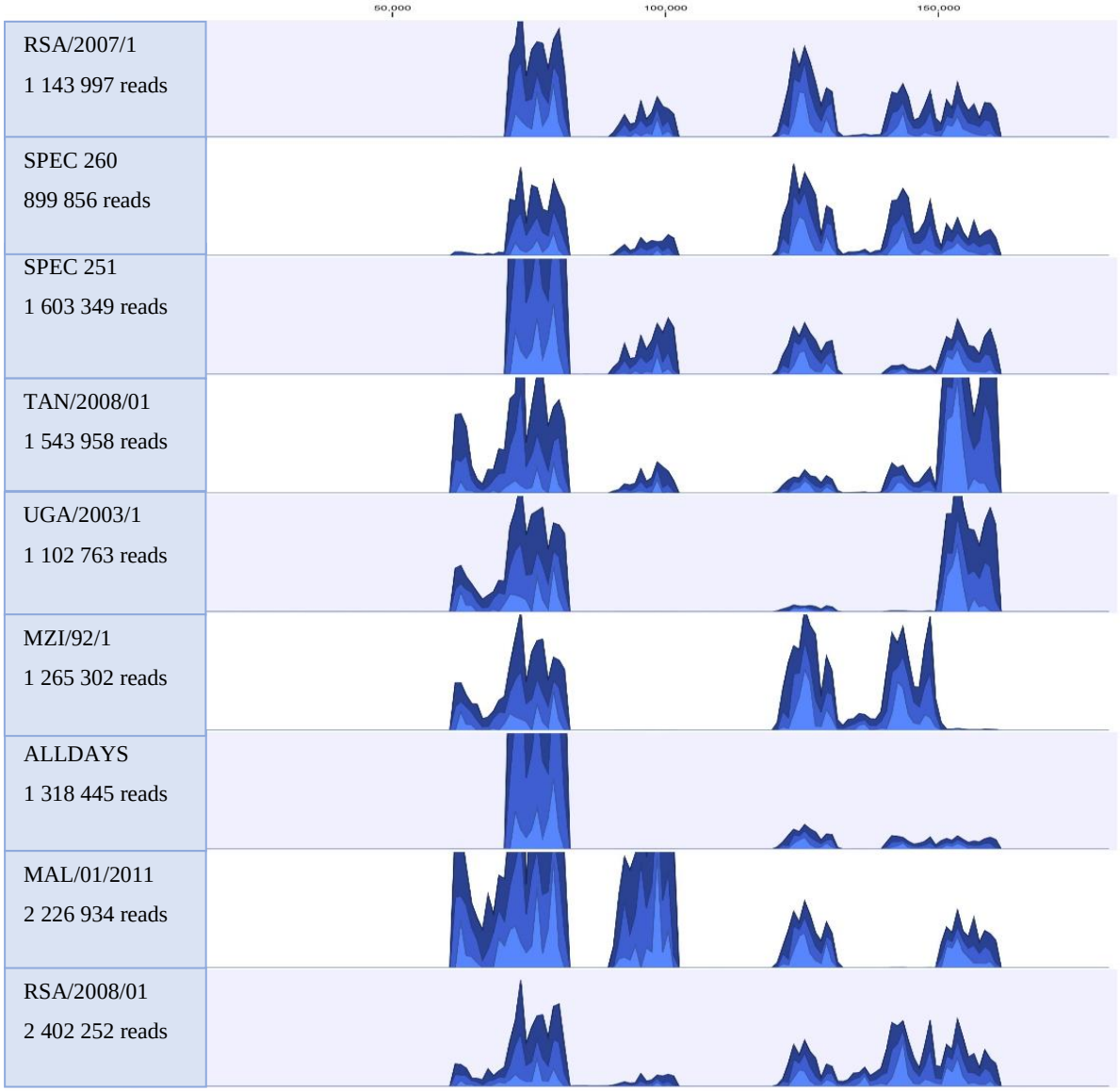

Figure 5.3: Visualization of sequencing read depth for long range PCR amplicons sequenced. The coverage plot demonstrates that significant bias in read depth still occurs between amplicons from long range PCR across and between genomes.

5.3.3 Sequencing RCA products on MiSeq

To determine whether the rolling-circle amplifications method could be used as an amplification method for sequencing of ASFV full genomes, the assay was performed with DNA extracted from cultivated virus isolates. Two viruses representing genotype 1 (Zaire) and South Africa genotype XIX (Alldays) were selected and the same DNA extract used for long range PCR testing (5.3.1) was used to ensure comparability. Reads were mapped against the E75 genotype I ASFV genome (Genbank Accession Number FN557520) which served as the reference genome (Table 5.6) with CLC genomics. The percentage of mapped reads was very low with just 2.2% and 0.29% of the total reads being mapped for Zaire and Alldays, respectively. Despite this, the number of bases mapped were sufficient to obtain coverage across the genome (Figure 5.4).

Table 5.6: Summary statistics of NGS sequencing of RCA products

	Total reads	Mapped	Number of bases mapped	Unmapped
Zaire	2 052 708	45 220 (2.20%)	13 611 220	2 0007 488 (97.80%)
Alldays	1 552 806	4 441 (0.29%)	1 336 741	1 548 365 (99.71%)

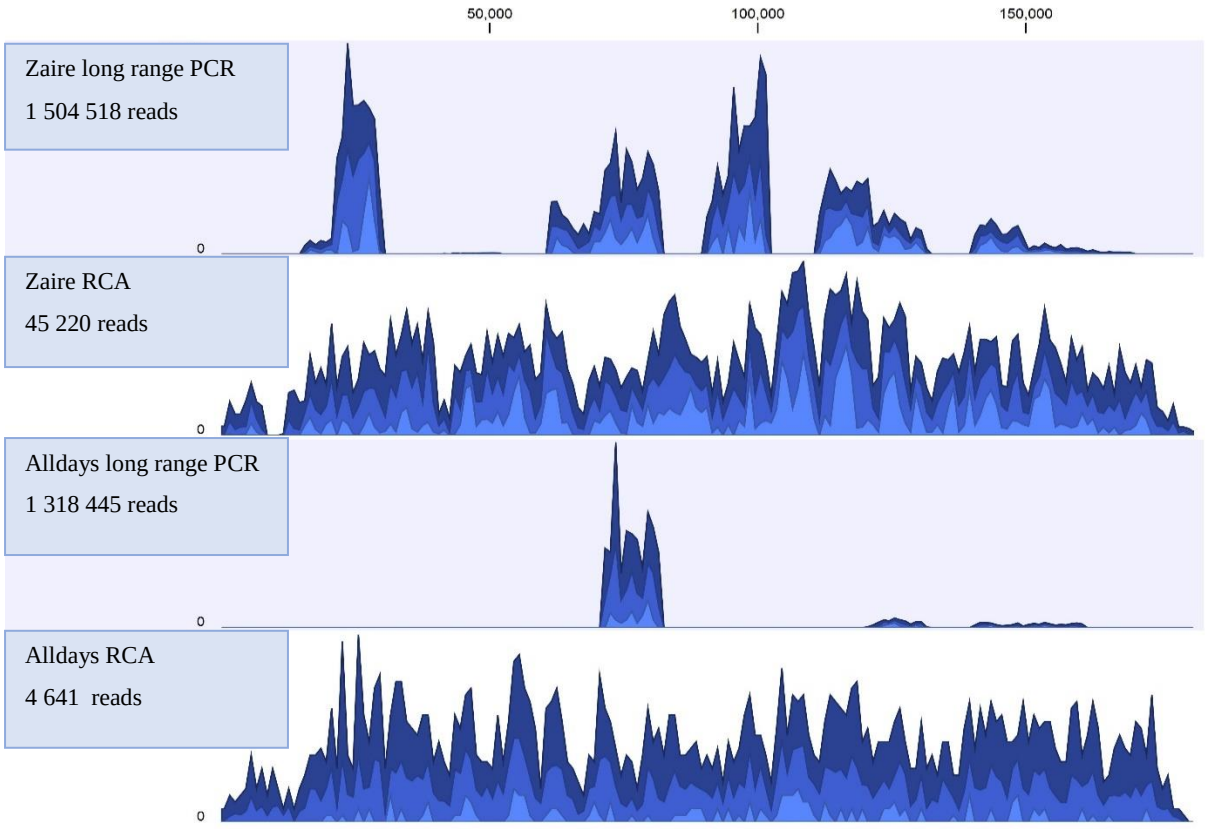

Figure 5.4: Visualization of the sequencing read depth for RCA versus long range PCR, for the Zaire and Alldays viruses.

5.3.4 Mauve alignment

The consensus sequence of each isolate (long range PCR as well as RCA sequences) was aligned with progressive Mauve algorithm aliment tool in Geneious R9 software and included virus isolate E75 (Genbank FN557520). Matches among the genomes are shown in coloured linked boxes (Figure 5.5). Each of these blocks corresponds to a region of the genome sequence that aligned to part of another genome and within each block a similarity profile of the genome is shown. The height of the bars above the sequence reflects the similarity profile and corresponds to the average level of conservation in that region of the genome sequence. For the genotype I Zaire virus long range PCR and RCA amplification methods resulted in similar coverage, however for Alldays coverage across the full genome was higher with RCA (Figure 5.4).

5.4 Discussion

Next generation sequencing has become a powerful tool and is useful for viral genome sequencing. Concerns however relate to low viral load within samples and presence of host genome that can represent up to 99% of the total nucleic acids in a sample (Dupinay et al., 2012). High levels of contaminating host genome sequences can render NGS ineffective for generating complete genome sequences if the viral nucleic acids are low relative to host nucleic acids. It is therefore important to limit / eliminate host nucleic acids and to enrich viral particles of interest. Despite the availability of a broad range of second generation sequencing technologies, sequencing of viral genomes and assembly of longer sequences such as entire genomes have not been straightforward. Several approaches have been developed to concentrate viral nucleic acid concentrations including long range PCR and rolling circle amplifications. Long range PCR was used to prepare specific DNA fragments for sequencing. The method was also used as a targeted enrichment application to ensure high levels of viral nucleic acid prior to NGS. The advantage of long range PCR is that it ensures specificity through amplification of virus genome fragments even in the presence of high levels of contaminating host nucleic acids, as in the case when ASF viruses are cultivated in PBM, or when residual amounts of ASF viral nucleic acids are present in the invertebrate *Ornithodoros* tick host and in clinical samples. This method is, however, constrained by the unavailability of primers that will ensure complete genome amplification and coverage, due to limited genomic information for virus genotypes and the high levels of sequence variation between ASFV genotypes.

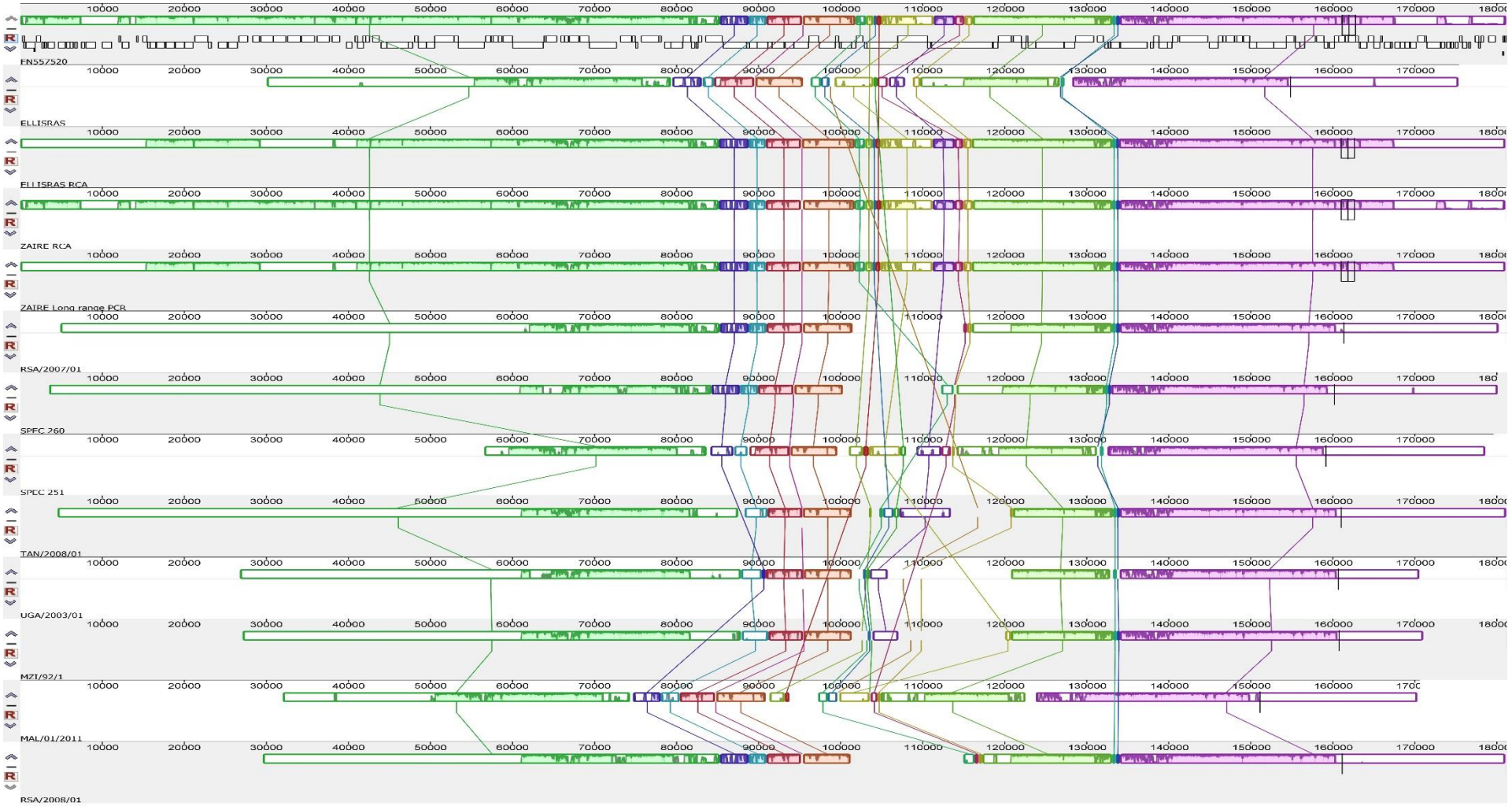

Figure 5.5: Schematic presentation of Mauve alignment, the coloured blocks in the first reference genome E75 (FN557520) are connected by lines to similarly coloured blocks in all the isolates genomes. These lines indicate the corresponding regions across genomes.

Despite extensive efforts in primer design and optimization, consistent amplification across multiple genotypes could not be achieved. In particular, the design of primer combinations to amplify across genotypes in the left and right variable regions of the ASFV genome based on available full genome data was not possible, but greater success was achieved for the conserved regions using reference genome data currently available on the genbank database (www.ncbi.nlm.nih.gov), with the exception of the central variable region of the genome (Figure 5.5) were gaps occurred. For the genotype I virus (Zaire) evaluated in this study, long range PCR and RCA resulted in similar genome sequence coverage results, most likely due to the high genotype I genome sequence representation in sequence databases, which biased the design of primers. The long range PCR approach can be operational only if conserved and specific genomic regions are available across genomes so that primers with a broad recognition range can be designed for amplification. In this study we compared 10 virus isolates across 10 genotypes to determine whether the long range PCR approach is suitable for NGS of ASFV and the results confirmed that only those fragments concentrated within the central conserved region of the genome could be amplified across genotypes. The extent of the underlying genetic diversity is underscored by the finding that no more than 54% of the reads could be mapped to the reference ASF genotype I genome, which seems to be more related to the bioinformatics software inabilities. High diversity in the different genotypes and lack of full genome reference sequences for each genotype, can also lead to problematic and inaccurate mapping. The bacterial contamination found, is a relevant issue often found as problematic and can have an impact on data analysis (Strong et al., 2014). It was concluded that the long range PCR method is only suitable for regions that are highly conserved, and thus cannot be used to achieve whole ASFV genome amplification, as the terminal ends contain large, highly variable regions and reference data are unavailable for the many of the 23 known genotypes.

Rolling circle amplification was performed with phi29 DNA polymerase to achieve isothermal amplification of low amounts of genomic viral DNA. This polymerase has a low error rate (Dupinay et al., 2012), contains a proof reading activity, and holds potential for exponentially amplifying viral genomes from limited starting material. This method produces more DNA than PCR-based methods (Mahmud et al., 2015), and is able to generate the micrograms of DNA required to enable *de novo* assembly of complete viral genomes. Although the proportion of virus genome reads obtained using this approach was lower than that obtained with the long range PCR approach, the 0.29% - 2.2% that was recovered could be mapped against the reference genome, ensuring genome coverage comparable to that of the long range PCR

approach. Although gaps in the full genome were observed, it remains to be confirmed with primer walking and Sanger sequencing, whether these are stretches of missing data, or whether they correspond to actual genome deletions. Due to the better genome coverage achieved with RCA, we propose that this approach should initially be used when attempting to generate representative genome data, across different strains and different genotypes, and that it be combined with primer design and Sanger sequencing to close gaps and/or verify deletions.

The results highlight some of the limitations of generating multi-genotype complete genome datasets from long range PCR amplifications. This method is also very labour and time intensive. In contrast, Rolling Circle Amplification trialled with two of the ten genotypes resulted in greater success in terms of genome coverage. These results indicate that additional investigations across a broad range of genotypes is needed in order to assess the full potential of RCA for generating divergent, multi-genotype comparative genome datasets. In addition to being less cost and time-intensive, the RCA method holds advantages over the PCR-based strategy due to the non-specific nature of the amplification process which ensures more equitable virus genome representation within the sequence data generated. The results thus indicate that RCA is likely the superior enrichment approach for ASFV genome characterisation using NGS.

Acknowledgement

We are grateful to Ingenasa, Spain for providing a long range PCR protocol.

Chapter 6

Experimental transmission of African swine fever between naturally infected sylvatic *Ornithodoros* tick vectors and domestic pigs: Transmission dynamics and genomic variation

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Abstract

In order to investigate transmission dynamics of ASF virus sylvatic cycle, naturally infected ticks sampled from Alldays in Limpopo province, South Africa, infected with a genotype XIX virus were allowed to feed on two healthy domestic pigs (pigs 1 and 2). Once ASF infection was confirmed in these pigs naïve ticks sampled from Dinokeng area in Gauteng Province were allowed to feed on the pigs. One uninfected in-contact pig (pig 3) was housed with the tick-infected pigs to assess direct contact transmission between domestic pigs. Pig 1, infected *via* the tick bite route showed clinical signs of infection, including pyrexia, 6 days post infection (dpi), ultimately leading to death on day 7. In contrast, pigs 2 and 3, exposed to infected ticks and to infected pigs, respectively, both displayed a rise in temperature on day 9, but showed no signs of gross pathology at necropsy (performed on day 10). The timing of the latter two infections suggest that both pigs were infected by horizontal pig-to-pig transmission. Infection with ASFV was confirmed in all three pigs by *p72* amplification and sequencing. Similarly, virus acquisition by the naïve ticks that fed on ASFV positive pigs, confirmed through PCR demonstrated completion of one full tick-pig-tick transmission cycle. Virus evolution was investigated by comparing NGS sequences for viruses sampled at different points in time with the aim of uncovering genetic signatures of virus adaptation to the different hosts. Comparative genomics revealed low levels of diversity between the homologous ~115 kbp genome fragment characterised in this study.

6.1 Introduction

African swine fever virus (ASFV) is maintained in a transmission cycle between suids (wild or domestic) and *Ornithodoros* ticks (Figure 6.1). *Ornithodoros* ticks are the most important maintenance hosts and vectors of ASFV in Africa (Thomson, 1985). African swine fever (ASF) causes high mortality rates in domestic pigs as well as the acute / peracute onset of the disease, thus antibodies against ASFV are rarely detected. Direct contact between domestic pigs and infected warthogs, rarely results in transmission of ASFV (Thomson, 1985). In South Africa warthogs live in burrows which are often infested with *Ornithodoros* ticks, an eyeless, burrow-dwelling argasid tick. Neonate warthogs first become infected with ASFV after being bitten by an infected *Ornithodoros* tick, following which the virus is amplified to high levels in the naïve neonatal warthogs that in turn act as a source of infection for uninfected ticks. These ticks play an important role in maintenance as the virus can persist for long periods in the vector population itself, possibly even indefinitely (Plowright, 1977). The most likely source of infection of domestic pigs in Africa is these ticks (Wilkinson, 1989). ASF virus is primarily transmitted *via* direct contact in domestic pigs through nuzzling and/or ingestion (Sánchez-Vizcaíno et al., 2012) as the virus is present in all physiological secretions including nasal, oral and faecal. ASF is challenging to control due to the fact that the ticks play an important role in the disease dynamics and eradication of the ticks are difficult.

When pigs become infected, the incubation period varies between 5 – 15 days, depending on the viral isolate and the dose. A rise in temperature is usually observed 48 - 72 hours after infection when pigs excrete virus mostly *via* the oral-nasal route (Greig and Plowright, 1970). The most consistently pathogenic effect of ASFV observed in domestic pigs is haemorrhage in multiple organs (Wilkinson, 1989).

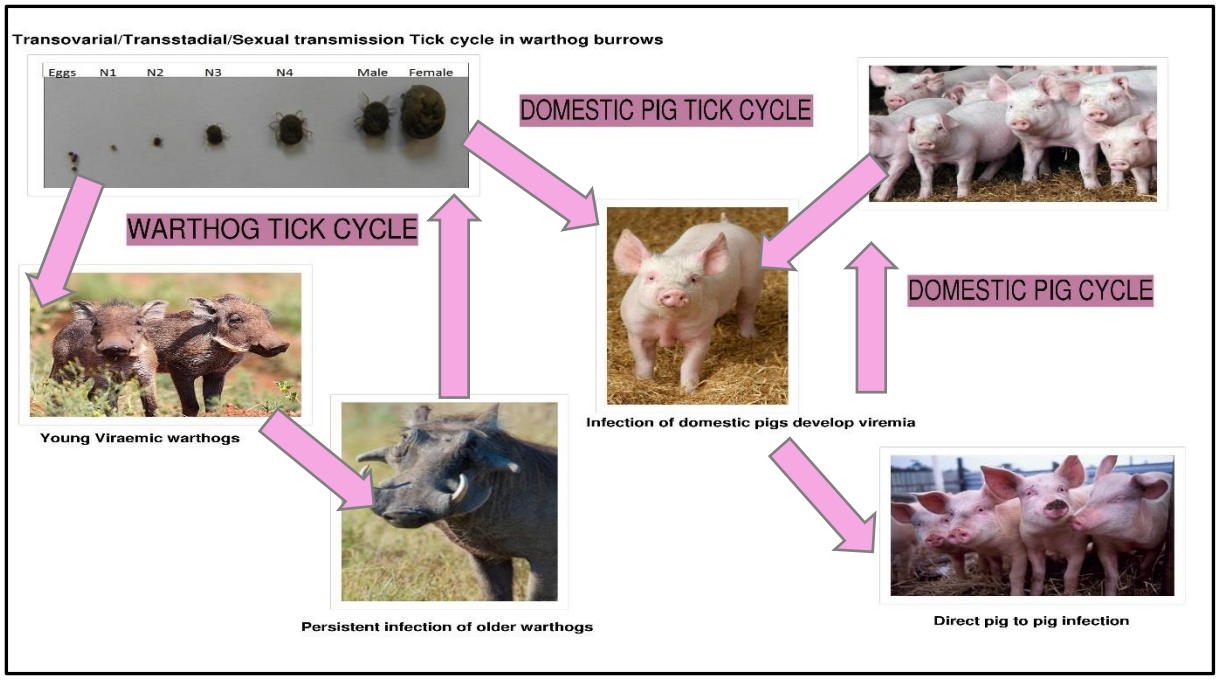

Figure 6.1: The three transmission cycles of African swine fever: (i) warthog-tick (sylvatic cycle), (ii) domestic pig-tick cycle and the (iii) domestic pig cycle

Ornithodoros ticks are rapid feeders and complete their blood meal in under an hour. Adult female ticks ingest on average 0.2 ml of blood per feed, almost 10 times higher than males and nymphs (Thomson, 1985). The minimum dose of virus required to infect ticks ranges from 10^1 to 10^4 HAD_{50} and depends on the tick origin and virus strain (Thomson, 1985). The first tick cells to become infected are phagocytic digestive cells of the mid-gut epithelium where viral replication occurs, with peak numbers of infected cells being present 21 days after ingestion of a blood meal. High levels of virus are found in the mid-gut with smaller amounts in salivary glands (Kleiboeker et al., 1998) and these infections result in a long-term persistent infection with ASFV that probably remain so for life (Plowright, 1977). African swine fever virus transmission to pigs occurs during tick feeding through saliva or coxal fluid secreted during or shortly after feeding. The most obvious route of infection is through saliva secreted by the tick into a feeding lesion in the suid host.

Literature suggests that ASFV infection of *Ornithodoros* ticks represents a well-adapted virus-host biological system (Kleiboeker and Scoles, 2001). This cyclical vertebrate-invertebrate infection-amplification event assists in the long-term maintenance of the virus in natural tick colonies. Many viruses exhibit evolutionary dynamics that facilitate rapid viral adaptation

under different selection pressures such as infecting a new host, infecting different cell types or infecting hosts with different immune responses (Pybus and Rambaut, 2009).

Predictable pathogenesis of ASF has been investigated in pigs as part of various studies *in vivo*, and natural routes of inoculation have been simulated such as intranasal (de Carvalho Ferreira et al., 2012, 2013), intra-oral (Boulanger et al., 1967; Colgrove et al., 1969) combined oro-nasal (Boinas et al., 2004; Hamdy and Dardiri, 1984) and direct contact/aerosol (de Carvalho Ferreira et al., 2013). ASF research *in vivo* has also been done on pigs utilizing intramuscular routes of inoculation with known ASFV isolates (Howey et al., 2013). These studies contribute to our understanding of viral dynamics and the mode of transmission among domestic swine, but arthropod-borne transmission via ticks of the *Ornithodoros* genus, as a relevant means of transmission of ASFV in South Africa has not been investigated. To our knowledge no detailed information is available about the transmission dynamics and virus-host interactions between *Ornithodoros* ticks and domestic pigs *in vivo*.

The ASFV has a linear double stranded DNA genome, ranging in length between 170 and 193 kbp (Chapman et al., 2008; de Villiers et al., 2010). The ASFV genome encodes for between 151 and 167 open reading frames (ORF's) and variations in genome length and gene number are mainly due to insertions or deletions of ORF's from the multigene families (MGF's) encoded by the virus. The gene families encoded are named according to the average number of amino acids in the proteins encoded by each family, the direction they are read in and position in that family from the left genome end (Dixon et al., 2013). The families include MGF100, 110, 300, 360 and 505/530. The virus MGF's are located within the left 40 kbp and right 20 kbp of the genome (Delavega et al., 1994, Yozawa et al., 1994). Isolates collected from Europe and Africa over a 40 year period were compared based on sequences of the B646L ORF, encoding the structural protein p72 and few if any changes in sequence were observed (Dixon et al., 2013). Most variations were due to the gain or loss of genes in the multigene families as well as in the number of tandem repeats, with variation mostly being detected in the 360 and 110 gene families (Dixon et al., 2013; Bishop et al., 2015). MGF 360 and MGF 505/530 seem to have important roles in host range and virulence (Dixon et al., 2013) but little is known with regard to the mechanisms leading to insertions or deletions of the multicopy families. Understanding of evolutionary relationships between isolates at the level of the genome is currently lacking. With the introduction of next-generation sequencing (NGS) technologies the experimental analysis of viral genetic diversity in response to cycling between

markedly different hosts has become possible. However a comparative analysis of full genome sequencing of a tick-derived (genotype X) as well as a pig-derived (genotype IX) virus both from Kenyan origin, indicated comparative similarity in overall sequence (Bishop et al., 2015). However, these strains differed markedly in virulence, with the Genotype IX being associated with a lethal outbreak of ASF in Western Kenya, while genotype X was derived from a tick from central Kenya (Bishop et al., 2015).

The usefulness of NGS for viral diversity studies depends on the quality of the samples and the procedures to prepare the samples (as discussed in chapter 5). The exact changes and adaption the ASFV genome undergoes when it cycles between its invertebrate host and vertebrate host are not known, but are likely to be great. An understanding of these dynamics is therefore relevant for understanding epidemiological dynamics and adaptations.

The aim of this study was two-fold:
To reconstruct a tick-suid-tick transmission cycle under experimental conditions by cycling the virus from naturally infected ticks to a vertebrate host (the domestic pig) and back to naïve uninfected ticks with the aim of:

(i) Recording clinical manifestation of the disease in domestic pigs infected *via* different routes of infection with the same virus strain, and
(ii) Assessing genomic evolution of an ASFV during tick - domestic pig - tick cycling, under experimental conditions.

6.2 Materials and Methods

6.2.1 Animals and housing

Three female Large-White pigs (*Sus scrofa domesticus*), ranging between 21 – 30 kg, were sourced from the Agriculture Research council (ARC)-Animal Production Institute (Irene, Pretoria). Animals were identified individually and housed together in a stable of 36 m^2 in a containment Level 3 facility at ARC-Onderstepoort Veterinary Institute, Transboundary Animal Diseases Programme (OVI-TADP). Animals were kept within the housing facility for seven days prior to the start of the experiment to allow adaptation to the new environment and environmental enrichment was provided. The animals were fed twice a day by the animal caretakers and water was provided at all times.

6.2.2 Sampling and identification of naturally infected Ornithodoros ticks

Ticks were collected from a private game farm in Limpopo Province near the town of Alldays. Warthog burrows were investigated for the presence of ticks (as described in Chapter 2). From the three burrows investigated only one burrow was infested with ticks and all visible ticks were collected. Ticks were pooled and kept alive in screw-capped plastic containers and transported to OVI-TADP under the necessary permits. All adult *Ornithodoros* ticks (N=12) were molecularly assessed for ASFV infection. Due to the small size of the nymphal stages, the limited number of ticks collected (less than 60) and the importance of keeping as many ticks alive as possible for the infection experiment, we did not test nymphal stages for ASFV genome presence. Assessment of ASFV infection was achieved by performing a DNA extraction on a single leg removed from the live adult ticks (Zsak and Kleiboeker, pers comm.), using a commercial kit (Roche High Pure Template Preparation kit). Briefly 100 µl lysis buffer and 20 µl reconstituted proteinase K was added to each tick leg and incubated for 24 hours at 55 °C following the supplier-prescribed DNA extraction method, with final elution in 50 µl of elution buffer. African swine fever virus genome presence was evaluated by amplification of the C-terminal end of the *p72* gene used for genotyping, with previously described primers and thermal cycling conditions (Bastos et al., 2003). The total reaction volume for each PCR was 50 µl and consisted of the following components; 3 µl DNA template, 0.2 mM dNTP, 0.5 µM of each primer and 1 U thermostable Go Taq G2 DNA polymerase (Promega Corp., Madison, WI, USA). PCR products were evaluated by 1.5% agarose gel electrophoresis against a 100 bp ladder (Promega Corp., Madison, WI, USA). In this assay, amplification of a 478 bp band confirms virus presence. The PCR products were purified and submitted to Inqaba Bio Technologies for cycle-sequencing and a Neighbor joining tree containing reference sequences for seven southern African ASF genotypes (Boshoff et al., 2007) was constructed in Mega 5.1 (Tamura et al., 2011).

In this manner naturally infected adult ticks were identified for use in the experimental infection. The nymphal stages were not tested for ASFV genome presence due to the small size of the ticks, and were combined with positive adult ticks as detailed in Table 6.1 for the infection experiment. This was done to increase the probability of viral transmission, as it was found that nymphal infection rates can be almost two-fold higher than that found in adults (Chapter 2).

Forty-two ticks ranging between N1 – N3 and adults were selected for the infection experiment (Table 6.1). These ticks were divided into two groups of 21 ticks each. As partial *p72* gene sequencing recovered identical sequences in adult ticks and as all ticks were obtained from a single burrow, we assumed that the same strain was present in all positive ticks as no evidence of mixed / multiple viral infections was found, on a per burrow basis, in any of the ASFV-positive burrows identified in Chapter 2. Thus, although the genotype of the virus in each field collected adult tick was determined, it was not possible to assess virus titres / load harboured by individual adult ticks, nor was the infection status of the nymphal stages known.

Table 6.1: The two tick pools used for the pig infection experiment

Tick stage	Pig 1	Pig 2
N1	10	10
N2 – N3	5	5
Adults	6 (4 Female and 2 Males)	6 (5 Females and 1 Male)

6.2.3 Naïve Ornithodoros ticks

Ticks were collected from private game reserves in the Dinokeng area, north of Pretoria, Gauteng Province, which forms part of the ASF free zone of South Africa. In total 16 warthog burrows were investigated for the presence of ticks, of which 50% were infested. Ticks were collected and kept alive in screw-capped plastic containers and transported to TADP. The adult ticks were assessed for ASFV infection as described in section 6.2.2. One hundred and eighty ticks, ranging from N1 to adult stages, were divided into three separate groups of 60 each and placed at 25 °C in separate plastic containers filled with a 2 cm layer of fine sifted sand.

6.3 Study design

6.3.1 Experimental infection and direct contact transmission

This experiment was performed to simulate transmission of ASF from the sylvatic cycle to domestic pigs, and once established to allow pig-to-pig transmission thereby permitting comparison of two routes of infection (tick-pig and pig-pig) under experimental conditions. Three ASF sero-negative pigs were used in this study. Two pigs were selected for tick infection, where the adult ASFV positive field ticks as well as nymphal stage ticks were allowed to feed on these pigs to facilitate viral transmission from the positive ticks to each pig. In this way, a challenge model representative of transmission from the sylvatic cycle was established (in which the naturally infected ticks are referred to as 1st stage ticks, Figure 6.2).

An in-contact pig was included to confirm horizontal transmission of the virus from a tick-infected pig to an uninfected pig.

After a seven day acclimation, all pigs were sedated with an intramuscular injection of Azaperione (40 mg/ml; 1-2 ml/20 kg). The field collected ASFV positive adult ticks as well as the nymphal stage ticks were put on the right hip of two pigs and covered and constrained to a 10 cm diameter area by a round plastic container (Figure 6.3) to prevent the ticks from moving around too much on the pigs. Feeding was allowed to proceed until the ticks dropped off or until a maximum feeding time of 60 minutes was reached. The day of tick feeding was assigned as day 0 of the infection experiment. The pigs were subsequently monitored on a daily basis for clinical signs of infection.

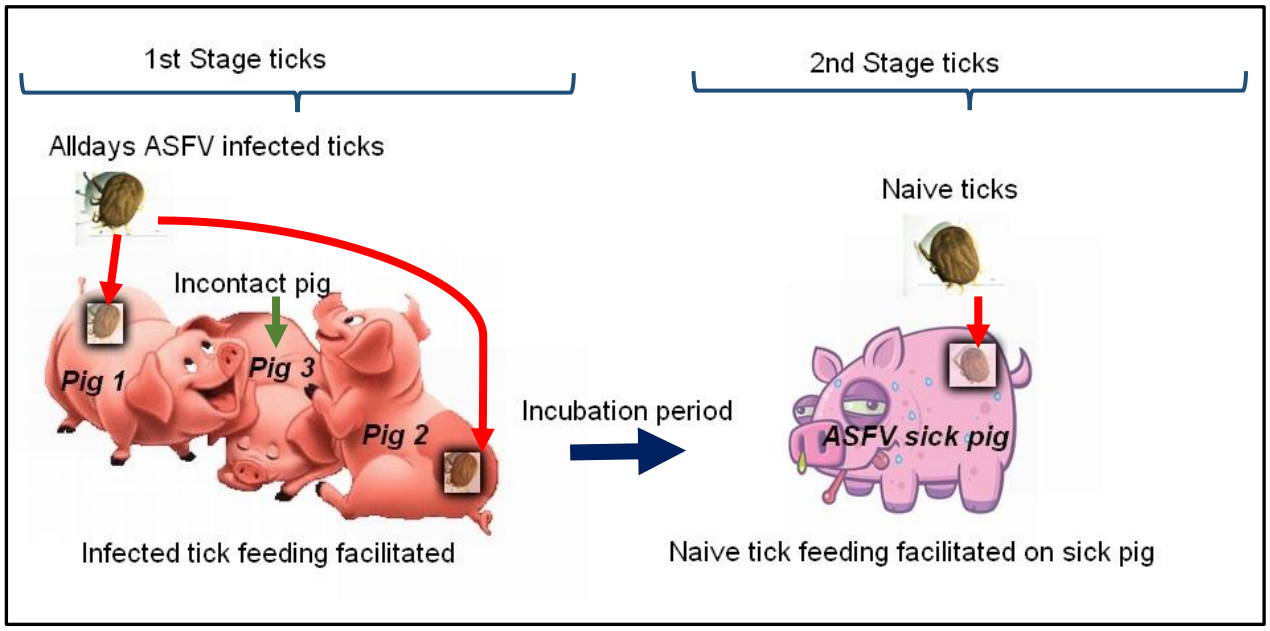

Figure 6.2: Schematic representation of transmission experiment

6.3.2 Tick infection study

Upon detection of pyrexia (rectal temperature greater than 40 °C) heparinised blood was drawn from the exposed pigs, to determine the presence of ASFV *via* C-terminal *p72* gene amplification (Bastos et al., 2003). On confirmation of a positive result, indicating ASFV circulation in the infected pig, tick feeding with 60 naïve ticks per pig was facilitated, as described in section 6.3.1 to enable virus acquisition of ASFV from the positive pig to the naïve ticks (Figure's 6.2 and 6.3) (called 2nd stage ticks for clarity purposes). Fully fed, the ticks were randomly divided into groups and stored in marked plastic containers containing sand for 21, 90 and 365 days post infection (dpi). No artificial tick feeding took place during the

experimental period from 0 dpi to 365 dpi. At the specific day post-infection (21, 90 and 365) the ticks were stored at -70 °C until they were used in the whole genome amplification component of the study.

6.3.3 Post-mortem performed on pigs

Post-mortems were performed on pigs that succumbed to ASF virus infection or euthanasia. The necropsy was performed by a veterinarian as soon as possible after death, and the gross pathology and macroscopic lesions were noted and photographed. Various tissue samples were collected and stored immediately at -70 °C.

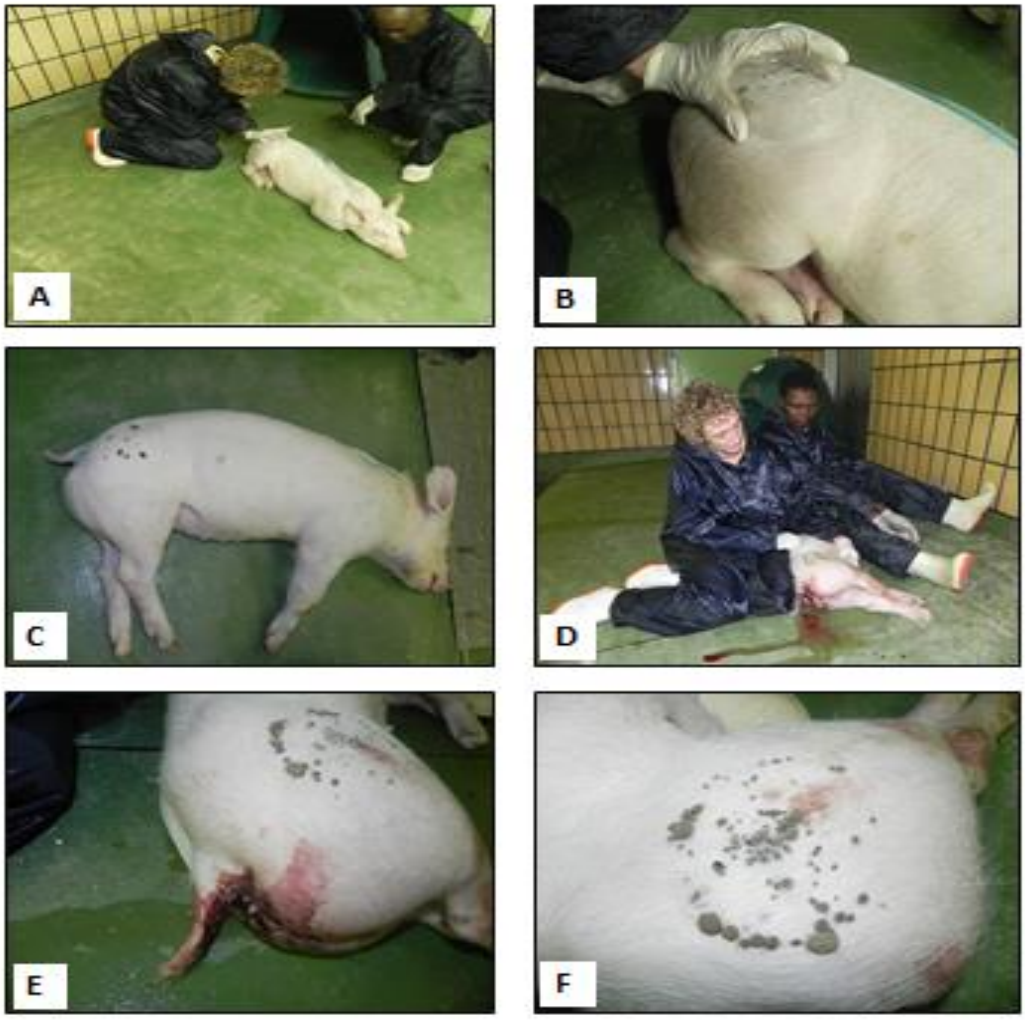

Figure 6.3: A - C: Feeding of naturally infected ticks (1st stage infection) on sedated pigs; D - F: Feeding of naïve ticks (2nd stage infection) on the ASF positive pig

6.3.4 Sample analysis

Samples were analysed by conventional *p72* PCR (Bastos et al., 2003) to detect ASFV genome. Tissue and ticks were homogenized and DNA was extracted with the QIAamp DNA Mini Kit (Qiagen). Bands of the expected 478 bp *p72* genome target size detected in positive organs were excised from the gel and purified. The purified products were submitted for nucleotide sequencing to Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) in order to verify that virus infection in all three pigs was due to the same virus. For antibody detection in serum, a highly sensitive and specific commercial ASFV-ELISA kit based on a competition format was used (INGEZIM ppa compac).

6.3.5 Virus isolation

Following the pig feeding experiment, all naturally-infected adult ASFV PCR-positive (first stage) ticks used in the initial pig infection experiment were immediately stored at -86 °C. All the ticks were pooled and a 10% (W/V) homogenate was prepared with PBS and subjected to viral isolation. Primary swine macrophage cultures prepared in 96 well plates, with slight modification (Malmquist and Hay, 1960) were inoculated together with a commercially available antibiotic-antimycotic (Gibco®) and fetal bovine serum, including only a negative cell culture control. The cells were examined after 24h, 48h and 72h for cytopathogenic effect or haemadsorption.

6.3.6 DNA preparation, whole-genome amplification and sequencing

The infected second stage ticks (N=60 / pig) were pooled into groups of 20 ticks and stored at -70 °C at 21, 90 and 365 dpi. Thus for each pig, three tick groups were stored, with each group contained 20 ticks from various stages. The spleen from Pig 1 was used as the reference material for initial infection. Preparations of pig tissue and second-stage tick pool homogenates were prepared with phosphate-buffered saline (PBS, pH 7.4). Sterile sand was added to 1 g of tissue sample and to each tick pool containing 20 ticks and ground with a pestle. The DNA was extracted from 200 µl of the resulting 10% w/v homogenate using the High Pure PCR template preparation kit (Roche). DNA was extracted directly from all pre- and post-infection (1^{st} and 2^{nd} stage ticks), with virus isolation attempts being limited to the 12 adult, naturally infected 1^{st} stage ticks (section 6.3.5). Virus isolation and passage for 2^{nd} stage ticks was avoided in order to prevent additional genome adaptations. The DNA extracted for each of the tick group samples was non-specifically amplified by following the standard protocol of illustra GenomiPhi V2 DNA amplification kits (Amersham Biosciences; GE Healthcare; Chapter 5).

In each reaction, one µl of purified DNA (concentration 10 ng/µl) served as starting material with a typical 20 µl gDNA yield of 4–7 µg gDNA (~ 250 ng/µl). This method uses whole genome amplification by isothermal strand displacement approach. Nucleotide sequencing of the amplified gDNA was determined by using Illumina sequencing (OVI-Biotechnology platform). The resulting sequence reads were analysed in CLC Bio Genomics Workbench 7. A quality control was performed by using CLC genomics workbench software and a data-analysis pipeline was followed for each individual sample to be able to do inter-sample comparison between the assembled genomes.

6.3.7 Alignment and phylogenetic analysis

A multiple sequence alignment was created using Geneious 9.0.2 software (Biomatters Ltd., Auckland) as well as CLC genomics workbench 7.0 (Qiagen bioinformatics) with published reference strains (Genbank accession numbers: AY261361; KM111295; KM111294; AY261360; AY261362; FN557520; AM712239; ASU18466: AM712240; AY261365; AY261363; AY261366). A neighbour-joining tree was inferred using Geneious 9.0.2 software using a dataset containing 22 sequences, with nodal support being assessed through 1000 bootstrap replications.

6.4 Results

6.4.1 Naturally infected Ornithodoros ticks

Adult ticks collected from the game farm near Alldays, tested positive for ASFV by means of the conventional *p72* genotyping PCR (Bastos et al., 2003; Figure 6.4). A phylogenetic tree which included reference sequences for seven genotypes occurring in South Africa (Boshoff et al., 2007) was constructed, based on partial *p72* sequences in order to determine the genotype and diversity of the viruses present in the collected ticks. A p-distance matrix indicated that based on partial *p72* amplification and sequencing of the 405 bp C-terminal region that the ASF strain present in each of the positive ticks was completely conserved across this gene region. The phylogenetic tree indicated that the tick virus falls within genotype XIX (Figure 6.5). This genotype comprises viruses previously isolated over a large part of Limpopo Province, corresponding to the area from which the ticks were collected. Positive virus isolation was performed on the pooled field ASFV positive tick samples after completion of the transmission experiment (stage 1 ticks) and the virus was named according to OVI-TADP register as RSA/12/15. For simplicity purposes this virus will henceforth be referred to as the Alldays virus, in this thesis, and is also designated as such in Chapter 5.

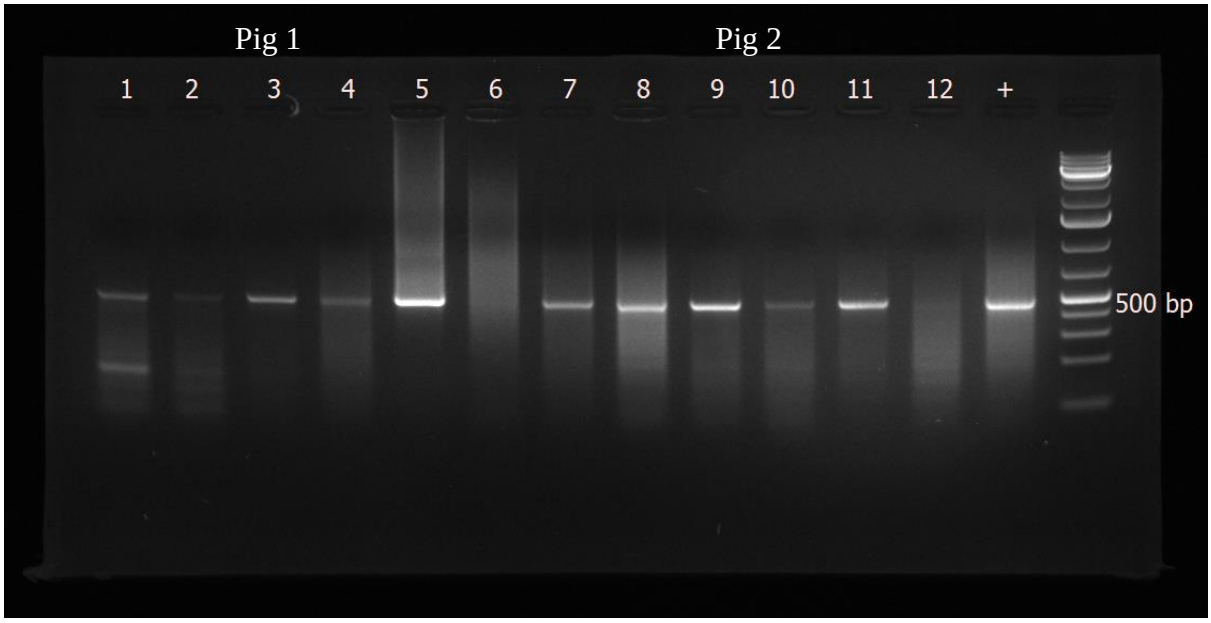

Figure 6.4: Gel electrophoresis of ASFV p72 PCR products from the adult ticks. Indicating the variable levels of p72 gene amplification and confirming that five of the six adult ticks in each of the pools was clearly PCR-positive. Ticks 1 – 6 (lane 1 – 6) were used for infecting Pig 1 and ticks 7 – 12 (lanes 7 -12) for infecting pig 2.

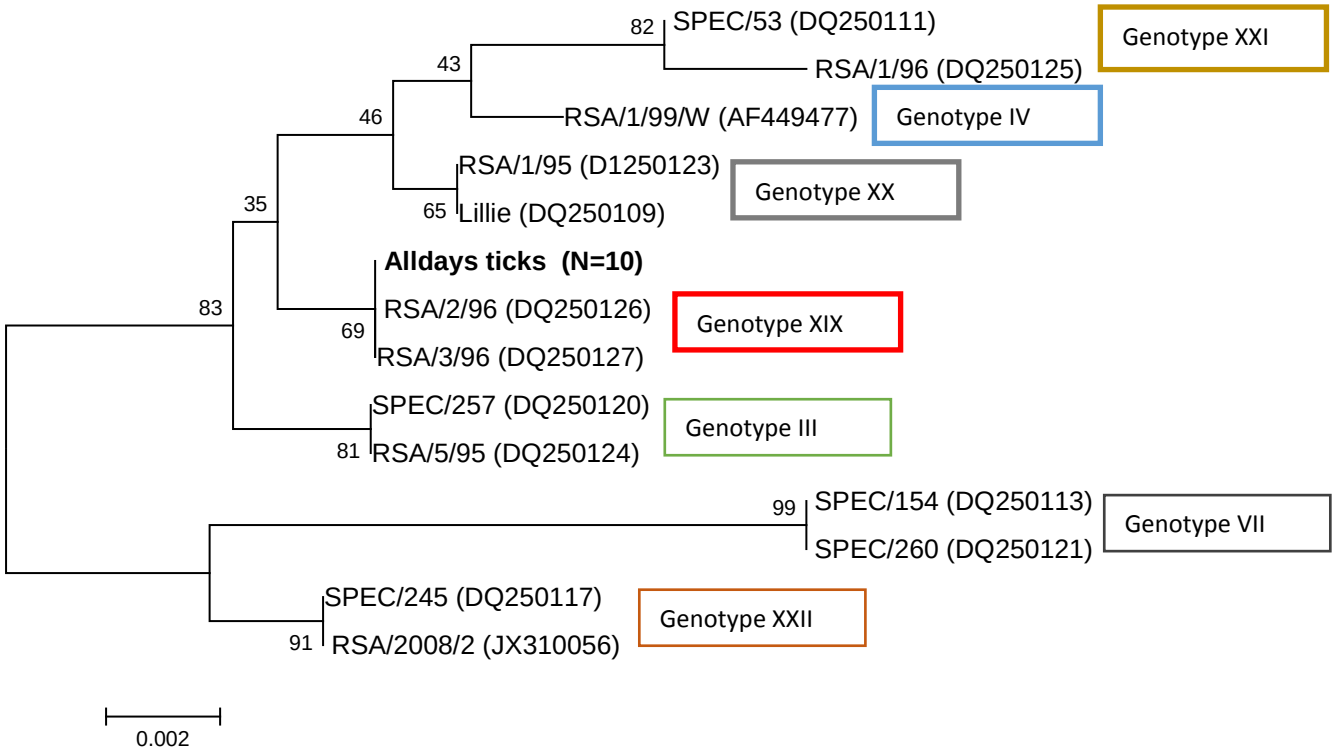

Figure 6.5: Neighbor-joining tree indicating genotypes from South Africa as well as the phylogenetic placement of the virus present in the all 10 ASFV positive field collected ticks that were used as the source of infection for the transmission experiment

6.4.2 Experimental infection and direct contact transmission

In the ASFV infected field tick feeding group, one out of the two pigs (named Pig 1) was successfully infected by tick feeding. Successful infection with ASFV in one pig was determined by the presence of characteristic clinical ASF signs, combined with detection of viremia within five days of tick exposure. A rise in rectal temperature was observed from 4 dpi, and at 5 dpi, the temperature increased above 40 °C (Table 6.2). Listlessness and huddling was seen from Pig 1 and cyanosis on the snout was observed (Figure 6.6). A nasal discharge and blood-stained faeces due to haemorrhagic enteritis was observed with fresh and blackened blood adhering to the tail and perineum (Figure 6.7).

On 6 dpi, the temperature of Pig 1 increased to 40.8 °C, while that of Pigs 2 and 3 remained normal. Viraemia was confirmed through testing DNA extracts prepared from whole blood *via* the ASFV genotyping PCR. Only Pig 1 tested PCR positive while Pig 2 and 3 remained negative for ASFV genome presence. Naïve ticks were allowed to feed on Pig 1 on day 6, just as soon as viremia was confirmed by PCR. This was done to ensure virus transmission occurred prior to the pig succumbing to infection as the survival time from onset of pyrexia to death is short. Pig 1 died 7 dpi and a necropsy was performed.

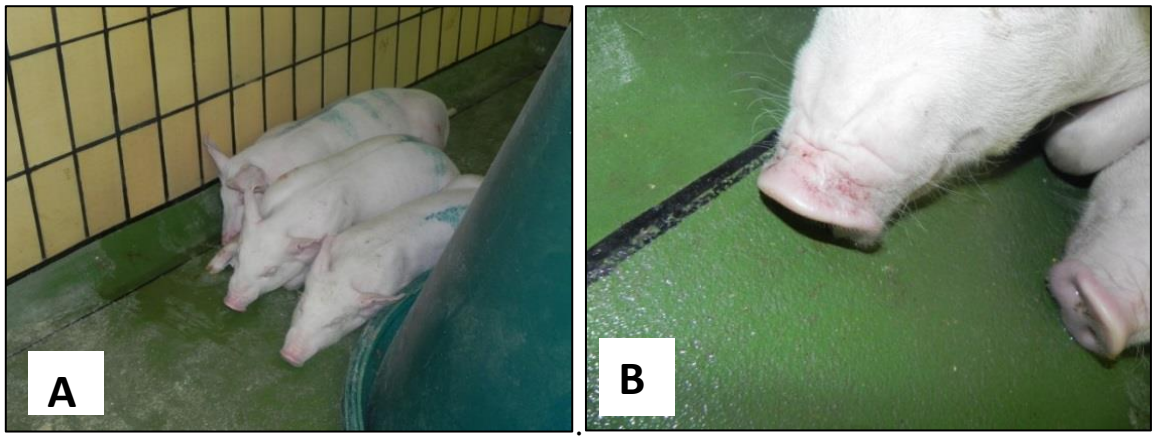

Figure 6.6 A and B: Indicating huddling of pig1 with the other 2 pigs and cyanosis on the snout

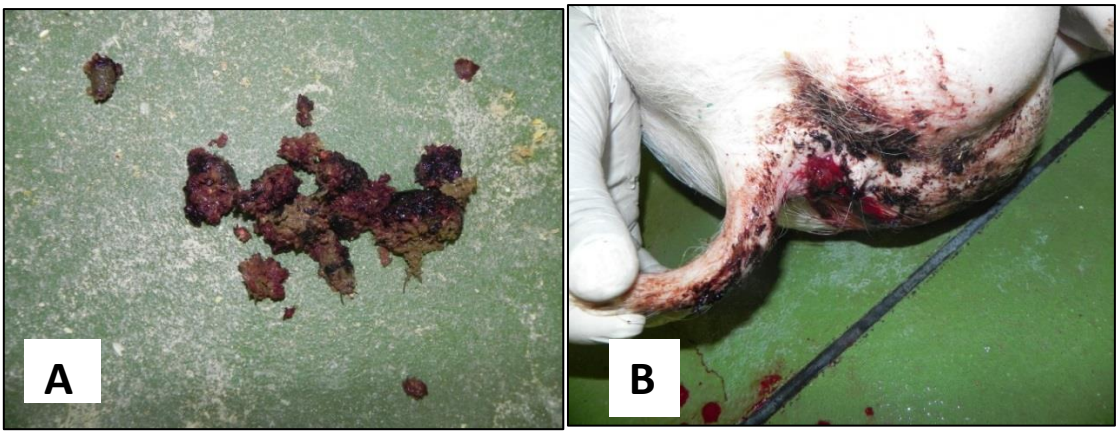

Figure 6.7 A and B: Indicating blood stained faeces from tick infected Pig 1

Although Pig 2 was part of the initial infection experiment in which two pigs were exposed to naturally infected ASFV positive (1st stage) ticks, it appears that initial infection did not take place as was only on 9 dpi that Pig 2 showed an increase in rectal temperature above 40 °C, coinciding with the time point at which the in-contact pig also displayed a similar rise in rectal temperature. Nucleic acid extraction from whole blood samples were tested with the *p72* genotyping PCR and both pigs tested positive for ASFV 9 dpi. At 10 dpi, the temperature of the pigs was 41.3 °C and 40.6 °C (Table 6.2), respectively and naïve ticks were allowed to feed on Pig 2 and 3 to facilitate virus transmission to the naïve (2nd stage) ticks. As pig 1 succumbed shortly after detection of a severe thermal response pigs 2 and 3 were euthanized, on ethical grounds, immediately following completion of tick feeding, and necropsies were performed. All sera collected throughout the experiment tested negative for the presence of ASFV antibodies (Table 6.2).

6.4.3 Pathology

Necropsy was performed on all three pigs. In Pig 1 typical acute ASF gross pathology was observed. External observations included a good overall body condition score, bloody diarrhoea in the perineum as well as multiple, purplish petechiae at the tick feeding sites on the surface of the pig skin, reddening to purplish cyanosis on the surface in the neck and head on the white-skinned pig was also observed (Figure 6.8).

Table 6.2: Summary of changes in rectal temperature (°C). ASFV PCR and ELISA results from 0 dpi until 10 dpi (termination of experiment) for the three pigs used in the study

		0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
PIG 1	Temp	39.3	39.4	39.5	39.7	39.9	40.5	40.8	PIG DIED			
	ASF PCR	NEG	-	-	-	-	POS	POS				
	ASF ELISA	NEG	-	-	-	-	NEG	NEG				
PIG 2	Temp	39.2	39.3	39.4	39.6	39.2	39.5	39.5	39.4	39.6	40.2	41.3
	ASF PCR	NEG	-	-	-	-	NEG	NEG	-	NEG	POS	POS
	ASF ELISA	NEG	-	-	-	-	NEG	NEG	-	NEG	NEG	NEG
PIG 3	Temp	39.3	39.4	39.9	39.5	39.3	39.3	39.8	39.2	39.4	40.0	40.6
	ASF PCR	NEG	-	-	-	-	NEG	NEG	-	NEG	POS *	POS
	ASF ELISA	NEG	-	-	-	-	NEG	NEG	-	NEG	NEG	NEG

POS – positive NEG – negative *Weak positive

Gross pathology on opening of the carcass of Pig 1 revealed accumulation of straw coloured fluid in the peritoneum (Figure 6.9). The left lobe of the lung showed interlobular oedema and the trachea was filled with froth (Figure 6.10 A - D). Gastro-hepatic, mesenteric and renal lymph nodes were swollen and haemorrhagic (Figure 6.11 A&B) giving the appearance of blood clots. The spleen and liver were markedly enlarged with dark red dicolourations (Figure 6.12 A&B). Petechiae and echymotic haemorrhages of the kidney cortex and pelvis were observed (Figure 6.13 A&B) as well as haemorrhages on the ventricles of the heart (Figure 6.14 A). The gastrointestinal tract appeared normal but haemorrhaging was present in the intestinal gastric mucosa (Figure 6.15 B).

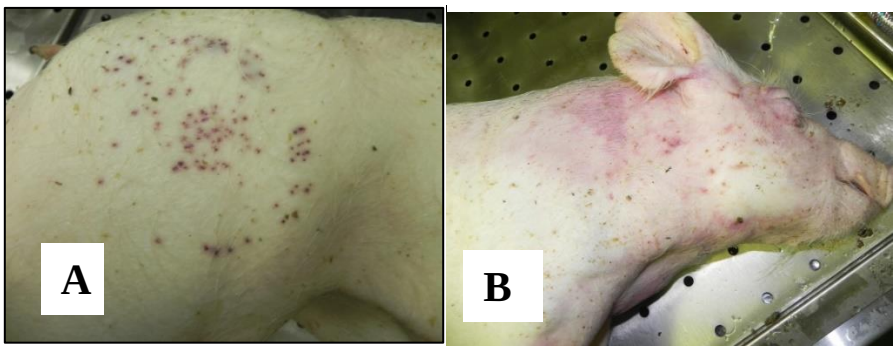

Figure 6.8: Development of lesions on white-skinned Pig 1. A) Lesions at the tick feeding site, and B) Reddening cyanosis of the skin in the head and neck region.

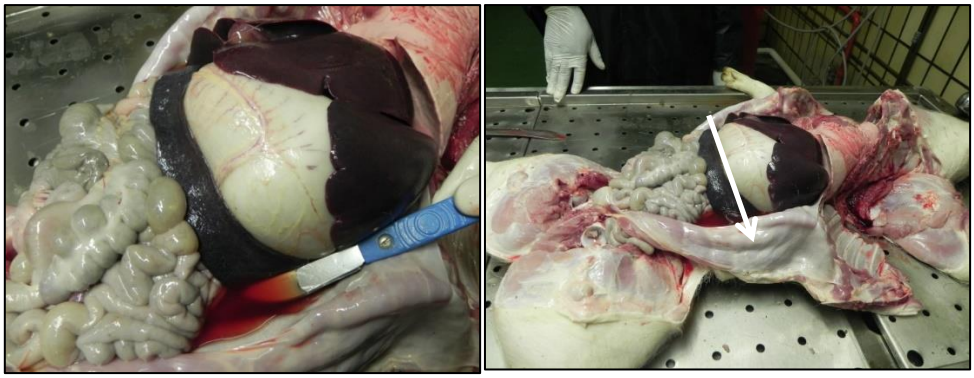

Figure 6.9: Straw coloured fluid in the body cavity of Pig 1

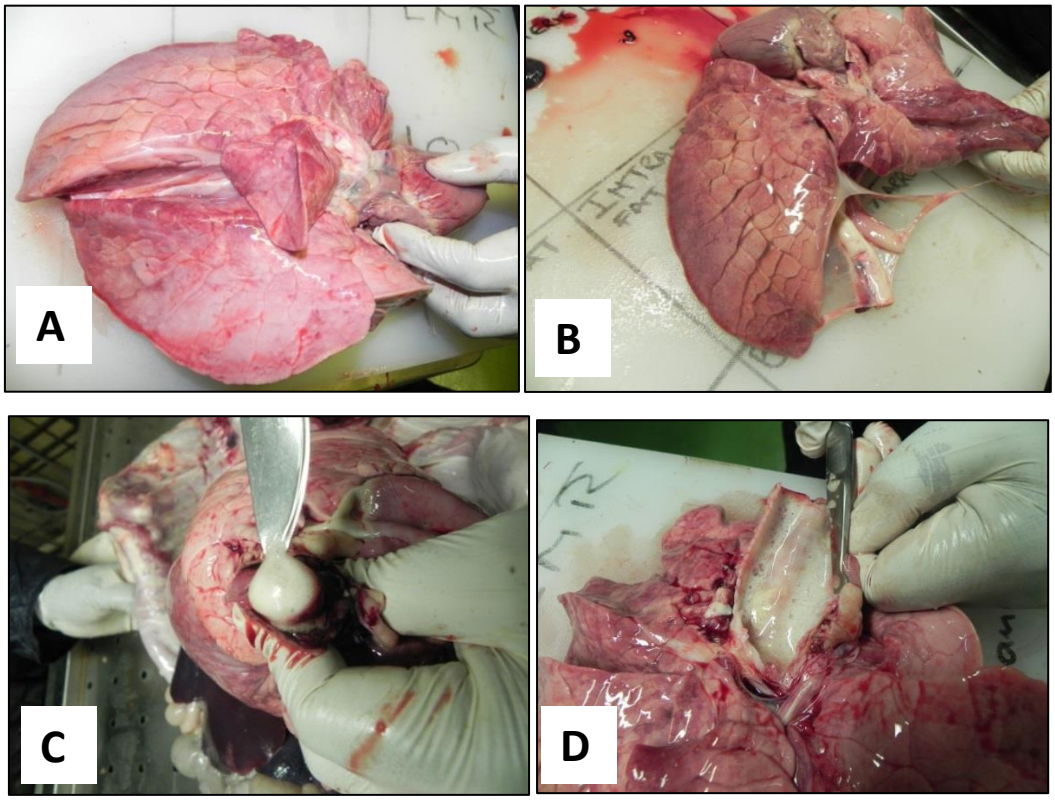

Figure 6.10 A - D: Oedema of the left lobe of the lung with froth in the trachea

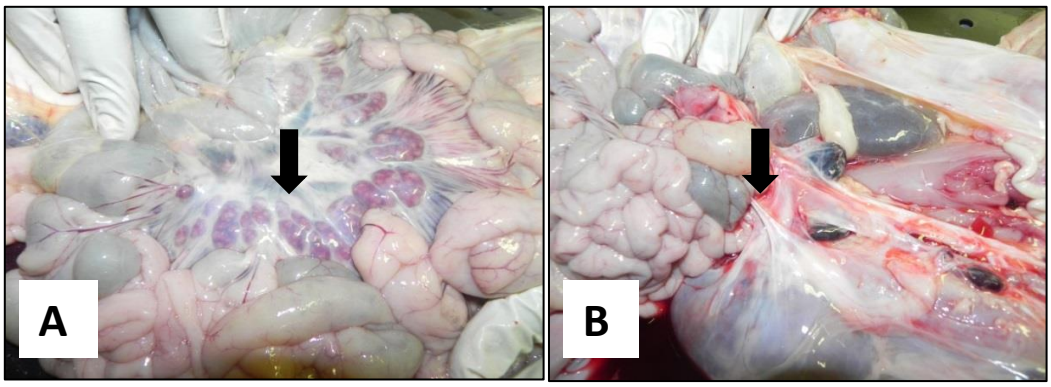

Figure 6.11 A and B: Haemorrhaging of the mesenteric and gastro-hepatic lymph nodes

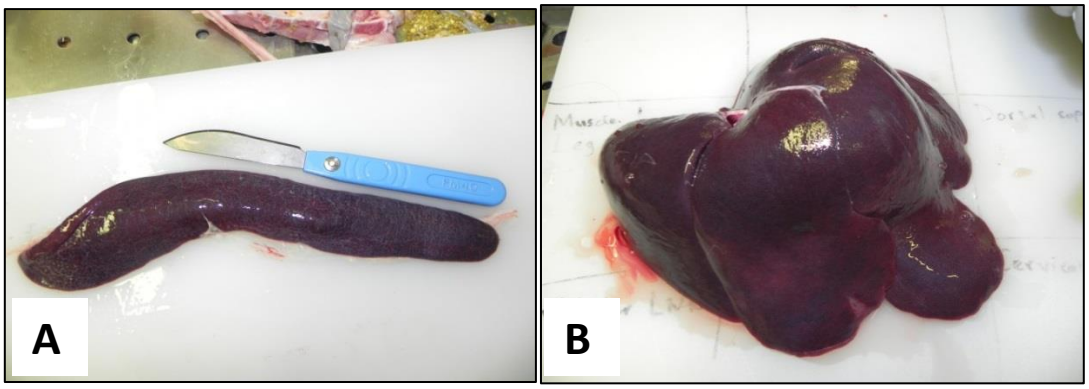

Figure 6.12 A and B: Moderate splenomegaly and hepatomegaly with red discolouration

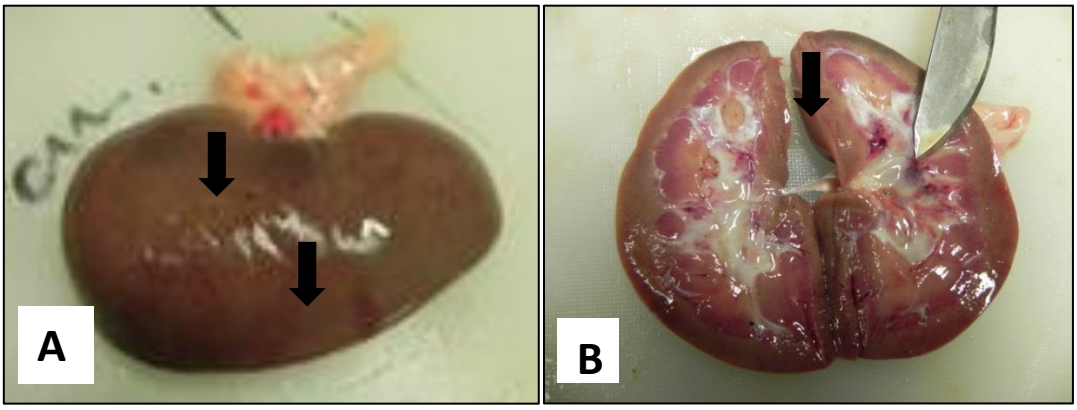

Figure 6.13 A and B: Haemorrhage of the kidney

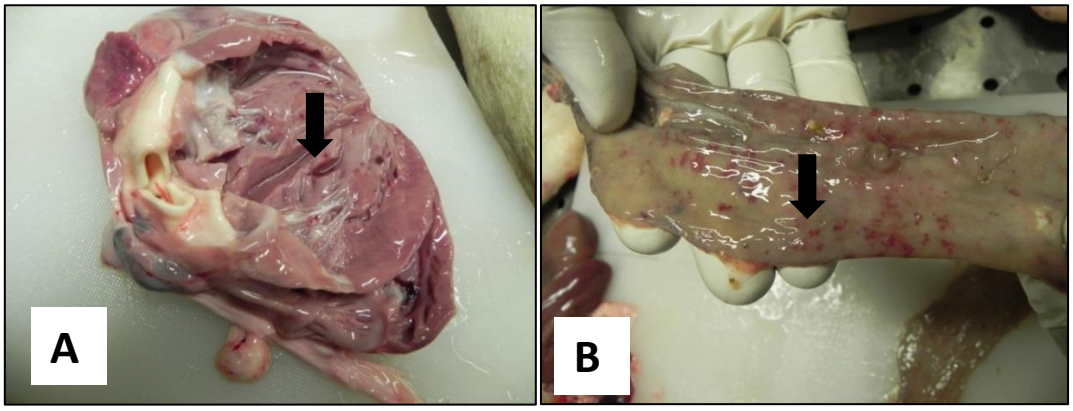

Figure 6.14 A and B: Haemorrhage of the heart ventricle and intestinal mucosa

Pig 2 and Pig 3 necropsies were performed on day 10 p.i., the pigs were euthanized and were not allowed to succumb to the virus infection as occurred with Pig 1. Both carcasses were in good condition. No external lesions or signs of ASF were detected and on gross pathology all organs appeared normal with no clinical signs or typical pathology of ASF (Figure 6.15 A - H).

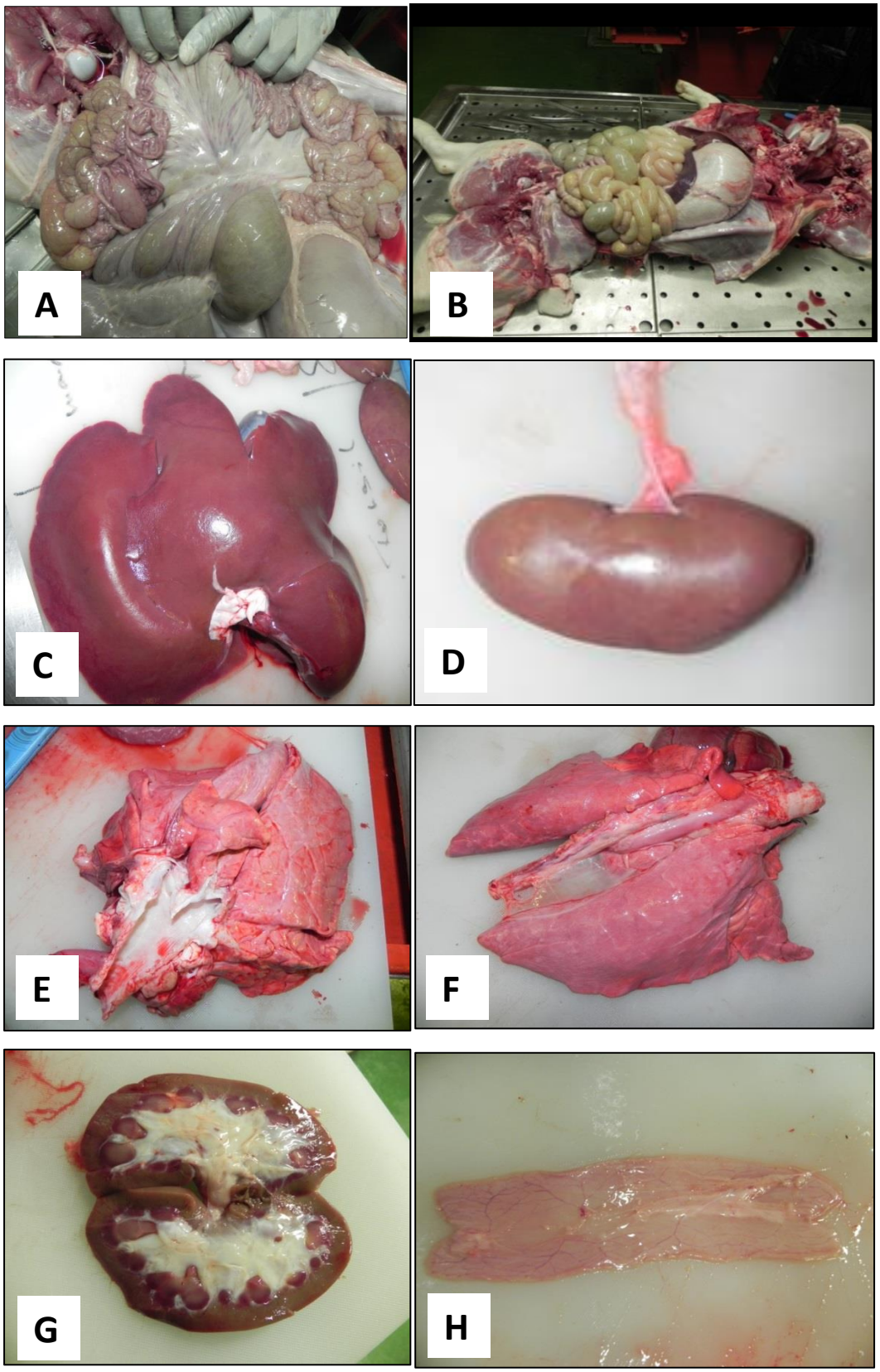

Figure 6.15 A - H: Normal necropsy, a) Straw-coloured fluid absent from body cavity, b) normal lymph nodes, c) normal liver, d) normal kidney, e) absence of froth in the trachea, f) normal lung; g) normal kidney; h) absence of haemorrhage in intestinal mucosa

6.4.4 p72 PCR and sequencing results of the tissue samples

All tissue samples taken during necropsy were tested by conventional PCR for presence of ASFV. Clinical pathology of Pig 1 showed signs of ASF and the PCR results confirm that all nine tissue samples were positive for ASFV genome presence. In Pig 2, seven out of nine tissue samples tested positive for ASFV, with the mesenteric lymph nodes and the heart being negative, while in Pig 3, six out of nine tissue samples tested positive for ASFV, with the mesenteric lymph nodes and the heart again being negative, as well as the renal lymph nodes (Table 6.3).

Table 6.3: Table indicating the tissue samples, ASFV and PCR results for the three pigs

TISSUE SAMPLE	PIG 1	PIG 2	PIG 3
Spleen	Positive	Positive	Positive
Liver	Positive	Positive	Positive
Kidney	Positive	Positive	Positive
Intestines	Positive	Positive	Positive
Gastric Lymph nodes	Positive	Positive	Positive
Hepatic Lymph nodes	Positive	Positive	Positive
Renal Lymph nodes	Positive	Positive	Negative
Mesenteric Lymph nodes	Positive	Negative	Negative
Heart	Positive	Negative	Negative

6.4.5 Analysis of NGS data quality, quantity and usability

6.4.5.1 Enrichment and sequencing of the viral genome

The extracted DNA from the different 2nd stage tick groups were used as template for rolling circle amplification (RCA) with DNA-dependent phi29-polymerase (GenomiPhi v2). DNA enrichment of the sample was observed when measured and compared to the quantity (ng/µl) of the starting material. All samples were diluted 1:10 with TE buffer and quantified, resulting in a range between 81.08 – 177.00 ng/µl falling above the minimum amount of 50 ng/µl required for NGS.

6.4.5.2 Reference mapping of the sequencing reads

For analyses of the quality and quantity of the obtained raw sequences, mapping of the reads was performed against the known reference sequence to access the purity and completeness of the reads obtained. The processed reads for each group were assembled and aligned against the Alldays virus genome sequence (Chapter 5) with CLC genomics workbench 7.0 software, as this was the virus isolated and cultivated from these ticks. On average about 1.58 million reads ~300 bp in length were generated for each sample (Table 6.4). The purity of the generated

DNA differed across the samples, with the lowest percentage reads mapped against the Alldays reference virus being 0.57% for the spleen sample from pig 1 and the highest being 52.26% for the 90 dpi tick pool from pig 2 (Table 6.4). The proportion of non-specific sequences of host nucleic acid was determined by mapping against the pig genome (Genbank accession number: CU466253) and indicated that 31.3% of the reads mapped to host nucleic acids. Despite the variation in mapping of the reads to the viral genome reference, all sequence attempts resulted in full-length coverage of an ~120 kbp region corresponding to the central conserved region of the ASFV genome and enabled assessment of genome variation in the different pigs and tick pools across the time-frame investigated. Accurate nucleotide sequence assembly is essential to estimate genetic diversity to determine viral genome adaptation to hosts over a period of time. Phred quality scores were generated in CLC genomics to assess base calling accuracy and sequencing data quality. The average phred score was 27 (range: 23-35) indicating an average base call accuracy of 99.7%.

Table 6.4: Summary of sequencing mapping percentages and quality

Virus isolate	Total reads	Reads Mapped to ASFV reference genome	Number of bases mapped	Phred Quality score of highest percentage reads
Pig 1 Spleen	2 400 178	13 709 (0.57%)	4 126 409	27
Pig 1 ticks 21 dpi	1 524 192	475 843 (31.22%)	143 228 743	31
Pig 1 ticks 90 dpi	1 680 340	159 288 (9.48%)	47 945 688	23
Pig 1 ticks 365 dpi	1 158 202	3 643 (2.30%)	1 096 543	35
Pig 2 ticks 21 dpi	2 061 810	439 413 (21.31%)	132 263 313	25
Pig 2 ticks 90 dpi	2 852 264	1 490 614 (52.26%)	448 674 814	28
Pig 2 ticks 365 dpi	1 910 194	5 594 (0.61%)	1 683 794	25
Pig 3 ticks 21 dpi	1 350 964	504 814 (37.37%)	151 949 014	24
Pig 3 ticks 90 dpi	2 620 676	1 232 769 (47.04%)	371 063 469	26
Pig 3 ticks 365 dpi	1 678 126	13 244 (1.95%)	3 986 444	24

Variation across the complete *p72* gene region (1912 nt in length) for the 10 samples detailed in Table 6.4 was assessed by extracting data for this gene from the genome sequence generated for each. All sequences were identical to each other across the full *p72* gene for the different tick pools sampled at 21, 90 and 365 dpi and were a 100% match to the pig spleen virus sequence.

6.4.9 Alignments, analysis, comparison and variation of the tick derived ASFV genomes

An alignment based on 121,585 nucleotides (nt) was created to compare the virus variation from the initial pig 1 spleen to 365 dpi at different time intervals for the infected ticks (2nd stage) originated from the three different pigs. Minor differences between the sequences from

pig 1 spleen, 21 dpi, 90 dpi and 365 dpi were observed with the differences mainly corresponding to nucleotide substitutions rather than to insertions or deletions.

Nucleotide changes occurred in 677 positions (Appendix E) out of the 121,585 nt compared against the Alldays virus, isolated from the original ticks after completion of the cycle experiment, indicating an overall 0.55% difference and resulting in 341 amino acid changes (results not shown). Out of the 10 samples compared only one was from pig origin, the remaining nine were all of tick origin. Out of the 677 SNPs detected 96 were evident in the pig 1 spleen. Among the remaining 9 tick samples Pig 1, 365 dpi tick pool had 232 and pig 2, 90 dpi tick pool had 288 nucleotide changes respectively. However none of these changes were observed in any of the other samples. The pig 3, 90 dpi tick pool and the 365 dpi tick pool shared 38 changes with the Pig 2, 90 dpi tick pool and Pig 3, 90 dpi tick pool, shared 7 of the 38 changes observed in the former groups. However, none of these changes were observed in any of the other 365 dpi tick pools (of pig 1 and 2). No changes were observed for the Pig 1, 21 and 90 dpi, the Pig 2, 21 and 365 dpi nor the Pig 3 21 dpi tick pools. Overall the data indicated that DNA sequences derived from the transmission experiment showed very low levels of variation with no mutations becoming fixed across the time-line within which ticks from each of the three pigs were assessed.

Complete genome sequences were not obtained for the ASFV genome directly from the ticks when referenced against the genotype I isolate from Spain E75 (Genbank accession number FN557520). Instead, the data generated for the infected tick pools aligned from position 12,515 to 134,848 against the full genome of 181,375 bp of FN557520. A multiple sequence alignment was created using published reference genomes and genomes from infected ticks derived at the different time stages. Based on the 121,462 bp used for the phylogenetic analysis, the tick derived viruses are most closely related to the published Warmbaths full genome (Gen bank accession: AY261365). This is shown in the phylogenetic neighbour joining tree (Figure 6.16) derived from the alignment data set.

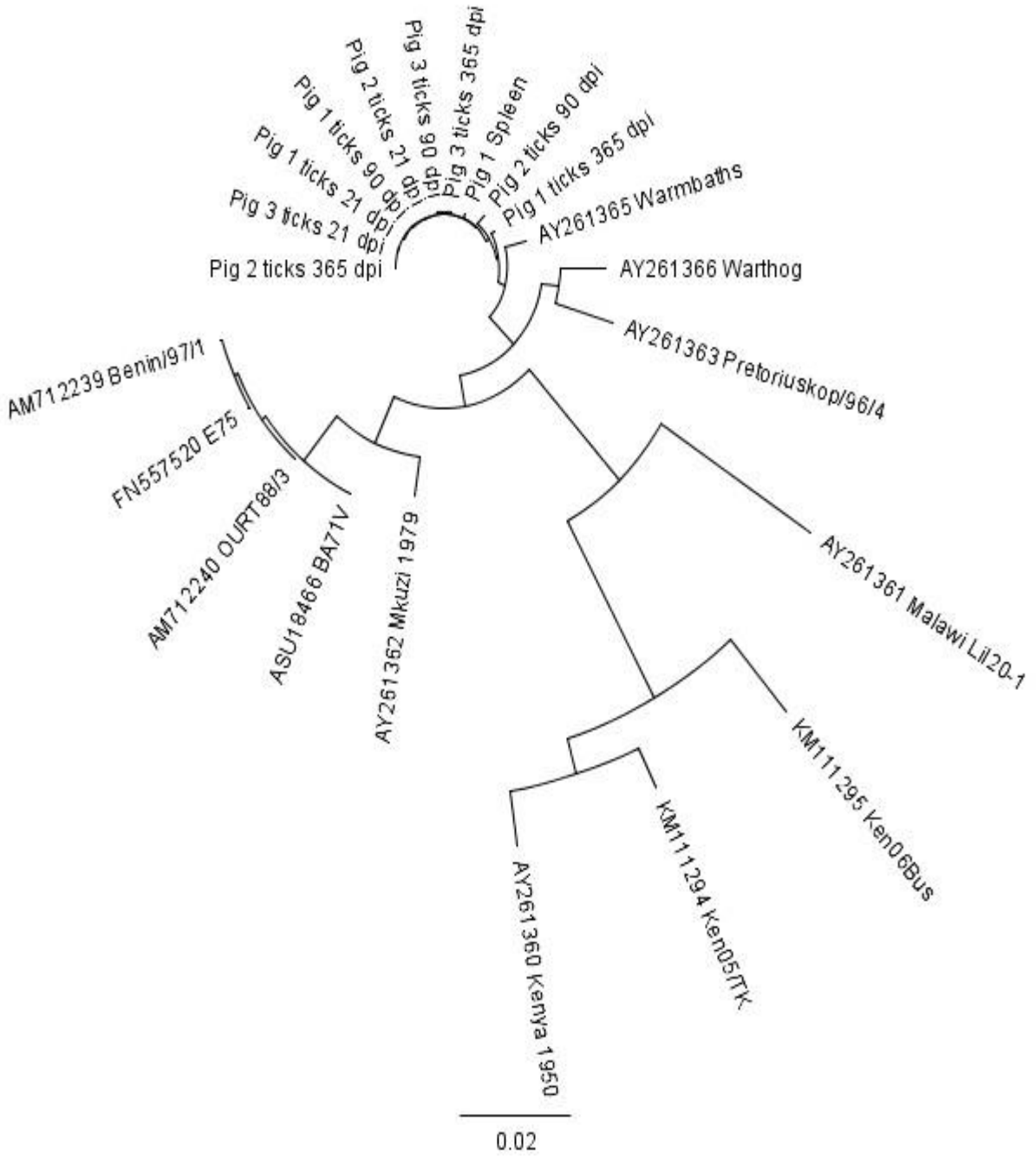

Figure 6.16: Unrooted Neighbor joining tree inferred from an ~121 kbp alignment of the ten genomes obtained from the transmission experiment, and published reference ASFV genomes.

6.5 Discussion

6.5.1 Transmission of African swine fever virus in domestic pigs

This is the first time where an ASFV transmission study was done under experimental conditions by using naturally infected *Ornithodoros* ticks as the mode of infection. The goal was to investigate the cycling of the virus, from the sylvatic vector, to domestic pigs and back to *Ornithodoros* ticks. Fever was successfully used as a marker to determine infection, confirming that it can be used as a transmission parameter (De Carvalho Ferreira et al., 2013). Pig 1 showed a temperature higher than 40 °C and bloody diarrhoea 5 dpi. Viremia was confirmed, and during necropsy, typical ASF pathology was observed, indicating successful transmission. Short onsets of disease from infection till death at 7 dpi. indicate a highly pathogenic strain. Due to starting off with an unknown concentration of virus in the ticks used for the initial infection, the reason for establishment of infection in only one out of the two pigs used could not be determined. Contact transmission from domestic pig to domestic pig was demonstrated, with the remaining two pigs showing a rise in temperature and viremia 9 days after the infected ticks were allowed to feed on two naïve pigs. Pigs 2 and 3 did not die due to ASFV infection but were euthanized on detection or fever, on ethical grounds, to limit discomfort and pain due to the viral infection. Typical ASF gross pathology was not observed during necropsy of these two pigs, but 77.7% and 66.6% of the organs sampled were ASF-positive by PCR for pig 2 and pig 3 respectively. The virus was transmitted back to naïve ticks, as all tick pools (2^{nd} stage ticks), after completion of the experiment, tested positive for the presence of ASFV by PCR. This indicated completion of the full transmission cycle from tick to pig and back to tick.

6.5.2 Genomic evolution of ASFV during tick-suid-tick cycling, under experimental conditions

Next Generation Sequencing (NGS) is valuable to study virus diversity, however to determine ASFV genome diversity by NGS is difficult due to large variation in the terminal variable regions of the genome. The usefulness of NGS for viral diversity estimation depends crucially on the quality of the sample (Beerenwinkel et al., 2012). It is technically challenging to prepare DNA for NGS directly from clinical samples and a critical point is the enrichment of viral genomes. The rolling circle amplification (RCA) method used in this study proved to be successful with certain limitations but can be investigated for future usage across genotypes for large fragment sequencing. Although complete genome sequences could not be obtained due to high levels of variation in the terminal ends and lack of reference sequences to map

against a large portion was used for genome comparison. Difficulties were also encountered due to working directly from clinical samples, resulting in low levels of viral DNA and over-representation of host DNA during the NGS process. In this study a small amount of template DNA was used directly from the infected ticks, and so all samples were contaminated with high amounts of tick host DNA. Large amounts of sequence data were generated for an accurate comparison study to confidently recover changes that may have occurred during host transmission in the central conserved region of the genome. The results provide evidence for the possible use of NGS to study virus evolution within and between the hosts. The partial genomes obtained from the different time stages of the ticks were similar in overall sequences with 0.5% difference across the genomes. The differences at nucleotide level between virus genomes of ticks sampled at different time internvals are however not consistent across time frames and could be due to sequencing errors. The Phred quality scores obtained indicate a possible 0.3% error rate, with Illuminia MiSeq error rate estimated at 0.8% (Ambardar et al., 2016), with the sequence variation falling within of what is expected for the MiSeq platform. To distinguish between mutations that truly represent the viral genome diversity from errors introduced by the NGS chemistry is very problematic. It is also difficult to assess the effects of point mutations observed during different time frames as the function of many ASFV genes are currently still unknown (Dixon et al., 2013).

Differences between ASFV genomes are due to insertions and deletions in the variable left and right regions of the viral genome (Chapman et al., 2008) and progressive deletion of genes in the left variable end of the ASFV genome was observed during adaptation of a Georgian ASFV isolate to Vero cells (Krug et al., 2015). With lack of sufficient data to analyse the variable terminal ends in the current study it is difficult to access adaptations, as such no evidence can be provided that adaptation between hosts specifically involves the terminal ends. Fundamental questions on the adaptation of ASFV to different hosts remain, and the adaptation and viral changes that occur during tick to pig transmission are poorly understood. Host adaptation and host-to-host transmissibility are likely independent properties that are associated with different selection pressures and genetically reflected responses, and will only be resolved as additional complete ASFV genomes of different species origin become available and the different gene functions are better understood.

Acknowledgments

We thank Dr Paidamwoyo Mutowembwa for assistance with the clinical animal aspects of the experiment as well as the animal caretakers who looked after the pigs. Mr Gerbrand van der Zel from Gauteng Provincial Government, for assisting with collecting of naïve ticks. The project was done under ethical approval from University of Pretoria project number EC011-13 and Agricultural Research council project number OV/24/01/P001. Permission to do research in terms of Section 20 of the Animal Diseases act, 1984 (Act no 35 of 1984), Department of Agriculture, Forestry and Fisheries was also obtained.

Chapter 7

Concluding remarks

African swine fever (ASF) is endemic in South Africa and is unlikely to ever be eradicated from this region (Penrith et al., 2013) due to its association with wildlife and *Ornithodoros* ticks and the importance of the sylvatic cycle. Reports of ASF in South Africa, date from as early as 1926 and a designated ASF control zone was implemented in 1935 (Magadla et al., 2016) to control the disease. By applying the legislation of the ASF control zone, the distribution of ASF has been well controlled for over 70 years. Recent research results indicate that: (i) *Ornithodoros* ticks were found in various provinces including Gauteng, an area which falls outside of the current ASF control zone (Magadla et al., 2016), (ii) ASFV-positive *Ornithodoros* ticks were found to be present at least 100 km south of the control zone in Gauteng (Penrith et al., 2009), (iii) an outbreak of ASF occurred south of the ASF control area in 2012 in Mpumalanga and Gauteng due to illegal movement of pigs out of the control zone (Fasina et al., 2015), (iv) a molecular survey of *Ornithodoros* ticks sampled in 2002 from Mkuze Game Reserve, a reserve in Kwa-Zulu Natal, previously identified as positive for ASF and part of the control zone, were all negative for ASFV, signalling the possible disappearance of the virus from this region (Arnot et al., 2009). All of these finding raised questions regarding the relevance of the current delineation of the ASF control area in South Africa and emphasised the importance of regular monitoring and surveillance of ASF reservoirs of infection. The last in-depth epidemiological studies, based on distributions of *Ornithodoros* ticks, as well as ASFV infection of these ticks, were carried out in the late 1970's and early 1980s (Thomson et al., 1983, Thomson 1985), leaving an almost 30 year gap between surveys. In the absence of a vaccine or treatment options, control of the disease relies strictly on regulating movement of pigs and pig products and on biosecurity measures that ensure efficient separation of domestic pigs and wild suids and their associated soft ticks. Given the epidemiological importance of the sylvatic cycle vector, it is crucial to assess vector-associated factors influencing ASF distribution and diversity in the southern African sub-region. Thus the research priorities of this study were aligned with the ultimate goal of gaining a better understanding of African swine fever virus maintenance and transmission dynamics in the sylvatic *Ornithodoros* vector.

The ASFV status of tick populations from different game parks across the ASF control zone in South Africa was assessed in Chapter 2. It was found that *Ornithodoros* ticks are absent from

Pilanesberg nature reserve and present in Madikwe nature reserve but that no ASF virus could be detected in soft ticks collected from the latter reserve. As both nature reserves are located in the North-West Province within the control zone, these results suggest that a more in depth evaluation of the northern part of the North-West Province is needed in order to determine its continued inclusion in the ASF control area is warranted. In Kruger National Park (KNP), although large numbers of *Ornithodoros* ticks were found, the overall infection rate of these ticks with ASFV was 1.3%, a value that corresponds well with those from two previous studies (Thomson and co-workers 1983; Jacquier et al. unpublished). Two novel genotypes (XXIV ad XXV) currently restricted to *Ornithodoros* ticks in the Kruger National Park were identified as well as known genotypes (II), previously only described from Mozambique (Bastos et al., 2004) and indicating for the first time, a cross-boundary genotype distribution. Thus, KNP remains an important potential source of ASF outbreaks, and the results of this study stress the importance of maintaining current recommendations of physical separations of pigs and wild suids in this area, as well as strict implementation of control measures in adjacent communities. This was also highlighted by the fact that ASFV positive ticks were found outside the southern border of KNP.

As a land-locked country, Swaziland is surrounded by the South African ASF control zone to the West and South and by ASF-positive Mozambique to the East, yet is considered to be free of ASF. As both sylvatic hosts (*Ornithodoros* ticks and common warthog) occur in this country, the first assessment of the ASF status of Swaziland (Chapter 3) focussed on seven nature reserves in evaluating *Ornithodoros* tick and ASF virus presence. A total of 562 ticks were tested for the presence of ASFV, but all results indicated absence of ASFV from Swaziland in the sylvatic cycle ticks evaluated in this study. The preliminary results highlight the need for additional research, including serological surveys of warthogs in all the parks and of domestic pigs across the country. Despite the limits of the study, the results obtained are the first to substantiate the current ASF-free status of Swaziland, whilst highlighting the need for more extensive studies due to the widespread presence of sylvatic cycle hosts. Of interest was that an inverse relationship between altitude and tick abundance was observed confirming previous suggestions that altitude may play a role in *Ornithodoros* ticks distribution (Haresnape and Mamu, 1986).

The *Ornithodoros* phylogeographical component (Chapter 4) was based on 16S rRNA gene characterisation of ticks sampled from eight game parks within the ASF control zone of SA,

together with neighbouring Mozambique and Swaziland. Previous data from Zimbabwe, Namibia, Tanzania and Uganda were also incorporated and the analyses recovered four distinct geographical *O. porcinus* clades, three of which occur in South Africa. No support for two subspecies within *O. porcinus, O. porcinus porcinus* and *O. porcinus domesticus* was obtained supporting previous suggestions of Bastos et al. (2009) that it may be more appropriate to denote the argasid vector for ASFV, as *O. porcinus* in future. At game park level, four geographically discrete haplotypes were recovered in Kruger National Park, with overlapping geographical ranges. This study represents the first investigation of the population dynamics of *Ornithodoros* ticks in South Africa, and suggests that further in-depth investigations of the haplotype and clade distribution boundaries are warranted.

With the advances made in next-generation sequencing technologies (NGS), opportunities to sequence complete genomes of ASFV isolates across genotypes is becoming possible. However pure and high concentrations of DNA are required for successful data generation and due to the fact that viruses are obligate intracellular organism, preparations are often contaminated with host nucleic acid. Two enrichment strategies, long range PCR and rolling circle amplification (RCA) were investigated to optimize the characterisation of ASF full genomes across different genotypes (Chapter 5). Ten virus isolates were compared across 10 genotypes to determine whether a long-range PCR approach is suitable for NGS of ASF viruses for which reference sequences are not currently available, and for genomes present in limited amounts, as is the case for clinical samples and tick vectors. It was concluded that this method is only suitable for regions that are highly conserved, and thus cannot be used for amplification of genomes containing large variable regions, as is the case for ASFV. Rolling circle amplification was performed with phi29, a DNA polymerase which has a low error rate (Dupinay et al., 2012). The sequencing reads obtained were generally much lower than that obtained with the long-range PCR approach, but the genome coverage was higher, indicating that RCA is the approach of choice for preparation of DNA for NGS of full ASF genomes present in clinical samples.

A sylvatic cycle transmission experiment was conducted by cycling the ASF virus from naturally infected *Ornithodoros* ticks to domestic suids and back to naïve uninfected ticks, under experimental conditions (Chapter 6), with emphasis on clinical observations and genomic variation between hosts and over time in the tick host. Under experimental conditions it was shown that the virus was transmitted from *Ornithodoros* ticks to domestic pigs exposed

to the infected ticks. Completion of the tick-suid-tick cycle was achieved when virus was transmitted back to naïve ticks that fed on each of the three ASF-positive pigs at a time when each was confirmed viraemic by PCRs performed on blood sample extracts. All ticks tested positive for the presence of ASFV up to 365 days dpi, confirming the full transmission cycle from tick to pig and back to tick. This was the first time where an ASFV transmission study was done under experimental conditions using naturally infected *Ornithodoros* ticks as the mode of infection. Full genome sequences can be used to look into viral evolution and viral diversity. NGS was performed on infected ticks sampled across a period of 21, 90 and 365 dpi, to establish genome variations across the period investigated. Based on partial genomes obtained, overall differences and variations between the different time frame samples were minor to none and as sequencing errors due to the NGS chemistry need to be taken into consideration, it is difficult to assess whether the observed SNP represent true mutations or not.

In summary, the main research aim of this study was to understand African swine fever virus maintenance and transmission dynamics in the sylvatic *Ornithodoros* vector in South Africa. Limitations, benefits as well as additional research priorities were identified as part of the reflection and are briefly summarised.

Several limitations were identified, that could have had an impact on the study:

- Tick collections are always challenging and as they rely on locating active warthog burrows in the field, in a range of highly diverse terrains encountered in the 16 game parks and nature reserves sampled in this study. Due to this and the large size of South African parks that are only accessibility via a limited number of main roads / arteries, large tracts of land are that possibly contain warthog burrows potentially infested with ticks can be easily overlooked. This skew in sampling to proximity to roads may influence infestation and infection rates.
- Very little census data are available on warthog's numbers, making it difficult to correlate warthog numbers to tick numbers. Warthogs serum samples to investigate ASFV sero-prevelance in all the parks were also not readily available to confirm results obtained in Chapter 2 and 3
- To date, 15 fully annotated complete genome sequences are available in the public domain but these only represent seven of the 23 currently published genotypes, making the standardization of a method that will work across all genotypes difficult.

The study generated a substantial body of novel information, the most important of which include:

- Despite the 30 year gap (from 1983 – 2013) between intensive surveillance of ASFV positive ticks and the current availability of newer, more sensitive detection methods, similar levels of overall prevalence were obtained for KNP. However the genetic characterization of tick viruses indicates that virus diversity in this park is high, and it is therefore a very important source of ASFV. Strict bio-security measurements in areas bordering the park must be given priority.
- The ASFV status of Swaziland in the sylvatic cycle was determined for the first time by conducting *Ornithodoros* tick surveys in seven game parks throughout the country. The presence of the tick vector, but apparent absence of the virus fills an important information gap as no ASFV information specific for Swaziland was available prior to this study.
- This study presents the first investigation of the population dynamics of *Ornithodoros* ticks in South Africa, indicating that the ticks have similarly high levels of variation based on mitochondrial16S rRNA gene sequencing, to that detected in the ASF viruses occurring within the ASF control zone of the country.
- A sylvatic cycle transmission experiment was successfully completed by cycling the ASF virus from naturally infected *Ornithodoros* ticks to domestic suids and back to naïve uninfected ticks, under experimental conditions.

Although key questions were answered additional research priorities were identified as being crucial to more fully understanding the epidemiology of ASF in South Africa. These include:

- A need for intensified investigations for the presence of *Ornithodoros* ticks, as well as serological surveys of warthogs in parks in the North-West Province to establish whether this province should continue to be included in the control zone.
- Additional research, including serological surveys in Swaziland on warthogs in all the parks and of domestic pigs across the country, in order to clarify all epidemiological aspects that impact the ASF status in Swaziland.
- Further investigations into the distribution patterns of the different *Ornithodoros* clades and haplotypes are needed in order to understand historical and contemporary drivers of host and virus variation.

- Delineating the boundaries of the geographical ranges of the ASFV-infected ticks is crucial to guiding ASF control zone adjustments as this influences spread of ASF virus in South and southern Africa.
- Clarification of the taxonomy of the sylvatic tick host and the phylogenetic position of *O. moubata* and taxonomic validity of *O. porcinus*, in particular.
- Expansion of the enrichment approach to a broader range of genotypes is needed in order to assess the full potential of RCA for generating divergent, multi-genotype comparative genome datasets.
- Adaptation of ASFV to different hosts as well as the modifications, which occur during tick to pig transmission are poorly understood, therefore more in-depth analyses are needed to confirm variations.

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Appendices

Appendix A

Information of the warthog samples tested origin

Original lab number	Sampling date	Place of capture	Age class	Sex
15/98	March 2015	Skukuza Staff village	Adult	Female
15/99	March 2015	Skukuza Golf course	Young adult	Female
15/17	February 2015	Skukuza	Young adult	Male
14/01	January 2014	Skukuza	Adult	Female
14/227	July 2014	Skukuza	Adult	Male
14/335	September 2014	Living Quarters Skukuza	Young adult	Male
14/343	September 2014	Skukuza	Adult	Female
14/344	September 2014	Skukuza	Young adult	Male
14/345	September 2014	Living Quarters Skukuza	Young adult	Female
14/348	October 2014	Skukuza Staff village	Adult	Male
14/349	October 2014	Skukuza Staff village	Adult	Female
14/350	October 2014	Skukuza Staff village	Juvenile	Female
14/351	October 2014	Skukuza Staff village	Juvenile	Male
14/352	October 2014	Skukuza Staff village	Adult	Female
14/353	October 2014	Skukuza Staff village	Adult	Male
14/354	October 2014	Living Quarters Skukuza	Adult	Female
14/355	October 2014	Skukuza Staff village	Juvenile	Male
14/356	October 2014	Skukuza Staff village	Juvenile	Female
14/357	October 2014	Skukuza Staff village	Juvenile	Female
14/358	October 2014	Skukuza Staff village	Adult	Female
14/359	October 2014	Skukuza Staff village	Adult	Male
14/360	October 2014	Skukuza Staff village	Young adult	Male
14/362	October 2014	Living Quarters Skukuza	Adult	Female
14/363	October 2014	Living Quarters Skukuza	Adult	Male
14/491	November 2014	Skukuza Staff village	Juvenile	Female
14/520	November 2014	Satara Buffalo camp	Young adult	Male
13/233	July 2013	Skukuza	Adult	Male
13/235	July 2013	Skukuza	Adult	Male
13/237	July 2013	Skukuza	Adult	Female
13/238	July 2013	Skukuza	Adult	Female
13/338	August 2013	Skukuza	Adult	Female
13/339	August 2013	Skukuza	Adult	Male
13/341	September 2013	Skukuza	Juvenile	Female

Original lab number	Sampling date	Place of capture	Age class	Sex
13/342	September 2013	Skukuza	Juvenile	Male
13/343	September 2013	Skukuza	Adult	Male
13/344	September 2013	Skukuza	Young adult	Female
13/345	September 2013	Skukuza	Young adult	Female
13/346	September 2013	Skukuza	Juvenile	Male
13/535	January 2014	Skukuza	Adult	Female
11/638	August 2011	Skukuza	Adult	Male
10/168	April 2010	Skukuza Staff village	Adult	Female
09/565	June 2009	Skukuza	Juvenile	Female
09/566	June 2009	Skukuza	Juvenile	Female
09/567	June 2009	Skukuza	Juvenile	Female
09/579	June 2009	Skukuza	Young adult	Female
09/580	June 2009	Skukuza	Adult	Male
09/589	June 2009	Skukuza	Young adult	Male
09/590	June 2009	Skukuza	Juvenile	Female
09/594	June 2009	Skukuza	Juvenile	Male
09/784	June 2009	Skukuza	Adult	Female
1	April 2015	Mauricedale	Adult	Male
2	April 2015	Mauricedale	Adult	Male
3	April 2015	Mauricedale	Adult	Male
4	April 2015	Mauricedale	Adult	Male
5	April 2015	Mauricedale	Adult	Male
6	April 2015	Mauricedale	Adult	Male
7	April 2015	Mauricedale	Adult	Male
8	April 2015	Mauricedale	Adult	Male
9	April 2015	Mauricedale	Adult	Male
10	April 2015	Mauricedale	Adult	Male

Appendix B

Information of the burrows investigated from Parks in South Africa

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
LB#1	Kruger National Park	31.5758	-23.7828	5
LB#2	Kruger National Park	31.5448	-23.7235	86
LB#3	Kruger National Park	31.4631	-23.6178	6
LB#4	Kruger National Park	31.4287	-23.5478	Absent
LB#5	Kruger National Park	31.3476	-23.5062	Absent
LB#6	Kruger National Park	31.3373	-23.5036	Absent
LB#7	Kruger National Park	31.3350	-23.5039	Absent
LB#8	Kruger National Park	31.3261	-23.5016	Absent
LB#9	Kruger National Park	31.3004	-23.4786	2
LB#10	Kruger National Park	31.2718	-23.4607	Absent
LB#11	Kruger National Park	31.2663	-23.4577	2
LB#12	Kruger National Park	31.2349	-23.4430	Absent
LB#13	Kruger National Park	31.3681	-23.4721	Absent
LB#14	Kruger National Park	31.3798	-23.4836	Absent
LB#15	Kruger National Park	31.6615	-23.9029	Absent
LB#16	Kruger National Park	31.6952	-23.9440	22
LB#17	Kruger National Park	31.6758	-23.9327	33
LB#18	Kruger National Park	31.6487	-23.9072	26
LB#19	Kruger National Park	31.6253	-23.9626	17
LB#20	Kruger National Park	31.6001	-23.9001	Present
LB#21	Kruger National Park	31.6047	-23.9167	68
LB#22	Kruger National Park	31.4952	-23.8353	Absent
LB#23	Kruger National Park	31.4811	-23.8230	Absent
LB#24	Kruger National Park	31.4857	-23.7908	Absent
LB#25	Kruger National Park	31.5082	-23.7691	Absent
LB#26	Kruger National Park	31.5189	-23.7759	3
LB#27	Kruger National Park	31.5214	-23.7781	64
LB#28	Kruger National Park	31.5568	-23.7845	Absent
LB#29	Kruger National Park	31.5649	-23.7926	7
LB#30	Kruger National Park	31.2242	-23.3092	4
LB#31	Kruger National Park	31.2210	-23.3126	2
LB#32	Kruger National Park	31.2162	-23.3511	2
LB#33	Kruger National Park	31.2127	-23.3769	Absent
LB#51	Kruger National Park	31.3878	-23.9277	Absent

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
LB#52	Kruger National Park	31.3655	-23.9295	Absent
LB#53	Kruger National Park	31.3665	-23.9206	63
LB#54	Kruger National Park	31.3665	-23.9312	6
LB#55	Kruger National Park	31.3206	-23.9238	8
LB#56	Kruger National Park	31.1641	-23.9233	5
LB#57	Kruger National Park	31.1617	-23.8812	3
LB#58	Kruger National Park	31.1596	-23.8538	9
LB#59	Kruger National Park	31.1447	-23.6620	22
LB#60	Kruger National Park	31.2943	-23.9312	Absent
LB#61	Kruger National Park	31.2930	-23.9310	Absent
LB#62	Kruger National Park	31.1714	-23.9618	4
LB#63	Kruger National Park	31.1786	-23.9730	2
LB#64	Kruger National Park	31.2136	-23.0088	2
LB#65	Kruger National Park	31.2155	-23.0078	91
LB#66	Kruger National Park	31.3277	-23.9670	3
LB#67	Kruger National Park	31.2264	-23.9611	Absent
LB#68	Kruger National Park	31.3192	-23.9639	55
LB#69	Kruger National Park	31.3005	-23.9449	2
LB#70	Kruger National Park	31.1172	-23.5207	Absent
LB#71	Kruger National Park	31.1082	-23.5158	13
LB#72	Kruger National Park	31.0915	-23.4893	Absent
LB#73	Kruger National Park	31.0868	-23.4801	5
LB#74	Kruger National Park	31.1285	-23.4589	86
N#1	Kruger National Park	31.3524	-23.1503	Absent
N#2	Kruger National Park	31.3490	-23.1570	Absent
N#3	Kruger National Park	31.2942	-23.1811	6
N#4	Kruger National Park	31.2585	-23.1880	3
N#5	Kruger National Park	31.2640	-23.1856	2
N#6	Kruger National Park	31.3962	-23.0969	Absent
N#7	Kruger National Park	31.4174	-23.0946	Absent
N#8	Kruger National Park	31.4276	-23.1146	2
N#9	Kruger National Park	31.0622	-22.7046	2
N#10	Kruger National Park	31.0911	-22.6865	2
N#11	Kruger National Park	31.1372	-22.6607	1
N#12	Kruger National Park	31.1600	-22.5635	1
N#13	Kruger National Park	31.1660	-22.6372	Absent
N#14	Kruger National Park	31.1995	-22.4028	14

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
N#15	Kruger National Park	31.1973	-22.4021	17
N#16	Kruger National Park	31.1863	-22.3912	52
N#17	Kruger National Park	31.1536	-22.3886	11
N#60	Kruger National Park	31.4038	-23.0669	Absent
N#61	Kruger National Park	31.3945	-23.0484	48
N#62	Kruger National Park	31.3929	-23.0432	35
N#63	Kruger National Park	31.3923	-23.0423	4
N#64	Kruger National Park	31.3902	-23.0395	82
N#65	Kruger National Park	31.2896	-22.9556	Absent
N#66	Kruger National Park	31.1414	-22.7512	18
N#67	Kruger National Park	31.1210	-22.7439	Absent
N#68	Kruger National Park	31.1152	-22.7432	Absent
N#69	Kruger National Park	31.1116	-22.7404	9
N#70	Kruger National Park	31.0833	-22.7301	16
N#71	Kruger National Park	31.0385	-22.7017	66
N#72	Kruger National Park	31.0302	-22.6828	Absent
N#73	Kruger National Park	30.9735	-22.6612	1
N#74	Kruger National Park	30.9646	-22.6621	Absent
N#75	Kruger National Park	31.1670	-22.7184	13
N#76	Kruger National Park	31.1639	-22.6944	29
N#77	Kruger National Park	31.1803	-22.6354	94
N#78	Kruger National Park	31.1737	-22.6102	81
N#79	Kruger National Park	31.1942	-22.5496	78
N#80	Kruger National Park	31.2208	-22.4772	Absent
N#81	Kruger National Park	31.2208	-22.4468	1
N#82	Kruger National Park	31.2724	-22.4475	3
N#83	Kruger National Park	31.2667	-22.4478	Absent
N#84	Kruger National Park	31.2639	-22.4468	63
N#85	Kruger National Park	31.2520	-22.4356	130
N#86	Kruger National Park	31.0809	-22.7321	Absent
N#87	Kruger National Park	31.0788	-22.7508	1
N#88	Kruger National Park	31.0758	-22.8019	Absent
N#89	Kruger National Park	31.0662	-22.8480	2
SK#1	Kruger National Park	31.5142	-25.0558	2
SK#2	Kruger National Park	31.5164	-25.0641	8
SK#3	Kruger National Park	31.5149	-25.0628	32
SK#4	Kruger National Park	31.5144	-25.0631	9

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
SK#5	Kruger National Park	31.4287	-24.9846	Absent
SK#6	Kruger National Park	31.4602	-25.0118	42
SK 7	Kruger National Park	31.6752	-24.6354	18
SK 8	Kruger National Park	31.6751	-24.6411	10
SK 9	Kruger National Park	31.6689	-24.6792	Absent
SK 10	Kruger National Park	31.6701	-24.6787	29
SK 11	Kruger National Park	31.6606	-24.6853	27
SK#12	Kruger National Park	31.6635	-24.7231	2
SK#13	Kruger National Park	31.6559	-24.6977	Absent
SK#14	Kruger National Park	31.6004	-24.5678	56
SK#15	Kruger National Park	31.5085	-25.4541	Absent
SK#16	Kruger National Park	31.4727	-24.4820	Absent
SK#17	Kruger National Park	31.4700	-24.4648	2
SK#18	Kruger National Park	31.5029	-24.4595	Absent
SK#19	Kruger National Park	31.5076	-24.4582	33
SK#20	Kruger National Park	31.5991	-25.0313	12
SK#21	Kruger National Park	31.5992	-25.0630	7
SK#22	Kruger National Park	31.6015	-25.0731	1
SK#23	Kruger National Park	31.6019	-25.0822	3
SK#24	Kruger National Park	31.5821	-25.1176	2
SK#25	Kruger National Park	31.5714	-25.1197	4
SK#26	Kruger National Park	31.5611	-25.0538	14
SK#27	Kruger National Park	31.5654	-25.0591	13
SK#28	Kruger National Park	31.6356	-24.9830	6
TS#20	Kruger National Park	31.9416	-24.6983	3
TS#21	Kruger National Park	31.8246	-24.7012	1
TS#22	Kruger National Park	31.7059	-24.7628	4
TS#23	Kruger National Park	31.7143	-24.7655	13
TS#24	Kruger National Park	31.7413	-24.7666	14
TS#25	Kruger National Park	31.9142	-24.8387	3
SAT#20	Kruger National Park	31.7894	-24.6000	5
SAT#21	Kruger National Park	31.7732	-24.4878	26
SAT#22	Kruger National Park	31.7491	-24.3172	76
ORP#20	Kruger National Park	31.5879	-24.2634	3
SUID#12	Kruger National Park	31.6167	-25.1615	Absent
SUID#13	Kruger National Park	31.6184	-25.1644	Absent
SUID#14	Kruger National Park	31.6203	-25.1777	Absent

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
SUID#15	Kruger National Park	31.6225	-25.1842	Absent
SUID#16	Kruger National Park	31.6230	-25.2214	Absent
SUID#17	Kruger National Park	31.6232	-25.2221	Absent
SUID#18	Kruger National Park	31.6195	-25.2420	Absent
SUID#19	Kruger National Park	31.6221	-25.2439	Absent
SUID#20	Kruger National Park	31.6280	-25.2639	Absent
SUID#21	Kruger National Park	31.5816	-25.3557	4
SUID#22	Kruger National Park	31.6428	-25.3950	Absent
SUID#23	Kruger National Park	31.4378	-25.3366	Absent
S#60	Kruger National Park	31.3877	-25.0244	7
S#61	Kruger National Park	31.4347	-25.1003	44
S#62	Kruger National Park	31.5610	-25.0593	10
S#63	Kruger National Park	31.5572	-25.0613	Absent
S#64	Kruger National Park	31.5508	-25.0692	15
S#65	Kruger National Park	31.5461	-25.0772	Absent
S#66	Kruger National Park	31.7691	-25.0881	16
S#67	Kruger National Park	31.8607	-25.3477	Absent
S#68	Kruger National Park	31.8636	-25.3565	Absent
S#69	Kruger National Park	31.8443	-25.3417	Absent
S#70	Kruger National Park	31.8398	-25.3406	Absent
S#71	Kruger National Park	31.3011	-25.3364	18
S#72	Kruger National Park	31.8233	-25.3363	Absent
S#73	Kruger National Park	31.4880	-25.1113	19
S#74	Kruger National Park	31.2976	-25.2007	Absent
S#75	Kruger National Park	31.3276	-25.2066	1
S#76	Kruger National Park	31.3358	-25.2074	Absent
S#77	Kruger National Park	31.5192	-25.3029	27
S#78	Kruger National Park	31.5201	-25.3195	39
S#79	Kruger National Park	31.5774	-25.3631	22
S#80	Kruger National Park	31.5183	-25.3863	Absent
Madikwe 1	Madikwe Nature Reserve	26.3803	-24.8168	Absent
Madikwe 2	Madikwe Nature Reserve	26.3878	-24.8201	Absent
Madikwe 3	Madikwe Nature Reserve	26.3901	-24.8211	Absent
Madikwe 4	Madikwe Nature Reserve	26.3902	-24.8211	Absent
Madikwe 5	Madikwe Nature Reserve	26.3902	-24.8212	Absent
Madikwe 6	Madikwe Nature Reserve	26.4303	-24.7822	Absent
Madikwe 7	Madikwe Nature Reserve	26.4285	-24.7800	Absent

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
Madikwe 8	Madikwe Nature Reserve	26.4275	-24.7797	Absent
Madikwe 9	Madikwe Nature Reserve	26.4159	-24.6861	Absent
Madikwe 10	Madikwe Nature Reserve	26.4039	-24.6810	Absent
Madikwe 11	Madikwe Nature Reserve	26.3952	-24.6848	Absent
Madikwe 12	Madikwe Nature Reserve	26.3450	-24.7093	Absent
Madikwe 13	Madikwe Nature Reserve	26.3453	-24.7502	PRESENT
Madikwe 14	Madikwe Nature Reserve	26.2852	-24.7085	Absent
Madikwe 15	Madikwe Nature Reserve	26.2741	-24.8112	Absent
Madikwe 16	Madikwe Nature Reserve	26.2699	-24.8125	Absent
Madikwe 17	Madikwe Nature Reserve	26.2603	-24.8153	Absent
Madikwe 18	Madikwe Nature Reserve	26.2549	-24.8153	Absent
Madikwe 19	Madikwe Nature Reserve	26.2542	-24.8151	Absent
BU #1	Mapungubwe National Park	29.4269	-22.2199	Absent
BU #2	Mapungubwe National Park	29.2075	-22.1922	Absent
BU #3	Mapungubwe National Park	29.2027	-22.1852	Absent
BU #4	Mapungubwe National Park	29.2334	-22.1819	42
BU #5	Mapungubwe National Park	29.2332	-22.1834	Absent
BU #6	Mapungubwe National Park	29.2157	-22.1920	5
BU #7	Mapungubwe National Park	29.2085	-22.1897	Absent
BU #8	Mapungubwe National Park	29.2084	-22.1896	Absent
Pilansberg 1	Pilansberg Nature Reserve	27.1945	-25.2507	Absent
Pilansberg 2	Pilansberg Nature Reserve	27.1829	-25.2490	Absent
Pilansberg 3	Pilansberg Nature Reserve	27.1704	-25.2497	Absent
Pilansberg 4	Pilansberg Nature Reserve	27.3284	-25.2514	Absent
Pilansberg 5	Pilansberg Nature Reserve	27.0522	-25.2548	Absent
Pilansberg 6	Pilansberg Nature Reserve	27.2663	-25.2309	Absent
Pilansberg 7	Pilansberg Nature Reserve	27.1099	-25.2497	Absent
Pilansberg 8	Pilansberg Nature Reserve	27.1099	-25.2496	Absent
Pilansberg 9	Pilansberg Nature Reserve	27.1179	-25.2551	Absent
Pilansberg 10	Pilansberg Nature Reserve	27.1219	-25.2541	Absent
Pilansberg 11	Pilansberg Nature Reserve	27.1278	-25.2523	Absent
Pilansberg 12	Pilansberg Nature Reserve	27.1300	-25.2516	Absent
Pilansberg 13	Pilansberg Nature Reserve	27.1358	-25.2474	Absent
Pilansberg 14	Pilansberg Nature Reserve	27.1309	-25.2513	Absent
Pilansberg 15	Pilansberg Nature Reserve	27.2116	-25.2551	Absent
Pilansberg 16	Pilansberg Nature Reserve	27.2134	-25.2554	Absent
Pilansberg 17	Pilansberg Nature Reserve	27.2141	-25.2555	Absent

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
Pilansberg 18	Pilansberg Nature Reserve	27.1512	-25.2505	Absent
Pilansberg 19	Pilansberg Nature Reserve	27.1058	-25.2739	Absent
Pilansberg 20	Pilansberg Nature Reserve	27.1087	-25.2698	Absent
Pilansberg 21	Pilansberg Nature Reserve	27.1091	-25.2663	Absent
Pilansberg 22	Pilansberg Nature Reserve	27.1122	-25.2390	Absent
Pilansberg 23	Pilansberg Nature Reserve	27.1366	-25.1976	Absent
Pilansberg 24	Pilansberg Nature Reserve	27.2074	-25.2617	Absent
Pilansberg 25	Pilansberg Nature Reserve	27.2165	-25.2561	Absent
MK #1	Marekele National Park	27.4942	-24.5313	Present
MK #2	Marekele National Park	27.4931	-24.5327	Present
MK #3	Marekele National Park	27.5328	-24.5328	Absent
MK #4	Marekele National Park	27.4942	-24.5313	Absent
MK #5	Marekele National Park	27.4989	-24.5186	Absent
MK #6	Marekele National Park	27.4985	-24.5172	Absent
MK #7	Marekele National Park	27.4942	-24.5313	Absent
MK #8	Marekele National Park	27.4836	-24.5248	Present
MK #9	Marekele National Park	27.4836	-24.5248	Absent
MK #10	Marekele National Park	27.4833	-24.5247	Absent
MK #11	Marekele National Park	27.4835	-24.5248	Absent
MK #12	Marekele National Park	27.4835	-24.5248	Absent
MK #13	Marekele National Park	27.4835	-24.5247	Present
MK #14	Marekele National Park	27.4835	-24.5248	Absent
MK #15	Marekele National Park	27.4835	-24.5248	Absent
MK #16	Marekele National Park	27.6897	-24.3326	Absent
MK #17	Marekele National Park	27.6894	-24.3324	Absent
MK #18	Marekele National Park	27.6894	-24.3323	Absent
MK #19	Marekele National Park	27.5460	-24.2978	Present
MK #20	Marekele National Park	27.5461	-24.3005	Absent
MK #21	Marekele National Park	27.5461	-24.3006	Absent
MK #22	Marekele National Park	27.4944	-24.3043	Absent
Met 1	Methethomusha Nature Reserve	31.2379	-25.4438	Absent
Met 2	Methethomusha Nature Reserve	31.2406	-25.4410	Absent
Met 3	Methethomusha Nature Reserve	31.2406	-25.4409	Absent
Met 4	Methethomusha Nature Reserve	31.2504	-25.4330	1
Met 5	Methethomusha Nature Reserve	31.2504	-25.4326	1
Met 6	Methethomusha Nature Reserve	31.2809	-25.4388	1
Met 7	Methethomusha Nature Reserve	31.2807	-25.4388	Absent

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
Met 8	Methethomusha Nature Reserve	31.2942	-25.4302	Absent
Met 9	Methethomusha Nature Reserve	31.3101	-25.4324	Absent
Met 10	Methethomusha Nature Reserve	31.3099	-25.4326	171
Nkom 1	Kwa Madwala Private Game Reserve	31.7103	-25.4957	Absent
Nkom 2	Kwa Madwala Private Game Reserve	31.7075	-25.4912	Absent
Nkom 3	Kwa Madwala Private Game Reserve	31.7053	-25.4900	3
Nkom 4	Kwa Madwala Private Game Reserve	31.7113	-25.4966	3
Nkom 5	Marloth Park	31.7785	-25.3465	Absent
Nkom 6	Marloth Park	31.7608	-25.3993	14
Nkom 7	Marloth Park	31.7580	-25.3728	Absent
Nkom 8	Mjejane Private Game Reserve	31.7029	-25.3860	Absent
Nkom 9	Mjejane Private Game Reserve	31.6968	-25.3888	Absent
Nkom 10	Mjejane Private Game Reserve	31.6892	-25.4188	13

Appendix C

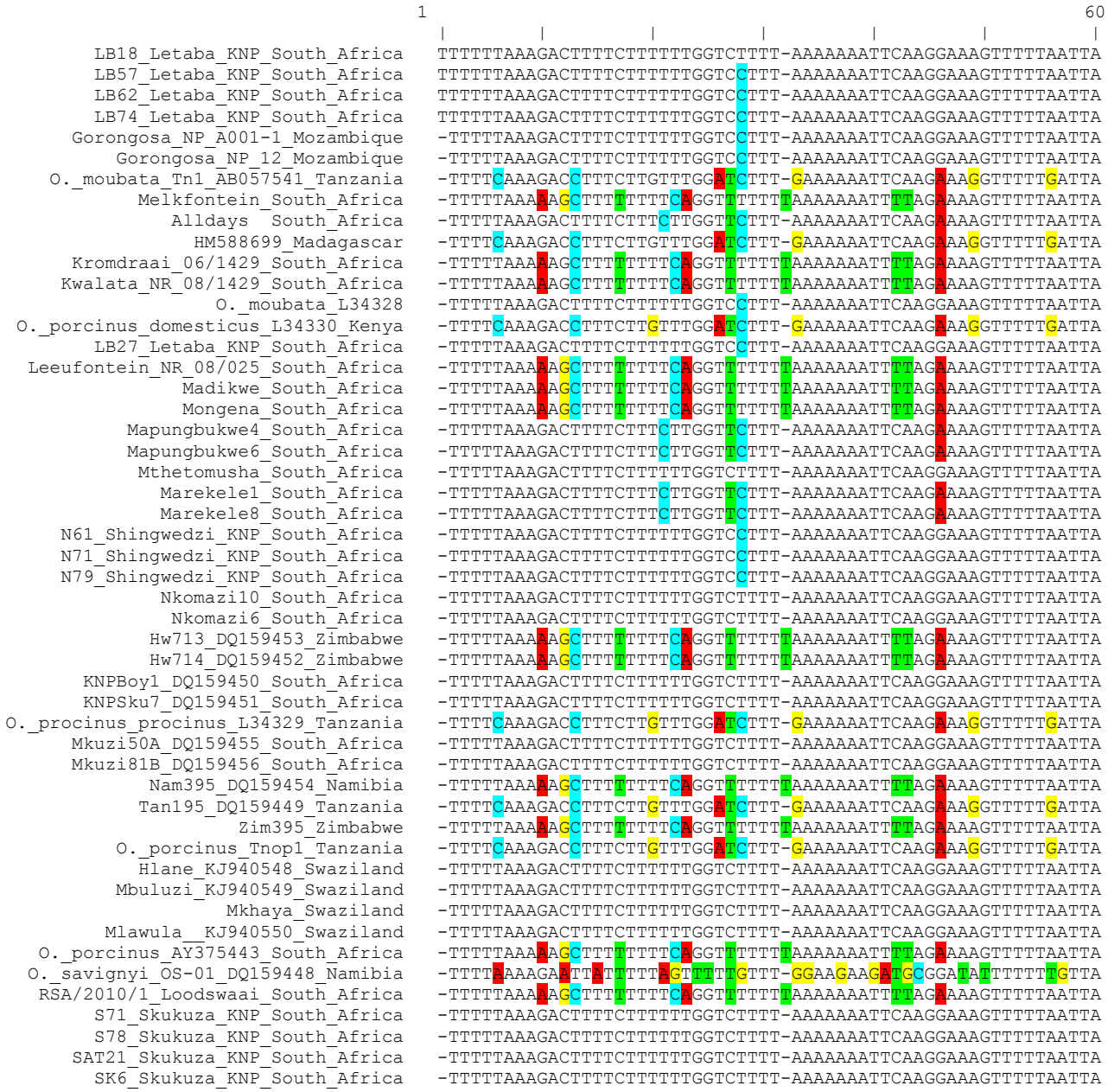
Information of burrows investigated from the 7 nature reserves in Swaziland

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
Swaziland 2012 #1	Mololotya NR	31.1927	-26.2226	Absent
Swaziland 2012 #2	Mololotya NR	31.0988	-26.1544	Absent
Swaziland 2012 #3	Mololotya NR	31.0965	-26.1589	Absent
Swaziland 2012 #4	Mololotya NR	31.0931	-26.1586	Absent
Swaziland 2012 #5	Mololotya NR	31.0930	-26.1590	Absent
Swaziland 2012 #6	Mololotya NR	31.0908	-26.1579	Absent
Swaziland 2012 #7	Mololotya NR	31.0918	-26.1586	Absent
Swaziland 2012 #8	Mololotya NR	31.0923	-26.1590	Absent
Swaziland 2012 #9	Mololotya NR	31.0829	-26.1603	Absent
Swaziland 2012 #10	Mololotya NR	31.1276	-26.1085	Absent
Swaziland 2012 #11	Mololotya NR	31.1269	-26.1072	Absent
Swaziland 2013 #12	Mkhaya Reserve	31.7964	-26.6659	Absent
Swaziland 2013 #13	Mkhaya Reserve	31.7957	-26.6303	Absent
Swaziland 2013 #14	Mkhaya Reserve	31.7939	-26.6370	7
Swaziland 2013 #15	Mkhaya Reserve	31.7969	-26.6351	7
Swaziland 2013 #16	Mkhaya Reserve	31.7959	-26.6239	Absent
Swaziland 2013 #17	Mkhaya Reserve	31.7959	-26.6387	Absent
Swaziland 2013 #18	Mkhaya Reserve	31.7997	-26.6420	24
Swaziland 2013 #19	Mkhaya Reserve	31.7946	-26.6312	3
Swaziland 2012 #35	Mbuluzi	31.9737	-26.1558	10
Swaziland 2012 #36	Mbuluzi	31.9927	-26.1465	94
Swaziland 2012 #37	Mbuluzi	31.9811	-26.1488	69
Swaziland 2012 #38	Mbuluzi	31.9987	-26.1491	20
Swaziland 2013 #39	Mbuluzi	31.9927	-26.1465	Absent
Swaziland 2013 #40	Mbuluzi	31.9877	-26.1519	20
Swaziland 2012 #41	Mlawula NR	32.0004	-26.1924	50
Swaziland 2012 #42	Mlawula NR	31.9991	-26.2017	42
Swaziland 2012 #43	Mlawula NR	31.9988	-26.2046	14
Swaziland 2012 #44	Mlawula NR	31.9745	-26.2691	5
Swaziland 2012 #45	Mlawula NR	31.9967	-26.2194	Absent
Swaziland 2012 #46	Mlawula NR	32.0034	-26.2094	17
Swaziland 2012 #47	Mlawula NR	32.0049	-26.2019	23
Swaziland 2012 #48	Mlawula NR	31.9993	-26.1968	104
Swaziland 2012 #49	Mlawula NR	31.9995	-26.1955	14
Swaziland 2013 #50	Mlilwane	31.1995	-26.4780	Absent
Swaziland 2013 #51	Mlilwane	31.8788	-26.2488	Absent
Swaziland 2013 #52	Mlilwane	31.1837	-26.4637	Absent
Swaziland 2013 #53	Mlilwane	31.1848	-26.4916	Absent
Swaziland 2013 #55	Hlane	31.8740	-26.2492	3
Swaziland 2013 #56	Hlane	31.8790	-26.2487	Absent
Swaziland 2013 #57	Hlane	31.8788	-26.2488	14

Reference number	Nature reserves Game Park	Longitude	Latitude	Number of ticks per burrow
Swaziland 2013 #58	Hlane	31.8791	-26.2484	Absent
Swaziland 2013 #59	Hlane	31.9662	-26.2287	Absent
Swaziland 2013 #60	Hlane	31.9671	-26.2293	Absent
Swaziland 2013 #61	Hlane	31.9668	-26.2295	3
Swaziland 2013 #62	Hlane	31.9664	-26.2274	Absent
Swaziland 2013 #63	Hlane	31.9643	-26.2264	Absent
Swaziland 2013 #70	Mbuluzi	31.9866	-26.1510	Absent
Swaziland 2013 #101	Mbuluzi	31.9928	-26.1455	6
Swaziland 2013 #102	Mbuluzi	31.9866	-26.1510	Absent
Swaziland 2013 #103	Mbuluzi	31.9866	-26.1511	Absent
Swaziland 2013 #104	Mbuluzi	31.9796	-26.1515	Absent
Swaziland 2013 #105	Mbuluzi	31.9811	-26.1488	4
Swaziland 2013 #106	Mbuluzi	31.9987	-26.1491	9
Swaziland 2013 #Nisela 1	Nisela	31.9294	-27.0035	Absent
Swaziland 2013 #Nisela 2	Nisela	31.9295	-27.0035	Absent
Swaziland 2013 #Nisela 3	Nisela	31.9270	-27.0066	Absent
Swaziland 2013 #Nisela 4	Nisela	31.9269	-27.0069	Absent

Appendix D

Alignment of 16S rRNA *Ornithodoros* dataset



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                  Nkomazi6_South_Africa  TGA-TATTTTGAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
                   Hw713_DQ159453_Zimbabwe  TGA-TACATTAATCAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
                   Hw714_DQ159452_Zimbabwe  TGA-TACATTAATCAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
            KNPBoy1_DQ159450_South_Africa  TGA-TATTTTAAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
            KNPSku7_DQ159451_South_Africa  TGA-TATTTTAAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
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           Mkuzi50A_DQ159455_South_Africa  TGA-TATTTTAAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
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                  Nam395_DQ159454_Namibia  TGA-TACATTAATCAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
                Tan195_DQ159449_Tanzania  TGA-TGATTTAAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
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        O._porcinus_AY375443_South_Africa  TGA-TACATTAATCAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
     O._savignyi_OS-01_DQ159448_Namibia  TGA-TAGATTGAATAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
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           S78_Skukuza_KNP_South_Africa  TGA-TATTTTGAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
         SAT21_Skukuza_KNP_South_Africa  TGA-TATTTTAAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
           SK6_Skukuza_KNP_South_Africa  TGA-TATTTTGAAAAAAATACTCTAGGGATAACAGC-GTTATTACTTTGGATAGTTCATA
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            LB62_Letaba_KNP_South_Africa   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTGATGAAGAAGT
            LB74_Letaba_KNP_South_Africa   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTGATGAAGAAGT
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              Gorongosa_NP_12_Mozambique   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAAGT
         O._moubata_Tn1_AB057541_Tanzania  TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAAGT
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                   Alldays _South_Africa   TAGATGAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAGGT
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            LB27_Letaba_KNP_South_Africa   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTGATGAAGAAGT
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                    Mongena_South_Africa   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAGTGAAGAAGT
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                Mapungbukwe6_South_Africa  TAGATGAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAGGT
                 Mthetomusha_South_Africa  TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAAGT
                   Marekele1_South_Africa  TAGATGAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAGGT
                   Marekele8_South_Africa  TAGATGAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAGGT
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                       Zim395_Zimbabwe     TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAGTGAAGAAGT
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            S78_Skukuza_KNP_South_Africa   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAAGT
          SAT21_Skukuza_KNP_South_Africa   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAAGT
            SK6_Skukuza_KNP_South_Africa   TAGATAAAGTAGATTGCGACCTCGATGTTGGA-TTAGGATACCTTTATTAATGAAGAAGT
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          LB74_Letaba_KNP_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTTAATTCCTACA
       Gorongosa_NP_A001-1_Mozambique   TATAAGAGGAGG-TTTGCTCAACC--TTTTAATTCCTACA
           Gorongosa_NP_12_Mozambique   TATAAGAGGAGG-TTTGCTCAACC--TTTTAATTCCTACA
     O._moubata_Tn1_AB057541_Tanzania   TATAAAAGGAGG-TTTGCTCAACC--TTCAAATTCCTACT
            Melkfontein_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
               Alldays _South_Africa   TATGAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
                 HM588699_Madagascar   TATAAAAGGAGG-TTTGCTCAACC--TTCAAATTCCTACA
       Kromdraai_06/1429_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
     Kwalata_NR_08/1429_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
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 O._porcinus_domesticus_L34330_Kenya   TATAAAAGGAGG-TTTGCTCAACC--TTCAAATTCCTACT
          LB27_Letaba_KNP_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTTAATTCCTACA
   Leeufontein_NR_08/025_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
                 Madikwe_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
                 Mongena_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
            Mapungbukwe4_South_Africa   TATGAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
            Mapungbukwe6_South_Africa   TATGAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
            Mthetomusha_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
              Marekele1_South_Africa   TATGAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
              Marekele8_South_Africa   TATGAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
       N61_Shingwedzi_KNP_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTTAATTCCTACA
       N71_Shingwedzi_KNP_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTTAATTCCTACA
       N79_Shingwedzi_KNP_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTTAATTCCTACA
              Nkomazi10_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
               Nkomazi6_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
            Hw713_DQ159453_Zimbabwe   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
            Hw714_DQ159452_Zimbabwe   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
        KNPBoy1_DQ159450_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
        KNPSku7_DQ159451_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
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      Mkuzi81B_DQ159456_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
            Nam395_DQ159454_Namibia   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
           Tan195_DQ159449_Tanzania   TATAAAAGGAGG-TTTGCTCAACC--TTCAAATTCCTACA
                    Zim395_Zimbabwe   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
         O._porcinus_Tnop1_Tanzania   TATAAAAGGAGG-TTTGCTCAACC--TTCAAATTCCTACA
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         Mbuluzi_KJ940549_Swaziland   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
                   Mkhaya_Swaziland   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
        Mlawula__KJ940550_Swaziland   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
   O._porcinus_AY375443_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTGCT
 O._savignyi_OS-01_DQ159448_Namibia   CGTGAAAGGGGG-TTTGCTCAACT--TTTAAATTCCTACA
  RSA/2010/1_Loodswaai_South_Africa   TATAAAAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
        S71_Skukuza_KNP_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
        S78_Skukuza_KNP_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
     SAT21_Skukuza_KNP_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTACA
        SK6_Skukuza_KNP_South_Africa   TATAAGAGGAGG-TTTGCTCAACC--TTTAAATTCCTAC
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Appendix E

Primer sequences for the amplification of segments of the ASFV genome, indicating the primer sequences provided by Ingenasa and primers designed in this study

Primer name	Primer Sequence	Tm (°C)	Region referenced against E75 (Genbank FN557520)
INGENASA PRIMERS			
#1F: ASFV 1577	5'-CTGATATTCATGTTAAAGTGC-3'	52.0	121..141
#1R: ASFV15488	5'-CCTAAAGACATTAAACAGTTAGC-3'	55.5	16159..16181 C*
#2F: ASFV 15138	5'-GTATTTCATCCATTCCACGTTCTC-3'	59.9	15831..15855
#2R ASFV 35942	5'-GTGGGGTCTGACCAGACCCGCATTG-3'	70.0	46546..46570 C
#3F ASFV 35822	5'GGTAGGTGGCAATCACAAGAAGAGAAG-3'	63.9	46450..46476
#3R ASFV 50739	5'-CCTGCTCGTGTTACTTATGAAAC-3'	60.4	61347..61373 C
#4F ASFV50532	5'-GGACTGCGACACGATCACAGAGTC-3'	65.5	61139.61162
#4R ASFV61047	5'-GTCCTACGACACCATGCGAACCAAG-3'	65.5	71692..71716 C
#5F ASFV60803	5'-CTATATTGGCAAACTGTTTCACGTC-3'	59.1	71448..17472
#5R ASFV70908	5'-CAATCACAACGGTCCTCCTGTTAAG-3'	61.8	81525..81549 C
#6F ASFV70513	5'-AGTTATAACCAGGTACAACAACATC-3'	58.2	81156..81180
#6R ASFV80490	5'-CATGATTATGCTTCAAAGCTGTTAG-3'	58.2	91093..91117 C
#7F ASFV80284	5'-GTTACGTAGATCACTGAGTTGCAATC-3'	60.5	90911..90936
#7R ASFV90848	5'-GAGCAAGCCAATTTGGAAGAACTTG-3'	62.5	101207..101231 C
#8F ASFV90696	5'-GTGTCCTCCATCGGATATACTATAC-3'	57.6	101296..101320
#8R ASFV100806	5'-AGTGTGCTCACCTATATCACGGAAC-3'	63.7	111379..111403 C
#9F ASFV100596	5'-CATTTCTGAACTGCGAGAGTTCTAG-3'	61.5	111193..111217
#9R ASFV110615	5'-TCGCTGTGCGTAATTTATCCCAATC-3'	62.5	121189..121213 C
#10F ASFV110371	5'-CATTAGATGCTTCTCTCCAGATTGAG-3'	59.8	120939..120964
#10R ASFV120442	5'-GTTCACAAGATATTCATTGTAGAAGTC-3'	57.2	131014..131040 C
#11F ASFV120249	5'-GAGAACAGGTCTTAGAATTACTTCATG-3'	58.1	130847..130873
#11R ASFV130332	5'-ACGCATCCGAAG GTGTTACAAGGAC-3'	66.4	140906..140930 C
#12F ASFV129898	5'-CTCTGAATGCGCAGAGCACCTTAC-3'	64.6	140496..140519
#12R ASFV139579	5'-GAACATGGGAATACGTGTGTCCAG-3'	62.1	150153..150176 C
#13F ASFV139484	5'-GATTAACCGCGACCATACCTATCGTC-3'	63.4	150081..150106
#13R ASFV149738	5'-GTTGAAGCACTAGGTCGGAGCAACAG-3'	66.0	160309..160334 C
#14F ASFV149634	5'-CTATCACCCAAGAAGCTGAAGCATAC-3'	62.0	160230..160255
#14R ASFV159505	5'-GAATCCATACTAATTCTTTACCTAAG-3'	54.1	170071..170096 C
#15F ASFV159147	5'-CTTATGAATAATTTCCGTAATCCGTG-3'	57.6	169738..169763
#15R ASFV166447	5'-CATATGAACCTTCTCTGTTATATACATC-3'	57.3	179677..179704 C
THIS STUDY			
ASFV8F	5'-GTCCTCGTTTTGATCGGTTT-3'	58.7	2593..2612
ASFV14F	5'-CATTTATAGGGTGTAGGTTGGGA-3'	57.8	4687-4709
ASFV8R	5'-ATAAATGCTTGACCGGGTT-3'	55.5	5636..5618 C
ASFV9F	5'-GATCTGCCCGACGTCAT-5'	56.2	7938..7954
ASFV9R	5'-CCCGAATAGGCAACGTCT-5'	57.1	9339..9322
ASFV11R	5'GCACACGGAGTATCTTTTAA-5'	56.6	14932..14913 C
RSA1AR	5'-GCACACGGAGTATCTTTTAA-3'	56.9	15652..15673 C

Primer name	Primer Sequence	Tm (°C)	Region referenced against E75 (Genbank FN557520)
RSA1BF	5'-GGGACTATGTGGTGAACTTT-3'	56.2	15392..15411
ASFV3F	5'-ATGGCGGGACTATGTGG-3'	58.6	15387..15403
ASFV2R	5'-CTAAAGCGATGAAAGACCCC-3'	55.0	16900..16920 C
ASFV13F	5'-GCAACATGCCTTTCCATCCT-3'	59.1	21062..21081
ASFV13R	5'-TCGTTGCCGCTCTCTCT-3'	59.9	25185..25201 C
ASFV10F	5'-GGGAAATGCTAAATGCGGTATGA-3'	62.8	29154..29176
RSA2AF	5'-ACATCTACTTTCTACCTGAC-3'	53.4	30907..30926
RSA1BR	5'-ACAGTCTTCGATTTGGTCAT-3'	57.2	31177..31196 C
ASFV12F	5'-TTGAGGGTACGGAAAATGTTCT-3'	57.5	32555..32576
ASFV14R	5'-ACACCATACTGAACCTAGCTT-3'	54.1	35540..35561 C
RSA2AR	5'-ATCAATGGTGTGGGAAAAAG-3'	56.1	41779..41798 C
RSA2BF	5'-CCTGAACATAAAGCCGTAAA-3'	52.9	41050..41069
ASFV12R	5'-TACTTTTAGCCCATACCAGACCT-3'	56.9	42837..42815
RSA2BR	5'-CAAAGATTGGAGTTCGTGATAG-3'	57.3	51770..51791 C
RSA3F	5'-TCGTTTACAAGTACAAAGCC-3'	54.6	50807..50826
RSA3R	5'-CCTACGCAAACTGTACCCAA-3'	56.9	61370..61389 C
RSA8F	5'-TCAGTACATACGCTATCGC-3'	55.0	101002..101020
RSA8R	5'-AACAAAATCGGGAAGAAGGA-3'	55.6	111362..111381 C
RSA10F	5'-TGTGTTTTACCTGTGGGTTT-3'	56.1	120831..120850
RSA15F	5'-CAATATCCTCTTCTATCTCGCA-3'	56.2	169602..169623
RSA15R	5'-TAGCTATAGTTCTTCTCCCT-3'	52.6	180289..180308 C

* = Compliment

Appendix F

Nucleotide changes in the compared genomes from the transmission experiment

Position	Polymorphism Type	Variant Sequences
336	SNP	Pig 3 365 dpi, Pig 3 90 dpi
351	SNP	Pig 3 365 dpi, Pig 3 90 dpi
374	SNP	Pig 3 365 dpi, Pig 3 90 dpi
1,203	SNP	Pig 2 90 dpi
1,214	SNP	Pig 2 90 dpi
1,217	SNP	Pig 2 90 dpi
1,222	SNP	Pig 2 90 dpi
1,240	SNP	Pig 2 90 dpi
1,242	SNP	Pig 2 90 dpi
1,270	SNP	Pig 3 365 dpi, Pig 3 90 dpi
1,300	SNP	Pig 3 365 dpi, Pig 3 90 dpi
1,358	SNP	Pig 3 365 dpi, Pig 3 90 dpi
1,475	SNP	Pig 3 365 dpi, Pig 3 90 dpi
2,162	SNP	Pig 1 365 dpi
2,188	SNP	Pig 1 365 dpi
2,203	SNP	Pig 1 365 dpi
3,076	SNP	Pig 1 Spleen
3,299	SNP	Pig 1 365 dpi
3,300	SNP	Pig 1 365 dpi
3,302	SNP	Pig 1 365 dpi
3,434	SNP	Pig 2 90 dpi
3,444	SNP	Pig 2 90 dpi
3,495	SNP	Pig 2 90 dpi
3,619	SNP	Pig 2 90 dpi
3,634	SNP	Pig 2 90 dpi
3,652	SNP	Pig 2 90 dpi
3,685	SNP	Pig 2 90 dpi
3,700	SNP	Pig 2 90 dpi
3,718	SNP	Pig 2 90 dpi
3,721	SNP	Pig 2 90 dpi
3,762	SNP	Pig 2 90 dpi
3,778	SNP	Pig 2 90 dpi
4,189	SNP	Pig 2 90 dpi
4,243	SNP	Pig 1 365 dpi, Pig 2 90 dpi
4,360	SNP	Pig 1 365 dpi, Pig 2 90 dpi
4,477	SNP	Pig 2 90 dpi
4,501	SNP	Pig 2 90 dpi
4,519	SNP	Pig 2 90 dpi
4,582	SNP	Pig 2 90 dpi
4,592	SNP	Pig 2 90 dpi
4,672	SNP	Pig 2 90 dpi
4,765	SNP	Pig 2 90 dpi
4,767	SNP	Pig 2 90 dpi
4,842	SNP	Pig 2 90 dpi
4,849	SNP	Pig 2 90 dpi
4,855	SNP	Pig 2 90 dpi
4,879	SNP	Pig 2 90 dpi
4,906	SNP	Pig 2 90 dpi
4,945	SNP	Pig 1 365 dpi, Pig 2 90 dpi
4,963	SNP	Pig 2 90 dpi
5,067	SNP	Pig 2 90 dpi
5,152	SNP	Pig 2 90 dpi

Position	Polymorphism Type	Variant Sequences
5,207	SNP	Pig 2 90 dpi
5,212	SNP	Pig 2 90 dpi
5,320	SNP	Pig 2 90 dpi
5,613	SNP	Pig 1 365 dpi
5,615	SNP	Pig 1 365 dpi
5,618	SNP	Pig 2 90 dpi
5,626	SNP	Pig 1 365 dpi
5,654	SNP	Pig 1 365 dpi
5,664	SNP	Pig 1 365 dpi
5,666	SNP	Pig 1 365 dpi
5,702	SNP	Pig 2 90 dpi
5,714	Indel	Pig 2 90 dpi
5,719	SNP	Pig 1 365 dpi
5,722	SNP	Pig 2 90 dpi
5,724	SNP	Pig 2 90 dpi
5,727	SNP	Pig 2 90 dpi
5,730	SNP	Pig 2 90 dpi
5,740	Indel	Pig 2 90 dpi
5,754	SNP	Pig 2 90 dpi
5,766	SNP	Pig 2 90 dpi
5,768	SNP	Pig 2 90 dpi
5,778	SNP	Pig 2 90 dpi
5,811	SNP	Pig 2 90 dpi
5,838	SNP	Pig 2 90 dpi
5,844	SNP	Pig 2 90 dpi
6,107	SNP	Pig 1 365 dpi
6,115	SNP	Pig 1 365 dpi
6,120	SNP	Pig 1 365 dpi
6,122	SNP	Pig 1 365 dpi
6,125	SNP	Pig 1 365 dpi
6,128	SNP	Pig 1 365 dpi
6,192	SNP	Pig 2 90 dpi
6,198	SNP	Pig 2 90 dpi
6,225	SNP	Pig 2 90 dpi
6,228	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,237	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,262	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,303	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,327	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,348	SNP	Pig 2 90 dpi
6,784	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,834	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,897	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,906	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,927	SNP	Pig 1 365 dpi, Pig 2 90 dpi
6,945	SNP	Pig 2 90 dpi
6,984	SNP	Pig 2 90 dpi
7,005	SNP	Pig 2 90 dpi
7,124	SNP	Pig 1 365 dpi
7,193	SNP	Pig 1 365 dpi
7,223	SNP	Pig 1 365 dpi
7,229	SNP	Pig 1 365 dpi
7,262	SNP	Pig 1 365 dpi
7,422	SNP	Pig 1 365 dpi
7,530	SNP	Pig 2 90 dpi
7,564	SNP	Pig 2 90 dpi
7,565	SNP	Pig 2 90 dpi

Position	Polymorphism Type	Variant Sequences
8,474	SNP	Pig 2 90 dpi
8,723	SNP	Pig 2 90 dpi
8,780	SNP	Pig 2 90 dpi
8,885	SNP	Pig 2 90 dpi
8,930	SNP	Pig 2 90 dpi
8,966	SNP	Pig 2 90 dpi
9,377	SNP	Pig 2 90 dpi
9,404	SNP	Pig 2 90 dpi
9,584	SNP	Pig 1 365 dpi
9,933	SNP	Pig 2 90 dpi
10,295	SNP	Pig 2 90 dpi
10,346	SNP	Pig 2 90 dpi
10,601	SNP	Pig 2 90 dpi
10,655	SNP	Pig 2 90 dpi
10,751	SNP	Pig 2 90 dpi
10,775	SNP	Pig 2 90 dpi
10,835	SNP	Pig 2 90 dpi
10,943	SNP	Pig 2 90 dpi
10,946	SNP	Pig 2 90 dpi
10,961	SNP	Pig 2 90 dpi
10,991	SNP	Pig 2 90 dpi
11,016	SNP	Pig 2 90 dpi
11,024	SNP	Pig 2 90 dpi
11,042	SNP	Pig 2 90 dpi
11,375	SNP	Pig 2 90 dpi
11,564	SNP	Pig 2 90 dpi
11,686	SNP	Pig 1 Spleen
12,162	SNP	Pig 1 Spleen
12,294	SNP	Pig 2 90 dpi
12,295	SNP	Pig 2 90 dpi
12,346	SNP	Pig 2 90 dpi
12,353	SNP	Pig 2 90 dpi
12,358	SNP	Pig 2 90 dpi
12,404	SNP	Pig 2 90 dpi
12,409	SNP	Pig 2 90 dpi
12,458	SNP	Pig 2 90 dpi
12,491	SNP	Pig 2 90 dpi
12,588	SNP	Pig 1 365 dpi, Pig 2 90 dpi
12,618	SNP	Pig 1 365 dpi
12,743	SNP	Pig 2 90 dpi
12,935	SNP	Pig 2 90 dpi
13,066	SNP	Pig 2 90 dpi
13,075	SNP	Pig 2 90 dpi
13,096	SNP	Pig 2 90 dpi
13,126	SNP	Pig 2 90 dpi
13,137	SNP	Pig 3 365 dpi, Pig 3 90 dpi, Pig 2 90 dpi
13,148	SNP	Pig 2 90 dpi
13,192	SNP	Pig 2 90 dpi
13,195	SNP	Pig 2 90 dpi
13,198	SNP	Pig 2 90 dpi
13,220	SNP	Pig 2 90 dpi
15,324	SNP	Pig 2 90 dpi
15,325	SNP	Pig 2 90 dpi
15,326	SNP	Pig 2 90 dpi
15,327	SNP	Pig 2 90 dpi
15,331	SNP	Pig 2 90 dpi
22,562	SNP	Pig 2 90 dpi

Position	Polymorphism Type	Variant Sequences
22,776	SNP	Pig 2 90 dpi
22,854	SNP	Pig 2 90 dpi
22,982	SNP	Pig 2 90 dpi
23,300	SNP	Pig 1 Spleen
31,119	SNP	Pig 1 365 dpi
31,132	SNP	Pig 1 365 dpi
31,161	SNP	Pig 1 365 dpi
31,171	SNP	Pig 1 365 dpi
31,182	SNP	Pig 1 365 dpi
31,184	SNP	Pig 1 365 dpi
31,188	SNP	Pig 1 365 dpi
31,191	SNP	Pig 1 365 dpi
31,195	SNP	Pig 1 365 dpi
31,196	SNP	Pig 1 365 dpi
31,201	SNP	Pig 1 365 dpi
31,205	SNP	Pig 1 365 dpi
31,206	SNP	Pig 1 365 dpi
31,210	SNP	Pig 1 365 dpi
31,219	SNP	Pig 1 365 dpi
31,236	Indel	Pig 1 365 dpi
31,282	SNP	Pig 1 365 dpi
31,286	SNP	Pig 1 365 dpi
31,290	SNP	Pig 1 365 dpi
31,294	SNP	Pig 1 365 dpi
31,326	SNP	Pig 2 90 dpi
31,360	SNP	Pig 2 90 dpi
31,448	SNP	Pig 1 365 dpi
31,450	SNP	Pig 1 365 dpi
31,473	SNP	Pig 2 90 dpi
31,481	SNP	Pig 2 90 dpi
31,790	SNP	Pig 1 365 dpi
31,859	SNP	Pig 2 90 dpi
31,861	SNP	Pig 2 90 dpi
31,874	SNP	Pig 2 90 dpi
31,898	SNP	Pig 2 90 dpi
31,900	SNP	Pig 2 90 dpi
31,901	SNP	Pig 2 90 dpi
31,905	SNP	Pig 2 90 dpi
31,911	SNP	Pig 2 90 dpi
31,916	SNP	Pig 2 90 dpi
31,923	SNP	Pig 2 90 dpi
32,385	SNP	Pig 1 365 dpi
34,182	SNP	Pig 1 365 dpi
34,346	SNP	Pig 2 90 dpi
34,352	SNP	Pig 2 90 dpi
34,599	SNP	Pig 2 90 dpi
37,049	SNP	Pig 2 90 dpi
37,063	SNP	Pig 2 90 dpi
37,242	SNP	Pig 2 90 dpi
37,245	SNP	Pig 2 90 dpi
37,249	SNP	Pig 2 90 dpi
37,256	SNP	Pig 2 90 dpi
37,258	SNP	Pig 2 90 dpi
39,245	SNP	Pig 1 365 dpi
39,247	SNP	Pig 1 365 dpi
39,249	SNP	Pig 1 365 dpi
39,259	SNP	Pig 1 365 dpi

Position	Polymorphism Type	Variant Sequences
39,274	SNP	Pig 1 365 dpi
39,280	SNP	Pig 1 365 dpi
39,313	SNP	Pig 1 365 dpi
39,341	SNP	Pig 1 365 dpi
39,396	SNP	Pig 1 365 dpi
39,397	SNP	Pig 1 365 dpi
40,002	SNP	Pig 1 365 dpi
40,819	SNP	Pig 2 90 dpi
40,823	SNP	Pig 2 90 dpi
40,829	SNP	Pig 2 90 dpi
41,032	SNP	Pig 1 Spleen
41,034	SNP	Pig 1 Spleen
41,043	SNP	Pig 1 Spleen
41,055	SNP	Pig 1 Spleen
41,057	SNP	Pig 1 Spleen
41,062	SNP	Pig 1 Spleen
41,084	SNP	Pig 1 Spleen
41,086	SNP	Pig 1 Spleen
41,099	SNP	Pig 1 Spleen
41,107	SNP	Pig 1 Spleen
41,109	SNP	Pig 1 Spleen
41,148	SNP	Pig 1 Spleen
41,150	SNP	Pig 1 Spleen
41,152	SNP	Pig 1 Spleen
41,154	SNP	Pig 1 Spleen
41,160	SNP	Pig 1 Spleen
41,168	SNP	Pig 1 Spleen
41,171	SNP	Pig 1 Spleen
41,193	SNP	Pig 1 Spleen
41,205	SNP	Pig 1 Spleen
41,211	SNP	Pig 1 Spleen
41,226	SNP	Pig 1 Spleen
41,238	SNP	Pig 1 Spleen
41,243	SNP	Pig 1 Spleen
41,250	SNP	Pig 1 Spleen
41,263	SNP	Pig 1 Spleen
41,264	SNP	Pig 1 Spleen
41,270	SNP	Pig 1 Spleen
41,271	SNP	Pig 1 Spleen
41,281	SNP	Pig 1 365 dpi
41,520	SNP	Pig 2 90 dpi
41,530	SNP	Pig 2 90 dpi
41,597	SNP	Pig 2 90 dpi
42,958	SNP	Pig 1 365 dpi
44,146	SNP	Pig 1 365 dpi
44,150	SNP	Pig 1 365 dpi
44,180	SNP	Pig 1 365 dpi
44,320	SNP	Pig 2 90 dpi
44,322	SNP	Pig 2 90 dpi
44,348	SNP	Pig 2 90 dpi
44,350	SNP	Pig 2 90 dpi
44,663	SNP	Pig 1 365 dpi
45,128	SNP	Pig 1 365 dpi
45,168	SNP	Pig 1 365 dpi
45,197	SNP	Pig 1 365 dpi
46,063	SNP	Pig 1 365 dpi
47,345	SNP	Pig 2 90 dpi

Position	Polymorphism Type	Variant Sequences
47,348	SNP	Pig 2 90 dpi
47,360	SNP	Pig 1 365 dpi
47,375	SNP	Pig 1 365 dpi
47,406	SNP	Pig 1 365 dpi
47,420	SNP	Pig 1 365 dpi
47,430	SNP	Pig 1 365 dpi
47,442	SNP	Pig 1 365 dpi
47,445	SNP	Pig 1 365 dpi
47,455	SNP	Pig 1 365 dpi
47,457	SNP	Pig 1 365 dpi
47,461	SNP	Pig 1 365 dpi
47,487	SNP	Pig 1 365 dpi
48,474	SNP	Pig 3 365 dpi, Pig 3 90 dpi, Pig 2 90 dpi
48,475	SNP	Pig 3 365 dpi, Pig 3 90 dpi, Pig 2 90 dpi
48,476	SNP	Pig 3 365 dpi, Pig 3 90 dpi, Pig 2 90 dpi
48,477	Indel	Pig 3 365 dpi, Pig 3 90 dpi, Pig 2 90 dpi
48,478	Indel	Pig 3 365 dpi, Pig 3 90 dpi, Pig 2 90 dpi
50,733	SNP	Pig 2 90 dpi
50,736	SNP	Pig 1 365 dpi
50,745	SNP	Pig 1 365 dpi
50,752	SNP	Pig 1 365 dpi
50,762	SNP	Pig 2 90 dpi
51,536	SNP	Pig 3 365 dpi, Pig 3 90 dpi
52,194	SNP	Pig 1 365 dpi
52,198	SNP	Pig 1 365 dpi
52,217	SNP	Pig 1 365 dpi
52,390	SNP	Pig 1 365 dpi
52,816	SNP	Pig 2 90 dpi
52,818	SNP	Pig 2 90 dpi
52,821	SNP	Pig 2 90 dpi
53,483	SNP	Pig 1 365 dpi
54,559	SNP	Pig 2 90 dpi
54,560	SNP	Pig 2 90 dpi
54,574	SNP	Pig 2 90 dpi
54,580	SNP	Pig 2 90 dpi
54,713	SNP	Pig 2 90 dpi
55,797	SNP	Pig 1 365 dpi
55,947	SNP	Pig 1 365 dpi
56,511	SNP	Pig 1 365 dpi
56,553	SNP	Pig 1 Spleen
56,633	SNP	Pig 1 Spleen
57,072	SNP	Pig 1 365 dpi
57,452	SNP	Pig 1 365 dpi
57,597	SNP	Pig 2 90 dpi
57,602	SNP	Pig 2 90 dpi
58,373	SNP	Pig 1 365 dpi
58,756	SNP	Pig 2 90 dpi
58,856	SNP	Pig 2 90 dpi
58,865	SNP	Pig 2 90 dpi
59,089	SNP	Pig 1 365 dpi
59,093	SNP	Pig 1 365 dpi
59,272	SNP	Pig 2 90 dpi
59,550	SNP	Pig 1 365 dpi
59,589	SNP	Pig 1 365 dpi
59,641	SNP	Pig 1 Spleen
59,648	SNP	Pig 3 365 dpi, Pig 3 90 dpi
59,651	SNP	Pig 1 Spleen

Position	Polymorphism Type	Variant Sequences
59,739	SNP	Pig 2 90 dpi
59,799	SNP	Pig 2 90 dpi
63,193	SNP	Pig 1 365 dpi
63,202	SNP	Pig 1 365 dpi
63,204	SNP	Pig 1 365 dpi
63,215	SNP	Pig 1 365 dpi
63,305	SNP	Pig 2 90 dpi
63,316	SNP	Pig 2 90 dpi
63,349	SNP	Pig 2 90 dpi
64,076	SNP	Pig 2 90 dpi
64,080	SNP	Pig 1 365 dpi
64,165	SNP	Pig 1 365 dpi
64,464	SNP	Pig 1 365 dpi
64,480	SNP	Pig 1 365 dpi
64,497	SNP	Pig 1 365 dpi
64,503	SNP	Pig 1 365 dpi
65,386	SNP	Pig 1 365 dpi
65,455	SNP	Pig 2 90 dpi
66,488	SNP	Pig 2 90 dpi
67,383	SNP	Pig 1 Spleen
67,450	SNP	Pig 1 Spleen
68,306	SNP	Pig 2 90 dpi
68,315	SNP	Pig 2 90 dpi
68,316	SNP	Pig 2 90 dpi
68,318	SNP	Pig 2 90 dpi
68,328	SNP	Pig 2 90 dpi
68,671	SNP	Pig 1 365 dpi
68,720	SNP	Pig 1 365 dpi
68,724	Indel	Pig 1 365 dpi
68,735	SNP	Pig 2 90 dpi
68,736	SNP	Pig 2 90 dpi
68,739	SNP	Pig 2 90 dpi
70,137	SNP	Pig 1 Spleen
70,140	SNP	Pig 1 Spleen
70,491	SNP	Pig 1 365 dpi
71,986	SNP	Pig 2 90 dpi
71,994	SNP	Pig 2 90 dpi
72,118	SNP	Pig 1 365 dpi
72,129	SNP	Pig 1 365 dpi
74,798	SNP	Pig 2 90 dpi
75,315	SNP	Pig 2 90 dpi
75,320	SNP	Pig 1 365 dpi
75,353	SNP	Pig 2 90 dpi
75,693	SNP	Pig 3 365 dpi, Pig 3 90 dpi
75,708	SNP	Pig 3 365 dpi, Pig 3 90 dpi
75,710	SNP	Pig 3 365 dpi, Pig 3 90 dpi
75,714	SNP	Pig 3 365 dpi, Pig 3 90 dpi
75,716	SNP	Pig 3 365 dpi, Pig 3 90 dpi
75,718	SNP	Pig 3 365 dpi, Pig 3 90 dpi
75,720	SNP	Pig 3 365 dpi, Pig 3 90 dpi
75,724	SNP	Pig 3 365 dpi, Pig 3 90 dpi
76,288	SNP	Pig 1 365 dpi
77,493	SNP	Pig 1 365 dpi
80,036	SNP	Pig 1 365 dpi
80,050	SNP	Pig 1 365 dpi
81,422	SNP	Pig 2 90 dpi
82,983	SNP	Pig 2 90 dpi

Position	Polymorphism Type	Variant Sequences
82,986	SNP	Pig 2 90 dpi
83,001	SNP	Pig 2 90 dpi
83,002	SNP	Pig 2 90 dpi
83,007	SNP	Pig 2 90 dpi
83,037	SNP	Pig 2 90 dpi
83,043	SNP	Pig 2 90 dpi
83,049	SNP	Pig 2 90 dpi
83,051	SNP	Pig 2 90 dpi
83,052	SNP	Pig 1 Spleen
83,055	SNP	Pig 2 90 dpi
83,061	SNP	Pig 1 Spleen
83,075	SNP	Pig 1 Spleen
83,076	SNP	Pig 1 Spleen
83,081	SNP	Pig 1 Spleen
83,085	SNP	Pig 2 90 dpi
83,529	SNP	Pig 1 365 dpi
83,710	SNP	Pig 2 90 dpi
83,903	SNP	Pig 1 365 dpi
83,930	SNP	Pig 1 365 dpi
83,943	SNP	Pig 1 365 dpi
84,566	SNP	Pig 2 90 dpi
84,569	SNP	Pig 2 90 dpi
84,570	SNP	Pig 2 90 dpi
85,476	SNP	Pig 2 90 dpi
86,143	SNP	Pig 3 365 dpi, Pig 3 90 dpi
86,162	SNP	Pig 3 365 dpi, Pig 3 90 dpi
86,181	SNP	Pig 2 90 dpi
86,253	SNP	Pig 2 90 dpi
87,065	SNP	Pig 1 365 dpi
89,376	SNP	Pig 2 90 dpi
89,404	SNP	Pig 2 90 dpi
89,449	SNP	Pig 3 365 dpi, Pig 3 90 dpi
89,592	SNP	Pig 1 365 dpi
90,260	SNP	Pig 2 90 dpi
91,331	SNP	Pig 1 Spleen
91,333	SNP	Pig 1 Spleen
91,348	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,358	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,359	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,361	SNP	Pig 1 Spleen, Pig 3 365 dpi, Pig 3 90 dpi
91,363	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,366	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,378	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,383	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,392	SNP	Pig 1 Spleen
91,394	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,404	SNP	Pig 3 365 dpi, Pig 3 90 dpi
91,409	SNP	Pig 3 365 dpi, Pig 3 90 dpi
92,122	SNP	Pig 1 Spleen
92,126	SNP	Pig 1 Spleen
92,184	SNP	Pig 3 365 dpi, Pig 3 90 dpi
92,193	SNP	Pig 1 365 dpi
92,194	SNP	Pig 1 365 dpi
92,195	SNP	Pig 1 365 dpi
92,196	SNP	Pig 1 365 dpi
92,197	SNP	Pig 1 365 dpi
92,197	SNP	Pig 3 365 dpi, Pig 3 90 dpi

Position	Polymorphism Type	Variant Sequences
92,319	SNP	Pig 2 90 dpi
92,323	SNP	Pig 2 90 dpi
92,338	SNP	Pig 1 365 dpi
92,349	SNP	Pig 1 365 dpi
92,371	SNP	Pig 1 365 dpi
92,377	SNP	Pig 1 365 dpi
92,942	SNP	Pig 2 90 dpi
92,966	SNP	Pig 2 90 dpi
92,972	SNP	Pig 2 90 dpi
92,973	SNP	Pig 2 90 dpi
92,974	SNP	Pig 2 90 dpi
92,994	SNP	Pig 2 90 dpi
93,177	SNP	Pig 2 90 dpi
93,184	SNP	Pig 2 90 dpi
93,187	SNP	Pig 2 90 dpi
93,195	SNP	Pig 2 90 dpi
93,198	SNP	Pig 2 90 dpi
93,202	SNP	Pig 2 90 dpi
93,219	SNP	Pig 2 90 dpi
93,797	SNP	Pig 1 365 dpi
93,808	SNP	Pig 1 365 dpi
93,841	SNP	Pig 1 365 dpi
93,874	SNP	Pig 1 365 dpi
93,882	SNP	Pig 1 365 dpi
94,296	SNP	Pig 2 90 dpi
97,930	SNP	Pig 1 365 dpi
97,932	SNP	Pig 1 365 dpi
99,159	SNP	Pig 1 365 dpi
99,177	SNP	Pig 1 365 dpi
99,201	SNP	Pig 1 365 dpi
99,343	SNP	Pig 2 90 dpi
99,388	Indel	Pig 3 365 dpi, Pig 3 90 dpi
99,548	SNP	Pig 2 90 dpi
99,551	SNP	Pig 2 90 dpi
99,938	SNP	Pig 1 Spleen
99,943	SNP	Pig 1 Spleen
99,957	SNP	Pig 1 Spleen
102,225	SNP	Pig 2 90 dpi
102,543	SNP	Pig 1 365 dpi
102,550	SNP	Pig 1 365 dpi
103,258	SNP	Pig 1 365 dpi
103,507	SNP	Pig 1 Spleen
103,526	SNP	Pig 1 Spleen
103,532	SNP	Pig 1 Spleen
103,554	SNP	Pig 1 Spleen
103,569	SNP	Pig 1 Spleen
103,586	SNP	Pig 1 365 dpi
103,590	SNP	Pig 1 Spleen
103,591	SNP	Pig 1 365 dpi
103,597	SNP	Pig 1 Spleen
103,606	SNP	Pig 1 365 dpi
103,623	SNP	Pig 1 365 dpi
103,626	SNP	Pig 1 Spleen
103,629	SNP	Pig 1 365 dpi
103,630	SNP	Pig 1 365 dpi
103,638	SNP	Pig 1 Spleen
103,641	SNP	Pig 1 Spleen

Position	Polymorphism Type	Variant Sequences
103,648	SNP	Pig 1 Spleen
103,666	SNP	Pig 1 Spleen
103,672	SNP	Pig 1 Spleen
103,769	Indel	Pig 1 365 dpi
103,774	SNP	Pig 1 365 dpi
104,050	SNP	Pig 2 90 dpi
104,051	SNP	Pig 2 90 dpi
104,052	SNP	Pig 2 90 dpi
104,056	SNP	Pig 2 90 dpi
104,442	SNP	Pig 2 90 dpi
104,479	SNP	Pig 2 90 dpi
105,949	SNP	Pig 2 90 dpi
106,021	SNP	Pig 1 365 dpi
106,712	SNP	Pig 1 365 dpi
106,719	SNP	Pig 1 365 dpi
106,721	SNP	Pig 1 365 dpi
106,725	SNP	Pig 1 365 dpi
106,728	SNP	Pig 1 365 dpi
106,730	SNP	Pig 1 365 dpi
106,749	SNP	Pig 1 365 dpi
106,753	SNP	Pig 1 365 dpi
106,770	SNP	Pig 1 365 dpi
107,547	SNP	Pig 2 90 dpi
107,552	SNP	Pig 2 90 dpi
107,575	SNP	Pig 2 90 dpi
107,577	SNP	Pig 2 90 dpi
109,297	SNP	Pig 2 90 dpi
109,386	SNP	Pig 1 365 dpi
109,389	SNP	Pig 1 365 dpi
109,927	SNP	Pig 2 90 dpi
110,001	SNP	Pig 3 365 dpi, Pig 3 90 dpi
110,501	SNP	Pig 1 365 dpi
110,529	SNP	Pig 1 365 dpi
110,533	SNP	Pig 1 365 dpi
110,548	SNP	Pig 1 365 dpi
110,549	SNP	Pig 1 365 dpi
110,552	SNP	Pig 1 365 dpi
110,561	SNP	Pig 1 365 dpi
111,984	SNP	Pig 2 90 dpi
112,106	SNP	Pig 1 365 dpi
112,703	SNP	Pig 1 365 dpi
112,872	SNP	Pig 1 365 dpi
112,968	SNP	Pig 1 Spleen
112,974	SNP	Pig 1 Spleen
112,977	SNP	Pig 1 Spleen
113,040	SNP	Pig 1 365 dpi
113,044	SNP	Pig 1 365 dpi
113,507	SNP	Pig 1 365 dpi
113,512	SNP	Pig 1 365 dpi
113,515	SNP	Pig 1 365 dpi
113,520	SNP	Pig 1 365 dpi
114,960	SNP	Pig 1 365 dpi
114,963	SNP	Pig 1 365 dpi
115,130	SNP	Pig 1 Spleen
115,138	SNP	Pig 1 Spleen
115,532	SNP	Pig 1 365 dpi
115,558	SNP	Pig 1 365 dpi

Position	Polymorphism Type	Variant Sequences
116,261	SNP	Pig 1 365 dpi
116,274	SNP	Pig 1 365 dpi
116,281	SNP	Pig 1 365 dpi
116,292	SNP	Pig 1 365 dpi
116,295	SNP	Pig 1 365 dpi
116,299	SNP	Pig 1 365 dpi
116,300	SNP	Pig 1 365 dpi
116,302	SNP	Pig 1 365 dpi
116,304	SNP	Pig 1 365 dpi
116,308	SNP	Pig 1 365 dpi
116,311	SNP	Pig 1 365 dpi
116,313	SNP	Pig 1 365 dpi
116,315	SNP	Pig 1 365 dpi
116,320	SNP	Pig 1 365 dpi
116,322	SNP	Pig 1 365 dpi
116,326	SNP	Pig 1 365 dpi
116,328	SNP	Pig 1 365 dpi
116,470	SNP	Pig 3 365 dpi, Pig 3 90 dpi
116,484	SNP	Pig 2 90 dpi
116,496	SNP	Pig 2 90 dpi
116,504	SNP	Pig 2 90 dpi
116,514	SNP	Pig 2 90 dpi
116,578	SNP	Pig 2 90 dpi
116,600	SNP	Pig 2 90 dpi
116,603	SNP	Pig 2 90 dpi
116,606	SNP	Pig 2 90 dpi
116,614	SNP	Pig 2 90 dpi
116,615	SNP	Pig 2 90 dpi
116,616	SNP	Pig 2 90 dpi
116,617	SNP	Pig 2 90 dpi
116,619	SNP	Pig 1 Spleen, Pig 2 90 dpi
116,622	SNP	Pig 2 90 dpi
116,625	SNP	Pig 2 90 dpi
116,627	SNP	Pig 2 90 dpi
116,630	SNP	Pig 2 90 dpi
116,632	SNP	Pig 2 90 dpi
116,642	SNP	Pig 2 90 dpi
116,644	SNP	Pig 2 90 dpi
116,811	SNP	Pig 2 90 dpi
116,812	SNP	Pig 2 90 dpi
116,813	SNP	Pig 2 90 dpi
116,814	SNP	Pig 2 90 dpi
116,815	SNP	Pig 2 90 dpi
116,816	SNP	Pig 2 90 dpi
116,817	SNP	Pig 2 90 dpi
117,540	SNP	Pig 1 365 dpi
117,576	Indel	Pig 1 365 dpi
117,577	Indel	Pig 1 365 dpi
117,578	Indel	Pig 1 365 dpi
117,844	SNP	Pig 1 365 dpi
117,849	SNP	Pig 1 365 dpi
117,853	SNP	Pig 1 365 dpi
117,858	SNP	Pig 1 365 dpi
117,861	SNP	Pig 1 365 dpi
118,139	SNP	Pig 3 365 dpi, Pig 3 90 dpi
119,105	SNP	Pig 1 365 dpi
119,298	SNP	Pig 1 365 dpi

Position	Polymorphism Type	Variant Sequences
119,299	SNP	Pig 1 365 dpi
119,300	SNP	Pig 1 365 dpi
119,426	SNP	Pig 1 Spleen
119,507	SNP	Pig 2 90 dpi
119,574	SNP	Pig 2 90 dpi
119,656	SNP	Pig 1 Spleen
119,657	SNP	Pig 1 Spleen
119,658	SNP	Pig 1 Spleen
119,664	SNP	Pig 1 Spleen
119,684	SNP	Pig 1 Spleen
119,685	SNP	Pig 1 Spleen
119,687	SNP	Pig 1 Spleen
119,692	SNP	Pig 1 Spleen
119,696	SNP	Pig 1 Spleen
119,704	SNP	Pig 1 Spleen
119,705	SNP	Pig 1 Spleen
119,706	SNP	Pig 1 Spleen
119,753	SNP	Pig 2 90 dpi
119,795	SNP	Pig 2 90 dpi
119,806	SNP	Pig 2 90 dpi
119,828	SNP	Pig 2 90 dpi
119,845	SNP	Pig 2 90 dpi
119,872	SNP	Pig 2 90 dpi
119,877	SNP	Pig 2 90 dpi
119,886	SNP	Pig 2 90 dpi
119,895	SNP	Pig 2 90 dpi
119,900	SNP	Pig 2 90 dpi
119,909	SNP	Pig 2 90 dpi
119,920	SNP	Pig 2 90 dpi
119,924	SNP	Pig 2 90 dpi
119,966	SNP	Pig 2 90 dpi
120,053	SNP	Pig 1 365 dpi
120,305	SNP	Pig 1 365 dpi
120,375	SNP	Pig 3 365 dpi, Pig 3 90 dpi
120,952	SNP	Pig 1 365 dpi
120,983	SNP	Pig 1 Spleen
120,985	SNP	Pig 1 365 dpi
120,987	SNP	Pig 1 365 dpi
120,992	Indel	Pig 1 365 dpi
120,993	Indel	Pig 1 365 dpi
120,994	Indel	Pig 1 365 dpi
120,995	Indel	Pig 1 365 dpi
120,996	Indel	Pig 1 365 dpi
121,013	SNP	Pig 1 Spleen
121,047	SNP	Pig 1 Spleen
121,117	SNP	Pig 1 Spleen
121,120	SNP	Pig 1 Spleen
121,147	SNP	Pig 1 Spleen
121,152	SNP	Pig 1 Spleen
121,159	SNP	Pig 1 Spleen
121,160	SNP	Pig 1 Spleen
121,165	SNP	Pig 1 Spleen
121,179	SNP	Pig 1 Spleen
121,180	SNP	Pig 1 Spleen

Appendix G

Ethical and Section 20 approvals

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	The role of *Ornithodoros* ticks in maintaining African swine Fever virus
PROJECT NUMBER	EC011-13
RESEARCHER/PRINCIPAL INVESTIGATOR	Mrs. C Boshoff

STUDENT NUMBER (where applicable)	92227661
DISSERTATION/THESIS SUBMITTED FOR	PhD

ANIMAL SPECIES	Pigs	
NUMBER OF ANIMALS	2	
Approval period to use animals for research/testing purposes		April 2013
SUPERVISOR	Prof. A Bastos / Dr L Heath	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 24 April 2013
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ANIMAL ETHICS COMMITTEE
Private Bag X04
0110 Onderstepoort

Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aec@up.ac.za

Ref: EC011-13 (Revised)

8 April 2013

Dr L Heath
Onderstepoort Veterinary Institute
(heathl@arc.agric.za)

Dear Dr Heath

EC011-13 (Revised) : The role of *Ornithodoros* ticks in maintaining African swine fever virus (C Boshoff)

Thank you for the revised application. The application for ethical approval dated 29 January 2013 is therefore approved by the Chairman of the Animal Ethics Committee on 5 April 2013.

Please provide the DAFF, Section 20 permit as soon as you have received it.

Kind regards

E Mostert

Elmarie Mostert

AEC Coordinator

Copy Mrs C Bosthoff

Mrs A O'Neil

ARC • LNR

APPROVED

ONDERSTEPOORT VETERINARY INSTITUTE
ANIMAL ETHICS COMMITTEE

AEC 19.12

Application for clearance to use vertebrate animals (including their embryos and foetuses) for an experimental study or in a standard operating procedure for diagnostic purposes

Project No: OV24/01/P001

Project Leader: Carin Boshoff

Project Title:
The role of *Ornithodoros* ticks in maintaining African swine fever virus

Where clearance is sought for a standard operating procedure (SOP), a separate clearance form must be completed per procedure

NOTE:

- Please read the Animal Ethics Categories form before you complete this application.
- This application must be typed.
- It must be signed by the Principal Investigator (the applicant) and other persons who are vouching for specialised aspects of the experimental design (i.e. statistician, safety officer, and persons responsible for supervising the use of scheduled medicinal substances).
- The application needs to be written simply but include all relevant detail.
- A score sheet must accompany all applications.
- If any animal during the experimental period gets sick/dies due to causes not related to the experimental work, a morbidity/mortality report must be submitted to the AEC accompanied by a full post mortem report.
- Once the experimental work starts a copy of the score sheet and a summary of the experimental work conducted must be visible at the experimental facilities in case of inspections by the AEC.
- Submitting applications:
  - An electronic copy of the application should be emailed to LopezL@arc.agric.za and a signed hard copy mailed to Dr Laura Lopez, Chairperson of the Onderstepoort Veterinary Institute – Animal Ethics Committee (OVI-AEC), Diagnostic Registration (Tel: 012-5299272).
  - **Deadline** for researchers to submit protocols will be the **1st of each month**. Late protocols go through to the next month.
    - If all the relevant signatures are present on the hard copies they will be distributed electronically to all AEC members for review and comments. Comments from AEC members will be submitted to Dr Lopez by the 10th of the month.
    - Dr Lopez will collate all the comments and send them back to the AEC members and applicants by the15th of the month.
    - Revised applications must be submitted by the 20th of the month.
    - AEC meetings will be held on the third Thursday of each month (except December) where all protocols and comments will be discussed, and final committee approval (or not) given, and communicated to applicants asap.
- Telephone enquiries on any animal ethics related matters may be directed to the Chairperson, Dr Laura Lopez (Tel: 012-529 9272, or LopezL@arc.agric.za).

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street, Riviera, 0084

From: Directorate Animal Health
Tel: 012 319 7532
Fax: 012 319 7470
E-mail: HenryG@daff.gov.za
Enquiries: Mr. Henry Gololo
Our Ref: 12/11/1/1
Your Ref No :

Carin Boshoff
Transboundary Animal Health Diseases Program
Private Bag X5
Onderstepoort
0110

Dear C. Boshoff

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

Your fax / memo / letter/ Email dated 06 February 2013 requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa.
2. Ticks collected from the ASF control area of the country, must be sealed in individual sample jars with screw caps as primary packaging. These individual sample jars must then be placed in self-sealed labelled biohazardous bags as secondary packaging, and these bags placed in biohazardous safety boxes with silicone gasket and side locks as tertiary packaging;
3. Ticks collected from the ASF control area of the country, must be transported from the ASF control zone directly to OVI-TADP under Red cross Permit issued by the responsible State Veterinarian;
4. Only extracted DNA may be supplied by OVI-TADP to Inqaba biotech, Hatfield, Pretoria
5. If histopathology is to be conducted, only samples in formalin may leave OVI-TADP
6. As ASF is a controlled animal disease in terms of the Animal Diseases Act, 1984 (Act No 35 of 1984), no information obtained during this study may be published or presented publically prior to approval by the Director Animal Health.

Title of research/study: "The role of *Ornithodoros* ticks in maintaining African swine fever virus. The aim of the study is to genetically characterise the genome of

an ASFV occurring in a naturally infected tick and following its evolution by cycling the virus to a vertebrate host (the domestic pig) and back to a naïve uninfected tick, under experimental conditions. (TADP-S-13/04)"
Researcher (s): Carin Boshoff
Institution: Transboundary Animal Health Diseases Program
Your Ref./ Project Number: TADP-S-13/04
Our ref Number: 12/11/1/1

Kind regards

DIRECTOR: ANIMAL HEALTH

DATE: ---------- 2013-04-22 ----------