

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

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African swine fever (ASF) is an economically significant haemorrhagic disease of domestic pigs. It is caused by the African swine fever virus (ASFV), a deoxyribonucleic acid (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 unknown, but this land-locked country is surrounded by ASF-positive countries, has a burgeoning 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% – 88.8% were recovered for the four *Ornithodoros*-infested reserves. A total of 562 ticks were screened for virus genome presence using a duplex Polymerase Chain Reaction (PCR) that targets the C-terminal end of the *p72* gene of the 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.

## 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 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 & 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 1a), and on strict bio-security measures that prevent contact between domestic pigs and *Ornithodoros* ticks (Penrith, Thomson & 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 1a). An important objective of livestock development in Swaziland is to increase animal production to meet the growing demand for animal protein. Recent increases in small-scale pig farming, particularly in communities in close proximity to game reserves containing sylvatic cycle hosts, has signalled the need for assessment of ASF status, as it is an important but unevaluated constraint for pig production. No prior surveys have been conducted; the ASF status of Swaziland is 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 present 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 Organization (FAO), with support of the European Union (EU), to reverse the country's declining agricultural productivity and to increase pig production (FAO 2011).



## Materials and methods

### Swaziland as the 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 km<sup>2</sup> and contains four separate geographical regions that run from north to south. These regions vary in altitude from 1800 m.a.s.l. – 200 m.a.s.l. 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, with an average altitude of 1200 m, lies on the edge of the escarpment; the middleveld has an average altitude of 700 m.a.s.l., with the lowveld of Swaziland being around 250 m.a.s.l. Swaziland's climate ranges from temperate 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 1000 mm – 2000 mm being recorded, with the lowveld in the east recording between 500 mm – 900 mm per annum (Atlapedia 1993–2011; Embassy of the Kingdom of Swaziland 2014).

### Study design

In Swaziland, there are national, private and community-owned protected areas including national parks, nature reserves, wildlife sanctuaries and game reserves. For the present study, access was granted for sampling in seven nature reserves and game parks (Table 1), and included parks located near the borders with South Africa and Mozambique. These parks form part of the Songimvelo-Malolotja Transfrontier Conservation Areas (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 unknown for the seven parks included in the present study.

In order to ensure consistency between sampling opportunities, a single team consisting of a sampling team leader and field assistant performed the collection under

the field guidance of a knowledgeable ranger at each specific 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 (3000 ha), respectively. Storm water pipes on dirt and tarred 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.

### Tick collection

Ticks were collected according to a modified manual collection method (Jori *et al.* 2013). Briefly, sand was collected from each burrow and spread in a thin layer across a large black plastic sheet, which was 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 min 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 the 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.

### Molecular analysis

Individual ticks were weighed and placed into 1.5 mL Eppendorf tubes 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 10% weight in volume (W/V) homogenate using a modified silica-guanidium thiocyanate method (Boom *et al.* 1990). A duplex polymerase chain reaction (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 those that target a conserved region of the mitochondrial 16S rRNA gene of *Ornithodoros* ticks, presence of virus genome and nucleic acid integrity could, respectively, be determined. Genomic amplification was performed in a final volume of

**TABLE 1:** Game parks and infestation rates of warthog burrows with *Ornithodoros* ticks in Swaziland.

Game Reserve or National Park	Latitude; longitude co-ordinates for the game reserves	Region in Swaziland	Approximate size of the game park (ha)	Altitudinal range across the park (m.a.s.l.)	Date sampled	Number of burrows with ticks	Number of burrows examined	Total number of ticks collected
Malolotya	26°08'49.0"S, 31°08'16.4"E	North-western	18 000	1830–640	November 2012	0	11	0
Mlilwane	26°28'40.1"S, 31°11'43.7"E	Western	4600	1450–670	March 2013	0	4	0
Nisela	26°58'20.3"S, 31°56'28.9"E	Southern	4000	230–140	April 2013	0	4	0
Hlane	26°16'59.3"S, 31°52'44.5"E	North-eastern	22 000	570–150	March 2013	3	9	20
Mkhaya	26°37'11.7"S, 31°45'13.3"E	South-eastern	10 000	310–225	May 2013	4	8	41
Mbuluzi	26°09'21.8"S, 31°58'56.5"E	North-eastern	3000	460–150	April 2013	8	13	232
Mlawula	26°12'26.7"S, 32°00'10.2"E	North-eastern	16 500	470–150	November 2013	8	9	269

50 µL 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, USA) and 3 µL of each DNA extract. Thermal cycling conditions prescribed for C-terminal *p72* gene amplification (Bastos *et al.* 2003) were used. The 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 and amplification of the 313 bp host gene target verifies DNA integrity. The DNA fragments of the expected size were excised from the gel and purified. Nucleotide sequences were generated for the tick mitochondrial gene target (Inqaba Biotechnologies, Pretoria, South Africa) of a geographically representative subset of tick samples. The resulting partial 16S rRNA sequences, submitted to Genbank under accession numbers KJ940548–KJ940550, were complemented with homologous data prior to phylogenetic inference in Mega 5 (Tamura *et al.* 2011).

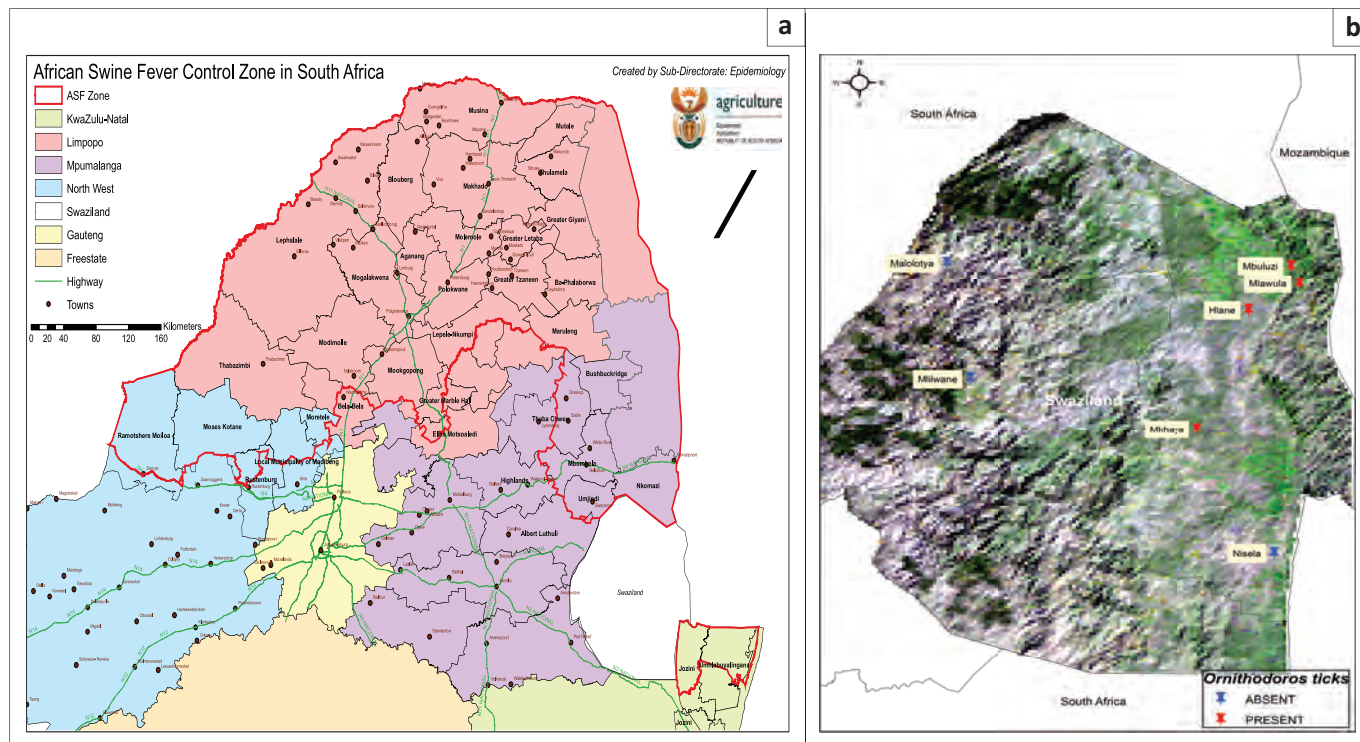
## Results

*Ornithodoros* ticks were found to be present in four of the seven game reserves that were sampled (Table 1 and Figure 1b). Tick infestation rates varied from 33.3% – 88.8% between the four tick-infested parks (Table 1). A total of 562 ticks were collected, of which 105 were female, 113 were male and the remaining 344 were nymphs. 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 *Ornithodoros* ticks in the game reserves that were evaluated.

## Discussion

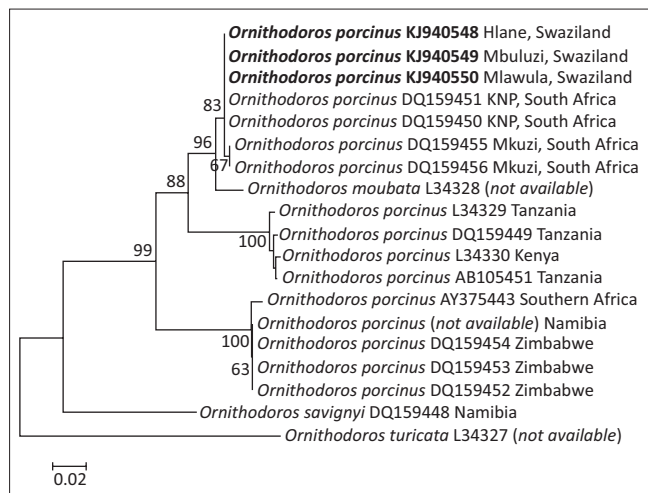
Ticks were only observed in parks situated in the eastern region of the country and were absent in the parks in the mountainous western region. Swaziland has a bowl-like terrain, being predominantly mountainous with forest escarpments in the north-western side, flattened throughout the centre of the country and becoming mountainous in the north-east along the Mozambique border. The game reserves that were positive for *Ornithodoros* tick presence all fell within the eastern savannah area and were associated with lower altitude localities (Figure 1b). 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 large differences in occurrence of *Ornithodoros* ticks were observed, leading the authors of that study to speculate that altitude may be an important factor influencing tick distribution (Haresnape & Mamu 1986).

The slight male bias in adult ticks collected in Swaziland is consistent with previous studies (Arnot, Du Toit & Bastos 2009; Thomson *et al.* 1983), 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



Source: (a) Department of Agriculture, Forestry and Fisheries (DAFF), Directorate Animal Health, n.d., *Disease maps*, viewed 14 January 2014, from <http://www.nda.agric.za/vetweb/Epidemiology/Disease%20Maps/ASFcopy.pdf>, (b) Map was drawn by Carin Boshoff with software ArcMap 10.2

**FIGURE 1:** Map indicating (a) Swaziland surrounded by the African Swine Fever control zone (indicated in red) and (b) the topography of Swaziland, the game reserves sampled in this study and *Ornithodoros* tick presence or absence.



Source: Neighbour joining tree was drawn by Carin Boshoff with MEGA 5.2

**FIGURE 2:** Neighbour joining tree depicting 16S rRNA gene relationships of *Ornithodoros* ticks sampled from three Swaziland wildlife reserves.

infection rate in adults versus nymphs (calculated based on results of the Thomson *et al.* 1983 study). This, together with the 1.4 fold higher sensitivity of PCR versus isolation (Basto *et al.* 2006), mitigates concerns regarding the adequacy of the Swaziland sample size and underscores the advantages of performing adult-tick-biased surveys (Arnot *et al.* 2009).

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 phylogeographical variation through comparison to reference data from southern and East Africa. Bastos *et al.* (2009) identified three geographically distinct *O. porcinus* lineages, with South African samples clustering within the south-east lineage. The gene phylogeny (Figure 2) confirmed that the sampled soft ticks from Hlane, Mlawula and Mbuluzi game reserves in Swaziland are identical to each other and all fall 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 Mkuze Game Reserve (MGR), respectively, across the gene region sequenced.

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, and 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, these negative results from Swaziland and Mkuze, once substantiated with additional,

more extensive studies, will assist in delineating, with greater confidence, the southernmost distributional range of ASF-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.

## 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 enzyme-linked immunosorbent assay (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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## Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

## Authors' contributions

C.I.B. (Agricultural Research Council-Onderstepoort Veterinary Institute) performed all of the experiments and prepared the manuscript. A.D.S.B. (University of Pretoria) assisted with the data analysis and preparation of the manuscript. M.M.D. (Central Veterinary Laboratory) was



responsible for sample collection approval and coordinating the fieldwork in Swaziland. L.H. (Agricultural Research Council-Onderstepoort Veterinary Institute) was responsible for funding and preparation of the manuscript.

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