A host-species informative internal control for assessing *African swine fever virus* infection rates in the African sylvatic cycle *Ornithodoros* tick vector

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Short title: ASFV in sylvatic *Ornithodoros* ticks

Abstract

African swine fever virus (ASFV) infection in adult *Ornithodoros porcinus* Walton (1962) ticks collected from warthog burrows in southern and East Africa was assessed using a duplex genomic amplification approach that is informative with respect to the invertebrate host species and infecting sylvatic cycle virus. DNA extracted from individual ticks was used as template for simultaneous amplification of a C-terminal 478 bp ASFV *p72* gene region and a ~313 bp fragment of the tick mitochondrial 16S rRNA gene, under optimised reaction conditions. Within-warthog-burrow infection rates ranged from 0 to 43 % using this approach, whilst phylogenetic analysis of 16S gene sequences revealed the presence of three geographically discrete *O. porcinus* lineages, but no support for recognition of *O. p. porcinus* and *O. p. domesticus* subspecies. False negatives are precluded by inclusion of host-species informative primers that ensure the DNA integrity of cytoplasmically-located genome extracts. In addition, infection rate estimates are further improved as false positives associated with carry-over contamination when performing a two-step nested PCR are negated by the one-step approach. Phylogenetic comparison of full-length virus gene sequences with the partial C-terminal *p72* gene target confirmed the epidemiological utility of the latter in a sylvatic setting. The method is therefore of particular value in studies assessing prevalence and diversity of ASFV in relation to the African sylvatic tick vector and holds potential for investigating the role of alternative tick species in virus maintenance and transmission.

Key words: *Ornithodoros porcinus*, African swine fever virus, PCR, *p72*, 16S rRNA, phylogeny

Introduction

The causative agent of African swine fever (ASF), a viral haemorrhagic disease of domestic pigs, is *African swine fever virus* (ASFV), a double-stranded DNA virus of the *Asfivirus* genus (Penrith et al. 2004). In southern and East Africa the virus is maintained in an ancient sylvatic cycle in which the warthog-associated, burrow-dwelling argasid tick of the genus *Ornithodoros*, plays a central role (Kleiboeker & Scoles 2001). The virus is believed to be maintained primarily by these eyeless, soft-shelled hosts which are able to transmit the virus trans-ovarially, trans-stadially and sexually (reviewed by Kleiboeker & Scoles 2001). In contrast, the vertebrate warthog host, *Phaecocoerus africanus*, is considered an end host as blood virus concentrations are generally low (< $10^{1.0}$ HAD_{50}/ml) in free-living adult animals and evidence for vertical and lateral transmission is lacking (reviewed by Thomson 1985). However, some naturally infected neonate warthogs have sufficiently high blood virus levels (> $10^{3.0}$ HAD_{50}/ml) to infect ticks and it has been proposed that the transient viraemia in these young animals may play a key role in maintaining virus levels and infection in the tampan hosts (reviewed by Thomson 1985). Virus transmission from the sylvatic tick-warthog cycle to domestic pigs occurs via the soft tick vector (Kleiboeker & Scoles, 2001; Penrith et al. 2004).

ASF although primarily confined to Africa, became widespread throughout Europe, the Caribbean and Brazil between 1960 and 1990, after its initial export to Portugal in 1957 and again in 1960 (Penrith et al. 2004). In these countries where the disease is exotic, and in West Africa, the viruses were shown to be genetically homogeneous across the C-terminal *p72* gene region characterised (Bastos et al. 2003) and a single virus genotype (genotype I), predominated prior to the recent introduction of a genotype II virus to Georgia in June 2007 (Oura & Dixon 2007).

Genetic homogeneity across the C-terminal region of the *p72* gene appears to be peculiar to areas in which a domestic pig cycle has become established in the absence of the soft tick vector and has thus far been associated with two viral genotypes, genotypes I and VIII (Bastos et al. 2003; Lubisi et al. 2007). The remaining 20 of the 22 distinct *p72* virus genotypes described to date (Bastos et al. 2003; Lubisi et al. 2005; Boshoff et al. 2007), occur in southern and East Africa in areas overlapping sylvatic host distribution, and are generally associated with higher levels of genetic heterogeneity across this gene region.

The persistence of ASF in exotic localities has been attributed to the presence of suitable vertebrate and invertebrate hosts (Perez-Sanchez et al. 1994) and it was the identification of ASFV in the Iberian soft tick *Ornithodoros erraticus* Lucas (Sanchez-Botija, 1963) that led to the seminal discovery of the virus in the natural *Ornithodoros* tampan host in Africa (Plowright et al. 1969). Subsequent experimental infection experiments have demonstrated that the virus can be transmitted by and/or maintained in a variety of non-sylvatic cycle Argasid ticks, including *O. erraticus*, *O. marocanus* Velu, *Carios puertoricensis* Fox, *O. coriaceus* Koch, *O. turicata* Dugès and *O. savignyi* Audouin (Sanchez-Botija, 1963; Mellor & Wilkinson, 1985; Hess et al. 1987; Endris et al. 1992). In the sylvatic-cycle-associated African *Ornithodoros* tick, ASFV pathogenesis is well documented and is characterized by a low infectious dose, lifelong infection and efficient transmission between ticks and suids (reviewed by Kleiboeker & Scoles 2001). In contrast, the taxonomy of this Argasid tick is variable in literature pertaining to its role as a vector for ASFV, being referred to either as *O. moubata*, *O. moubata porcinus*, *O. porcinus* or *O. porcinus porcinus* (reviewed by Kleiboeker & Scoles 2001).

Walton (1962, 1979) originally proposed recognition of four *Ornithodoros* species within the *O. moubata* complex, namely *O. compactus*, *O. apertus*, *O. moubata* and *O. porcinus*. However, Van der Merwe (1968) did not consider *O. porcinus* to be a separate species, but rather a subspecies of *O. moubata*, which she termed *O. moubata porcinus*. It was this alternative subspecies designation and that resulted in ASF sylvatic vector literature becoming fraught with taxonomic inconsistencies, and it is only in recent years that the original classification of Walton (1962, 1979) has started receiving due precedence. Walton (1962, 1979) recognized two *O. porcinus* subspecies, viz. *O. p. domesticus* (associated with human dwellings) and *O. p. porcinus* (a warthog burrow dwelling tick), with the latter subspecies being considered the reservoir of African swine fever (ASF). The role of *O. moubata*, a species confined to xeric areas with annual rainfalls of 50mm or less and from which the complex derives its name, was not known in this respect. These past taxonomic inconsistencies underscore the need for renewed investigations into ASFV tick host taxonomy particularly in light of the undisputed central role played by *Ornithodoros* sylvatic cycle ticks in the epidemiology of the disease in Africa, and the recent 2007 incursion of an East African virus genotype into Europe.

Precise estimates of virus infection rates in warthog-feeding African *Ornithodoros* ticks have remained elusive due mainly to technical difficulties experienced with virus isolation that arises from the low virus load and the limited amount of starting material. *In vitro* isolation of ASFV from ticks by conventional methods has, in the past, required that ticks be pooled prior to culture inoculation and that multiple blind passages be performed. Alternatively, *in vivo* isolation, which is now ethically untenable, was performed and involved injection of ground tick homogenate into domestic pigs (Plowright et al. 1969; Plowright 1977; Thomson et al. 1983).

Pooling can result in an underestimation of infection rate, whilst the execution of numerous, successive blind passages increases the risk of laboratory contamination (and false positives), particularly when performed in open-well, plate-format. Both impact negatively on the accuracy of infection rate estimates.

Sensitive PCR-based techniques provide a rapid and alternative means of confirming ASFV presence, and are increasingly being applied due to their success with difficult clinical samples, such as those that have been formol-inactivated (Bastos et al. 2004), and those dried and stored on filter paper (Michaud et al. 2007). The *p72* gene has been the focus of many molecular studies, being advocated for virus genome detection (OIE manual; King et al. 2003), pre-clinical diagnosis (Zsak et al. 2005) and for genotype delineation (Bastos et al. 2003). A nested PCR targeting this gene has also been prescribed for molecular assessment of ASFV infection in *Ornithodoris erraticus* (Basto et al. 2006), the European tick counterpart of *O. porcinus.* Whilst the latter approach has been valuable in confirming the suitability of PCR-based approaches for screening ASF tick vectors, the recommended two-tube nested PCR format lends itself to carry-over contamination and false positives, whilst the epidemiological potential of the *p72* viral gene target, that was designed to be virus-confirmatory, was not evaluated.

In light of the taxonomic inconsistencies of the African tick host, the plethora of *p72* gene-targeting primer sets and past difficulties associated with confirming virus presence in the African invertebrate host, this study set out to establish a one-step, duplex PCR that is host-species and virus-genotype informative, in addition to being virus-confirmatory. This was achieved by simultaneous amplification of the taxonomically informative 16S rRNA mitochondrial gene (Black & Piesman, 1994; Teglas et al. 2005) of the soft tick host and a suitable ASFV *p72* partial gene target with sylvatic genotype discriminatory power. The latter was determined by comparing the

genotype delineation capabilities of the different partial gene regions targeted with available primers, against that of a full-length *p72* gene dataset (Zsak et al. 2005).

Materials & Methods

Comparative genotype delineation utility of partial p72 gene fragments

Complete *p72* gene sequences of 16 taxa, namely AY578689, AY578690, AY578692, AY578694, AY578695, AY578696, AY578698, AY578699, AY578702, AY578704, AY578705, AY578706, AY578708, L00966 (Zsak et al. 2005), L27499 and L76727 (Yu et al. 1996) were used to compile the reference, full-length gene dataset. The five most widely used/cited *p72* gene primer sets and those prescribed for ASFV detection in *O. erraticus* ticks (Table 1), producing a dataset ≥ 200 nucleotides (nt) in length, and therefore of possible phylogenetic utility, were compiled from the 16 taxon full-length sequence dataset. Neighbor-joining (NJ) trees were inferred using Mega4 (Tamura et al. 2007) with nodal support being evaluated by 10 000 bootstrap replications (Fig. 1). Basic sequence statistics, including proportion of variable and parsimony informative sites, base composition and transition:transversion ratio (R) were also estimated using Mega4 (Table 1). The resulting complete *p72* gene phylogeny (Fig.1, (i)), was then compared to the gene trees inferred from partial sequence datasets (ii)-(vi) arising from different published gene targets (summarised in Table 1), to identify the partial gene region best suited to resolving sylvatic genotype lineage relationships.

Primer selection

Primers targeting the 478 bp fragment corresponding to the C-terminal end of the *p72* gene of the ASF virus genome (Bastos *et al*. 2003), shown to be the optimal gene target for sylvatic genotype resolution (Fig. 1), were selected to target the viral genome. The invertebrate host primers were designed to target completely conserved regions within the aligned mitochondrial 16S rRNA gene of ten argasid sequences (Black & Piesman 1994), and to have an annealing temperature similar to that of the *p72* primers. The host gene primers, 16S-FArg 5'-GGACAAGAAGACCCTATGAAT-3' and 16S-RArg 5'-CCGGTCTGAACTCAGATCA-3' targeting an amplicon of approximately 313 bp, were identified on this basis.

Tick collection and DNA extraction

Ticks were collected from 12 warthog burrows at six localities (summarised in Table 2) in East and southern Africa. Briefly, sand was removed from warthog burrows using a shovel and then sieved over a plastic sheet, following which the ticks were individually picked from the sieve and placed in 100 % ethanol. On return to the laboratory, ticks were sorted into two categories, namely adults and nymphs, following which, individual adult ticks were placed in a 1.5 ml Eppendorf tube and submerged in liquid nitrogen for 1 minute prior to adding phosphate-buffered saline (PBS) and sterile sand. 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-based DNA capture method (Boom *et al*. 1990). DNA was eluted in a final volume of 30 µl 1X Tris-EDTA buffer and stored at -20°C until further use.

ASF prevalence determination

The ideal primer concentration range for the duplex PCR was determined by maintaining the virus primer set at a constant at 0.5 μM and allowing the 16S primer concentration to range from 0.25 to 1.0 μM (in 0.25 μM intervals), and vice-versa to identify the optimal primer concentration for duplex PCR. All subsequent genomic amplifications were performed in a final reaction volume of 50 μl containing 0.5 μM of the p72-U and p72-D virus primers (Bastos et al. 2003), 0.5 μM of the 16S-FArg and 16S-RArg host gene primers, 3μl of template DNA and 1U of *Taq* polymerase (Biotools). Thermal cycling conditions prescribed for *p72* gene amplification (Bastos et al. 2003) were used and PCR products were evaluated by 1.5 % agarose gel electrophoresis against a 100 bp ladder (Promega) and 1 kb GeneRuler (Fermentas). If both the host and virus targets amplified then the sample was scored as positive for ASFV. If only the 16S gene amplified then the sample was scored as negative for ASFV presence. If neither band amplified, then the duplex PCR was repeated with a DNA concentration range of 1 μl through to 5 μl, in 1 μl intervals. Following two consecutive negative results, the sample was considered to be a false negative and was excluded from estimations of warthog burrow infection rates.

Phylogenetic analysis of the 16S rRNA tick host gene

In order to assess geographical variation within the ASFV-associated invertebrate host, the 16S rRNA gene of at least one *Ornithodoros porcinus* tick sampled from warthog burrows in South Africa, Namibia, Zimbabwe and Tanzania was purified using the High Pure PCR Product Purification kit (Roche) and sequenced by means of an automated cycle sequencing approach with BigDye v3.1 (Perkin-Elmer). Partial 16S nucleotide sequences generated in this study for 11 argasid ticks within the

genera *Ornithodoros* and *Otobius*, and one ixodid tick, *Amblyomma herbraeum* Koch, 1844 were submitted to Genbank under the accession numbers indicated in Table 2. These sequences were complemented with 14 homologous tick sequences from the Genbank database, resulting in a final dataset comprising four soft tick genera (*Ornithodoros, Otobius, Antricola* and *Carios*) and the *Amblyomma* outgroup. Sequences were aligned in ClustalX (v 1.82; Thompson *et al.* 1997) and adjusted according to the 16S rRNA secondary structure model determined with RNAfold (Vienna RNA Package v 1.4; Hofacker *et al.* 1994). Maximum parsimony (MP) analyses were performed in PAUP* 4.0b10 (Swofford 2003), neighbor-joining (NJ) with Mega4 (Tamura et al. 2007) and Bayesian Inference (BI) with MrBayes v3.04 (Huelsenbeck & Ronquist 2001). MP trees were generated through heuristic searches with tree bisection-reconnection (TBR) branch swapping and randomization of starting options (100 replicates). Parsimony trees were initially inferred using equal weighting of characters, following which successive weighting of characters (Farris 1969) with the rescaled consistency index (RCI) was used to generate a phylogeny. In both cases, gaps inserted for alignment purposes were treated as a fifth character state. The degree of character support for each node was assessed following 10000 nonparametric bootstrap replicates for MP and NJ analyses. Bayesian inference was conducted under the best-fit HKY+I+Γ model identified under the Akaike Information Criterion (AIC) in Modeltest v3.06 (Posada & Crandall 1998), and the Markov chain Monte Carlo (MCMC) process started from random starting points with 4 chains run simultaneously for $5x10^6$ generations, using default heating and swap parameters. Three independent runs were conducted to assess whether all converged to stationarity at similar likelihood scores. Trees and parameters were recorded every 100 generations and the first 500 000 trees (10 %) were discarded as the ‘burn-in’.

Results

Comparative utility of partial p72 *gene targets for sylvatic cycle virus resolution*

The full-length gene phylogeny (Fig. 1 (i)) recovered eight *p72* genotypes and three main ASFV evolutionary lineages, coinciding with those previously identified following C-terminal gene sequencing (Bastos et al. 2003; Lubisi et al. 2005; Boshoff et al. 2007). However, in contrast to the C-terminal gene target (Fig. 1 (v)), the full-length (1941 nt) gene tree displayed higher levels of heterogeneity for genotype I viruses. This higher within-genotype I heterogeneity of the full-length gene phylogeny was best reflected in the partial gene tree of the 330 nt dataset (Fig. 1 (iii)), amplified with the external PCR primers of Basto and co-workers (2006), attesting to the appropriateness of these primers for resolving virus relationships of the *O. erraticus*-genotype I virus infected tick host, for which they were designed. However, the same 330 nt partial gene target was homogeneous between sylvatic cycle virus genotypes, with genotypes III and XIX viruses being identical to each other across this gene region. Similarly, the OIE diagnostic PCR target of 238 nt (Fig. 1 (ii)) displayed poor between-genotype resolution, being incapable of discerning between viruses assigned to distinct genotypes (III, IV, XIX and XX) in the complete gene phylogeny. Within-genotype-I resolution of the OIE primer target was intermediate to that of datasets (iii) and (v). The remaining two partial gene datasets (iv) and (vi) which are nested within datasets (iii) and (v) respectively, reflected the same tree topology as their larger dataset counterparts, but generally had lower levels of bootstrap support. Sequence statistics indicated that whilst the truncated datasets essentially reflected the features of the full-length gene dataset (i), that extreme bias occurred in the smaller data subsets (iv) and (vi), as exemplified by

the 205 nt (King et al. 2003) primer target (dataset vi) that had an R value > 10 (Table 1).

ASFV burrow infection rates and evaluation of tick primer recognition range

Amplification of both gene targets occurred across all the primer concentration ranges tested (Fig. 2), however, 16S rRNA primer concentrations above 0.5 μM were shown to dampen amplification of the *p72* virus gene (lanes 2-5 in Fig. 2). In contrast, virus gene primer concentrations above 0.5 μM did not affect 16S rRNA gene amplification to the same extent (Fig. 2, lanes 6-9). The primer concentration combination that resulted in optimal amplification of both gene targets in a single reaction was an equimolar 0.5μM:0.5μM, virus-primer concentration to tick-primer concentration (Fig. 2). On confirmation that the 16S host and *p72* virus PCR primers could be combined within a single amplification reaction, a total of 142 adult ticks from 12 warthog burrows were screened using this method at the optimal primer concentrations. Results (summarised in Table 3) indicated that within-warthog burrow infection rates of adult *Ornithorodos* ticks ranged from 0 % (in seven burrows) to 42.8 % (in a single burrow in western Zimbabwe). The mean infection rate was 6.3 % when all ticks screened were included in the estimation, and 6.9 % when tick samples that failed to amplify the host 16S gene (N=12) were excluded from the calculation (Table 3).

The broader tick species recognition range of the 16S rRNA primers was confirmed by amplification and sequencing of the 16S gene target in two additional species within the Ornithodorinae subfamily, namely the eye-bearing *O. savignyi* congeneric, and the more distantly related *Otobius megnini* Dugès (Table 2). In addition, species within the Argasinae subfamily and two ixotid ticks, *Ixodes uriae*

White (results not shown) in addition to *Amblyomma herbraeum*, amplified with the host internal control primers. The latter specimen which was recovered from one of the warthog burrows was used for outgroup purposes in this study (Table 2).

16S rRNA tick gene sequence statistics

The alignment of 16S rRNA gene sequences of *Ornithodoros*, *Carios*, *Antricola* and *Otobius* to the outgroup *A. herbraeum* resulted in a homologous dataset of 284 sites containing 140 variable and 121 parsimony informative characters. Estimation of HKY+I+Γ model parameters in ModelTest produced a ti/tv ratio of 1.5107, a gamma-shape parameter (α) of 0.5172 and proportion of invariant sites (I) of 0.2523. Estimated mean base frequencies under this model (A = 0.3814, C = 0.0581, G = 0.1420 and T = 0.4185) were consistent with the A-T richness documented for 16S rRNA of ixodid ticks (Black & Piesman 1994).

16S rRNA tick gene phylogeny

All three methods of phylogenetic reconstruction applied to the 16S rRNA dataset resulted in topologically similar trees. Nodes with high posterior probability values from the Bayesian algorithm generally also received high bootstrap support in the MP and NJ analyses (Fig. 3), suggesting that the evolutionary associations are reliable (Hillis 1995). An unweighted parsimony analysis recovered two trees (365 steps, CI = 0.688, HI = 0.321, RI = 0.857, RCI = 0.589), whilst reweighting with the RCI resulted in a single tree (216.18 steps, CI= 0.815, HI= 0.185, RI= 0.921, RCI=0.750).

Three major clades confirming the monophyly of the soft tick genera *Otobius*, *Ornithodoros* and *Carios* were recovered with high bootstrap support and significant (p

< 0.05) posterior probabilities across all methods of analysis (Fig. 3). Similarly, the monophyly of *O. savignyi* and the *O. moubata* species complex, as well as the sister-taxon status of these two lineages was well supported. Genetic characterisation of warthog-dwelling ASFV-associated ticks, which in accordance with Walton (1979) were designated *Ornithodoros porcinus porcinus* in this study (Table 2), were shown to cluster according to geographical origin within three distinct clades (Fig. 3) rather than by host-species preference/association. The three *O. porcinus* lineages denoted (i) to (iii) in Fig. 3 comprised, (i) a south-western clade incorporating *O. p. porcinus* from Namibia and Zimbabwe and a single *O. porcinus* sequence (Genbank entry AY375443) from southern Africa, (ii) a north-eastern clade in which four East African specimens clustered, viz. two *O. p. porcinus* (Genbank entries L3432928 and AB105451) both from Tanzania, one *O. p. domesticus* (Genbank entry L34330) from Kenya and an *O. p. porcinus* sequence generated in this study, and (iii) a south-eastern clade, sister to the Tanzania-Kenya north-eastern clade (ii), in which exclusively *O. p. porcinus* ticks from two South African localities (Mkuze and the Kruger National Park) clustered. A single *O moubata* sequence (Genbank entry L34328) occurred basal to the South African *O. p. porcinus* lineage, with high levels of support (83-98 %). The results therefore did not support the monophyly of a xeric *O. moubata* species distinct from mesic *O. porcinus*, nor was there any support for recognition of the *O. p. porcinus* and *O. p. domesticus* subspecies designations. All analyses did however consistently indicate *Ornithodoros savignyi* to be basal and sister to the eyeless *O. moubata* species complex clade.

Discussion

Screening of individual ticks for ASFV by conventional cell-culture isolation is time-consuming and costly, particularly as infection rates are generally very low.

Previous isolation-based estimates of ASFV tick infection rates ranged from 0.017 % to 1.35 % in an East African study (Plowright 1977) and from 0.06 % to 1.4 % at different South African localities (Thomson et al. 1983). The duplex PCR method detailed here provides a rapid pre-screening approach for identifying ASFV-positive tick homogenates, on which to focus isolation efforts. The host 16S rRNA gene primers have the dual diagnostic function of acting as an internal PCR control and as an indicator of successful DNA extraction of cytoplasmically located genomes. This is particularly valuable as this mitochondrial genome target which like the ASFV genome occurs in multiple copies in the cytoplasm, provides a means of assessing the integrity of nucleic acids occurring outside of the nucleus. As burrows contain both live and dead ticks, it is likely that some of ticks collected by means of sand-sifting followed by the immediate freezing or storage in ethanol, may lack viable virus and intact DNA due to the rapid nucleic acid degradation that occurs following death of an organism. This is a generally overlooked aspect that can result in an under-estimation of tick infection rates, and one that is not addressed in molecular-based approaches in which the addition of foreign DNA is advocated for internal PCR control purposes. The duplex PCR described here incorporates a host-DNA internal control that provides a means of identifying those samples containing no detectable host DNA and by implication no viable virus. Exclusion of these non-viable samples from the calculation of infection rates leads to more accurate estimations of within-burrow infection rates as false negative results arising from DNA degradation or a failed DNA extraction are precluded. Between-burrow estimations are also improved due to the ability to screen individual ticks by PCR, instead of pooling samples (Plowright 1977; Thomson et al. 1983). In this study, warthog burrow infection rates ranged from 0 % to 43 %, which concurs with previous estimates based on virus isolation in which infection rates

obtained from pooled adult ticks, ranged from 0 % to 33 % between burrows (Plowright et al. 1969).

The recent availability of complete *p72* gene sequences (Zsak et al. 2005) made it possible to critically assess the usefulness of different partial *p72* gene targets in discrete epidemiological settings. This was achieved by benchmarking the gene phylogenies obtained using published primers targeting partial gene regions, against that obtained with the full-length gene dataset, which served as the 'gold standard' gene phylogeny. The previously described *O. erraticus* PCR tick screening gene target (Basto et al. 2006) was shown to be best-suited to resolving within-genotype I gene relationships. However, the between-genotype homogeneity for sylvatic viruses renders this partial *p72* gene region of limited use for epidemiological studies on ASFV-positive, warthog-associated African *Ornithodoros* ticks. In contrast, the C-terminal gene region (Bastos et al. 2003) which aside from being incapable of resolving intra-genotype I virus relationships, best reflected the between-genotype delineation power of full-length gene sequences and was therefore deemed to be of greater epidemiological utility in a sylvatic setting. In addition, these C-terminal targeting primers which have a demonstrated broad virus recognition range, have been successfully applied in genetic characterisation studies of ASF viruses from a variety of geographically disparate localities (Bastos et al. 2003; Bastos et al. 2004; Lubisi et al. 2005; Nix et al. 2006; Wambura et al. 2006; Boshoff et al. 2007) resulting in an extensive and valuable sequence database for comparative studies.

The 16S gene phylogeny revealed no support for the recognition of two distinct subspecies within *O. porcinus.* Instead, three geographically distinct *O. porcinus* lineages were recovered. Based on this, it may be more appropriate to denote the African argasid warthog- and ASFV-associated species as *O. porcinus* hence-forth. The

phylogeny also highlighted a need for further studies into the validity of the *O. moubata* species designation, which on the basis of a single sequence, appears to be sister to one of the three geographically-discrete *O. porcinus* lineages. This is in sharp contrast to the expectation that *O. moubata* would be sister to, but distinct from all *O. porcinus* lineages and implies that the xeric adaptation may have been recently derived. If confirmed following through extensive studies, this would imply that *O. moubata* is a subspecies of *O. porcinus*, and would invalidate the *O. moubata porcinus* subspecies designation proposed by van der Merwe (1968).

Changing climatic conditions and concomitant changes in the distributional ranges of ticks and tick communities (Cumming & van Vuuren 2006) has implications for the role of additional tick species in disease maintenance and vectoring. The range of many African tick species with recognised host-feeding preferences does not appear to be limited by host distributions (Cumming 1999) implying that host switching is likely to occur when preferred hosts are unavailable. This together with increased translocation of wildlife species means that new vertebrate-tick and new tick-disease relationships may become established. It is therefore important to have suitable methods in place that permit evaluation of the role of known and alternative tick species in transmitting DNA arboviruses, such as ASFV.

The one-step duplex PCR detailed here was shown to be better-suited to sylvatic cycle studies involving African *Ornithodoros* ticks, than the molecular-based approach described previously (Basto et al. 2006). This is due to the epidemiological utility of the viral gene target and the preclusion of false-negatives linked to non-viable virus, as well as false-positives associated with a two-step nested PCR approach. In addition, the internal control primers which target the host mitochondrial genome proved valuable for clarifying the taxonomy of the tampan hosts that are associated with the ASFV sylvatic

cycle in Africa, and have a sufficiently broad species recognition range to permit evaluation of the role of other soft tick species in the transmission ASFV, and other disease agents. Nucleotide sequencing of the simultaneously amplified host species and virus genotype informative genes facilitates further expansion of the extensive, but very much domestic pig-focussed *p72* ASFV genotype reference sequence database and will ensure that virus variability in relation to sylvatic invertebrate host diversity can be addressed more effectively in future.

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TABLE 1 Comparison of the sequence statistics and features of the full-length *p72* gene dataset (i) comprising 16 taxa, *versus* that of partial gene datasets (ii-vi) of the same 16 taxa, with the latter datasets corresponding to *p72* gene fragments targeted by published primer sets prescribed for detection and/or molecular epidemiological clarification of *African swine fever virus* relationships

Dataset number and nucleotide (nt) length	Gene position	Primer names	Reference(s) for the primer sequences	Va sites (% of total)	Pi sites (% of total)	% Va sites that are Pi	R (Ti/Tv)	%AT (T/C/A/G)
(i) 1941 nt	1-1941	F + R	Zsak et al. 2005 Yu et al. 1996	168 (8.7 %)	108 (5.6 %)	64.3 %	3.5	55.7 % (27/24/29/20)
(ii) 238 nt	792-1029	1 + 2	OIE Manual	18 (7.6 %)	11 (4.6 %)	61.1 %	5.0	54.2 % (25/28/31/17)
(iii) 330 nt	918-1247	72ARs + 72ARas	Basto et al. 2006	26 (7.9 %)	16 (4.8 %)	61.5 %	2.5	54.6 % (30/25/27/18)
(iv) 201 nt	984-1184	72Ns + 72Nas	Basto et al. 2006	18 (9.0 %)	13 (6.5 %)	72.2 %	1.9	54.1 % (31/25/23/21)
(v) 437 nt	1482-1918	p72-D + p72-U	Bastos et al. 2003	43 (9.8 %)	30 (6.9 %)	69.8 %	4.6	55.8 % (30/24/27/19)
(vi) 205 nt	1653-1857	F + R	King et al. 2003	24 (11.7 %)	15 (7.3 %)	62.5 %	10.7	55.5 % (27/23/28/22)

Va=variable; Pi=Parsimony informative; R=transition/transversion (Ti/Tv) ratio

TABLE 2 Summary of samples for which 16S rRNA data were generating using the host-species informative internal PCR control primers

Species	Sample name	Geographical origin	Genbank Accession No	Reference
Otobius megnini	--	New Mexico	L34327	Black & Piesman 1994
O. megnini	OtM01	South Africa	DQ159447	This study
Antricola mexicanus	--	NK	L34323	Black & Piesman 1994
Antricola marginatus	--	NK	L34324	Black & Piesman 1994
Carios capensis	TexasB	Texas, USA	AB076082	Ushijima et al. 2003
C. capensis	Hawaii1	Hawaii	AB057538	Ushijima et al. 2003
Carios sawaii	CS Miyazaki 1	Miyazaki, Japan	AB242430	Kawabata et al. 2004
C. sawaii	CS Kyoto 1	Kyoto, Japan	AB242438	Kawabata et al. 2004
Ornithodoros turicata	--	NK	L34327	Black & Piesman 1994
Ornithodoros savignyi	OS-01	Namibia	DQ159448	This study
Ornithodoros moubata	--	NK	L34328	Black & Piesman 1994
Ornithodoros porcinus	Tnop1	Tanzania	AB105451	Mitani et al. 2004
O. porcinus	TD02-052	Southern Africa	AY375443	Scoles 2004
O. porcinus domesticus	--	Digo District, Kenya	L34330	Black & Piesman 1994
O. porcinus porcinus	--	Shiyanga, Tanzania	L34329	Black & Piesman 1994
O. p. porcinus	Mkuzi50A	Mkuze, South Africa	DQ159455	This study
O. p. porcinus	Mkuzi81B	Mkuze, South Africa	DQ159456	This study
O. p. porcinus	KNPBoy1	Kruger NP, South Africa	DQ159450	This study
O. p. porcinus	KNPSku7	Kruger NP, South Africa	DQ159451	This study
O. p. porcinus	Zim395	Zimbabwe	NA	This study
O. p. porcinus	Hw713	Hwange NP, Zimbabwe	DQ159453	This study
O. p. porcinus	Hw714	Hwange NP, Zimbabwe	DQ159452	This study
O. p. porcinus	Nam395	Namibia	DQ159454	This study
O. p. porcinus	Tan195	Serengeti, Tanzania	DQ159449	This study
Amblyomma hebraeum	--	NK	L34316	Black & Piesman, 1994
A. herbraeum	AH-KNP01	Kruger NP, South Africa	DQ159446	This study

NA: Not available; NK: Not known; NP: National Park

TABLE 3 Within-burrow infection rates of adult *Ornithodoros* ticks determined by duplex PCR screening

Burrow Locality	**Number of adult ticks with DNA of confirmed integrity (Total number screened)**	**No of ASFV-positive ticks**	**ASFV infection rate / warthog burrow**
Kruger NP, Skukuza, South Africa	8 (8)	1	12.5 %
Kruger NP, Crocodile Bridge, South Africa	15 (15)	3	20.0 %
Kruger NP, Balule, South Africa	16 (16)	0	0 %
Kruger NP, Boyela, South Africa	11 (11)	1	9.1 %
Mkuze NP, Burrow M27, South Africa	21 (21)	0	0 %
Mkuze NP, Burrow M50, South Africa	5 (5)	0	0 %
Mkuze NP, Burrow M81, South Africa	28 (28)	0	0 %
Hwange NP, Burrow A, Zimbabwe	7 (7)	3	42.8 %
Hwange NP, Burrow B, Zimbabwe	7 (7)	1	14.3 %
Zimbabwe 395	6 (12)	0	0 %
Namibia	1 (2)	0	0 %
Serengeti NP, Tanzania	5 (10)	0	0 %
TOTAL	**130 (142)**	**9**	**6.9 %** (*6.3 %)

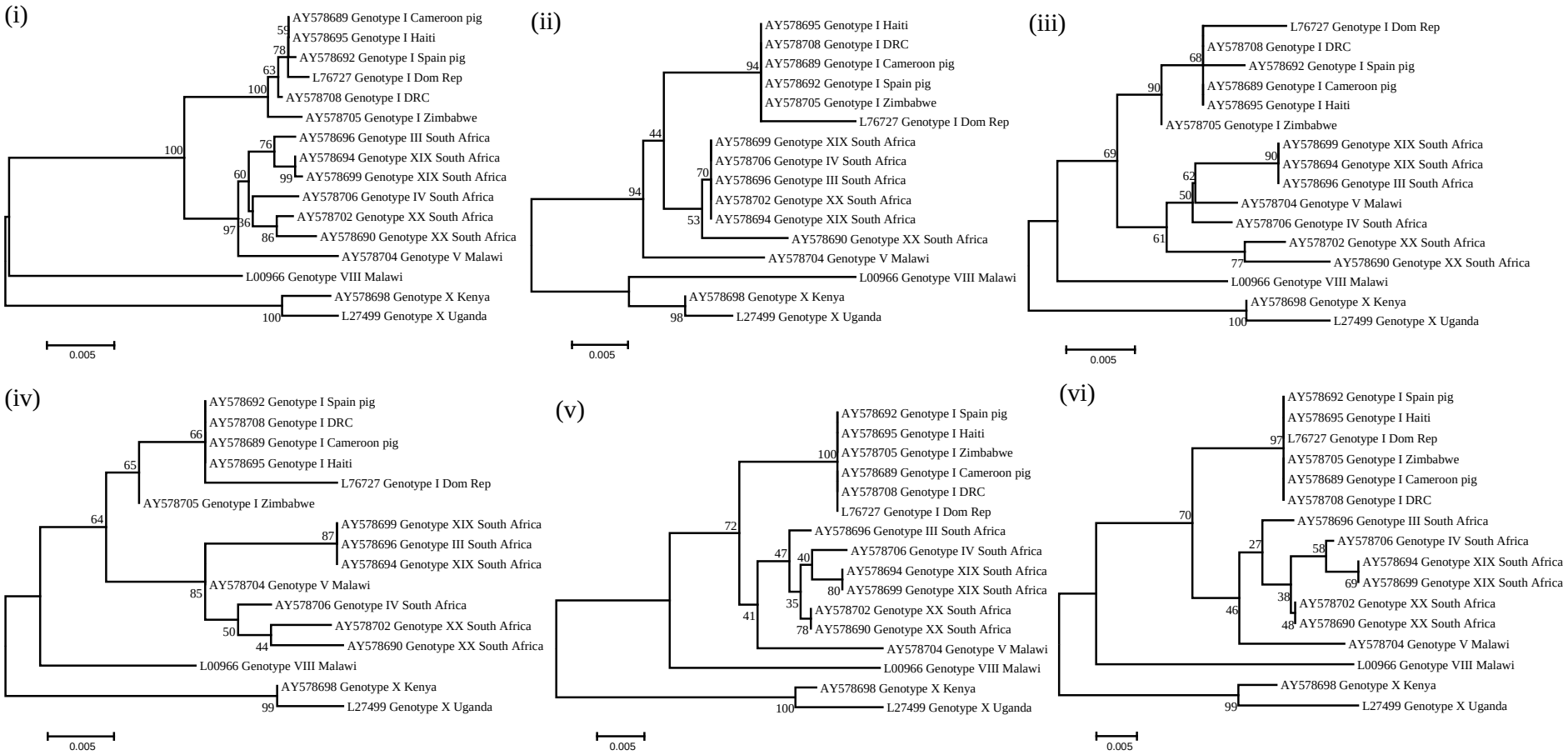
NP: National Park; *Warthog burrow infection rate estimate based on the total number (142) of ticks screened

Figure Legends

Fig. 1 Neighbor-joining (NJ) trees inferred from (i) complete gene data (1941nt), (ii) OIE diagnostic primer target (238nt), (iii) Basto et al. 2006 external PCR primers (330nt), (iv) Basto et al. 2006 internal PCR primers (201nt), (v) Bastos et al. 2003 genotyping primers (437 nt) and (vi) King et al. 2003 TaqMan PCR primers (205nt). Taxon names comprise the Genbank accession number, followed by genotype designation and sampling locality. Bootstrap support values are based on 10000 replicates.

Fig. 2 Multiplex PCR illustrating co-amplification of the host mtDNA gene target (~313bp) and the African swine fever virus gene target (478 bp). Lane 1: 100 bp ladder (Promega); Lanes 2-5 contain PCR reactions in which the virus primers were held constant at 0.5µM with the 16S tick primers ranging from 0.25-1.0 µM (in 0.25 µM intervals, i.e. lane 2 = 0.25 µM, lane 3 = 0.50 µM, lane 4 = 0.75 µM and lane 5 = 1.0 µM); Lanes 6-9 are those reactions where the 16S host primers were maintained at a constant 0.5 µM , and the virus primers ranged in 0.25 µM intervals, from 0.25-1.0 µM. The template DNA volume and sample was identical across the reactions loaded in lanes 2-9 and was extracted from an ASFV-positive tick collected in the Kruger National Park, South Africa.

Fig. 3 Mitochondrial large ribosomal subunit (16S rRNA) neighbor-joining (NJ) tree based on an aligned, partial, homologous dataset of 284 nucleotides. Percentage bootstrap support values ≥ 55 % are those obtained following 10000 replications for NJ and maximum parsimony (MP), whilst Bayesian inference (BI) estimates of posterior probability, expressed as percentages and are indicated NJ/MP/BI next to the relevant nodes. Bootstrap values and posterior probabilities < 55 % are denoted by a '--'. Taxon names include the species designation of each Genbank entry, followed by the sample name (if available), the Genbank accession number in bold and lastly the country of origin. Sequences generated in this study are indicated with grey shading.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AY578690 Genotype XX South Africa
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L27499 Genotype X Uganda
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(ii)
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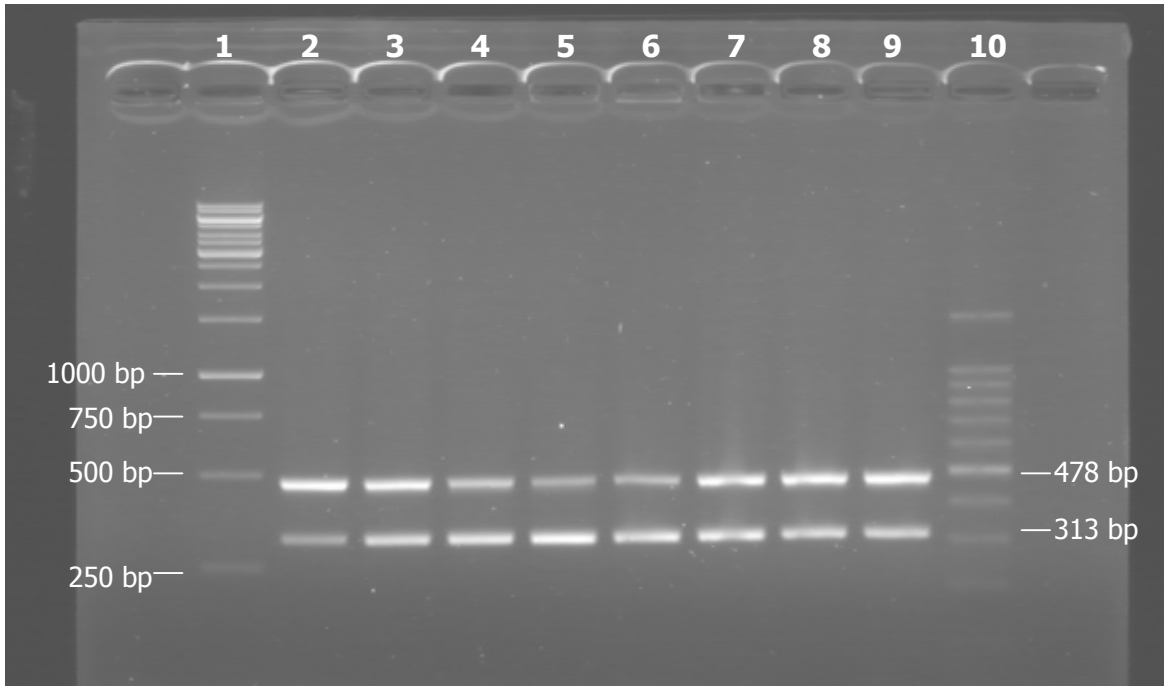
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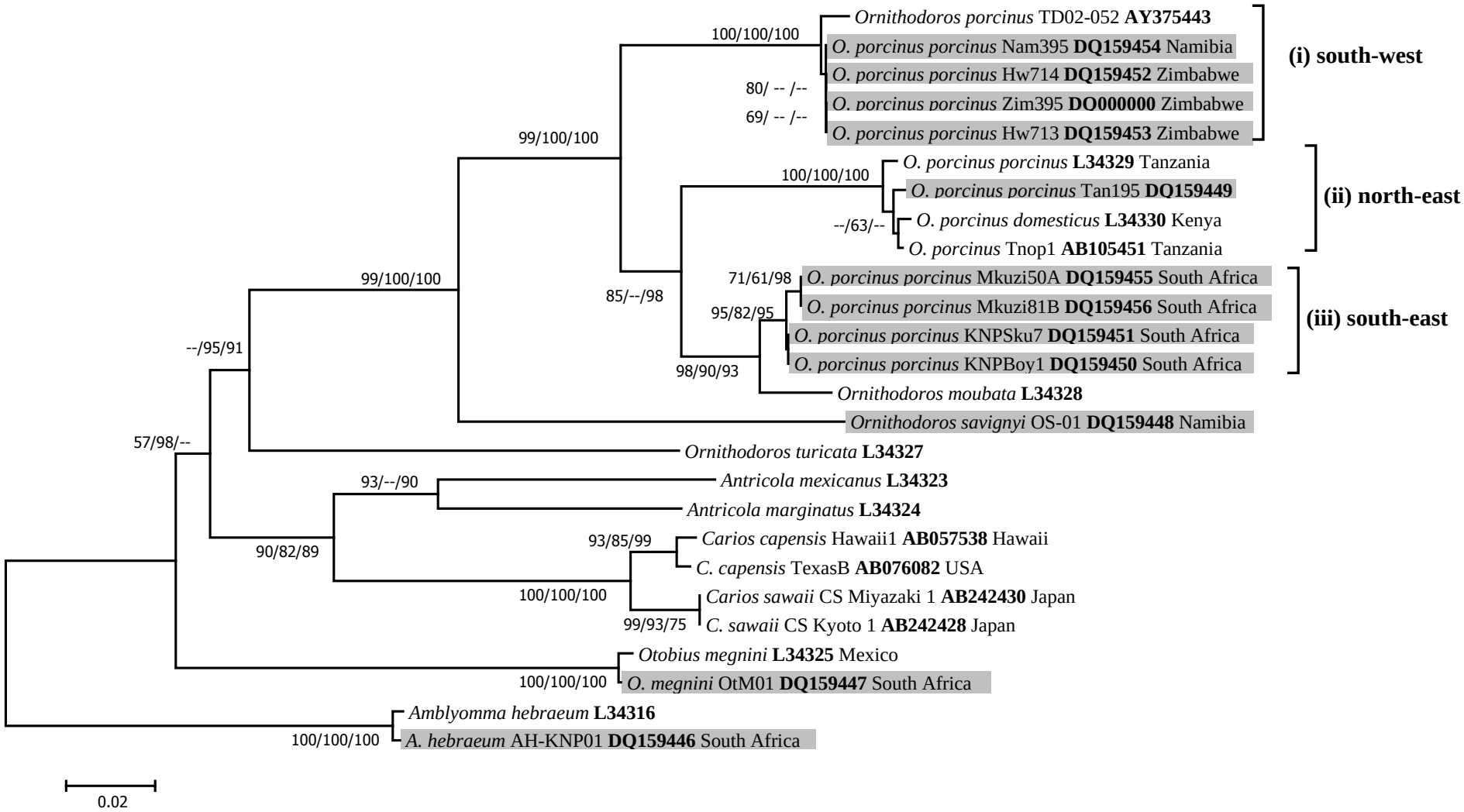
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O. porcinus porcinus Zim395 DQ000000 Zimbabwe
O. porcinus porcinus Hw713 DQ159453 Zimbabwe
(i) south-west
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O. porcinus porcinus Tan195 DQ159449
O. porcinus domesticus L34330 Kenya
O. porcinus Tnop1 AB105451 Tanzania
(ii) north-east
O. porcinus porcinus Mkuzi50A DQ159455 South Africa
O. porcinus porcinus Mkuzi81B DQ159456 South Africa
O. porcinus porcinus KNPSku7 DQ159451 South Africa
O. porcinus porcinus KNPBoy1 DQ159450 South Africa
(iii) south-east
Ornithodoros moubata L34328
Ornithodoros savignyi OS-01 DQ159448 Namibia
Ornithodoros turicata L34327
Antricola mexicanus L34323
Antricola marginatus L34324
Carios capensis Hawaii1 AB057538 Hawaii
C. capensis TexasB AB076082 USA
Carios sawaii CS Miyazaki 1 AB242430 Japan
C. sawaii CS Kyoto 1 AB242428 Japan
Otobius megnini L34325 Mexico
O. megnini OtM01 DQ159447 South Africa
Amblyomma hebraeum L34316
A. hebraeum AH-KNP01 DQ159446 South Africa
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