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Multi-locus sequence analysis of *Anaplasma* in the common warthog (*Phacochoerus africanus*) from South Africa

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ABSTRACT

The prevalence and diversity of *Anaplasma* in common warthog (*Phacochoerus africanus*) was investigated using a multi-locus sequence analysis (MLSA) approach targeting the 16S rRNA, citrate synthase (*gltA)* and heat-shock operon (*groESL*) genes. PCR screening of 100 warthog samples from the Kruger National Park in South Africa with eight published assays identified 50 positive animals, all of which were initially identified with the 16S rRNA assay. In contrast, the *gltA* and six *groESL* assays recovered PCR-positivity rates of 2 % and 0 %-4 %, respectively. As optimisation did not improve *Anaplasma* detection rates, an alternative *groESL* assay targeting a 923 bp region was designed. This new assay detected 45 positive animals, all of which were positive with the 16S rRNA assay. Nucleotide sequencing of the three MLSA gene targets confirmed that 50 % (50/100) of warthogs were *Anaplasma*-positive. Juvenile warthogs displayed a significantly higher infection rate (15/18; 83.3 %) than adults (35/82; 42,68 %). Phylogenetic analyses of individual and concatenated gene datasets confirmed that the *Anaplasma* species in warthogs is closely related to the species detected in *Ornithodoros* soft ticks from Zambia. This, together with the high levels of nucleotide sequence identity (≥98.97 %), suggests the likely existence of a host-restricted cycle involving warthogs and the soft ticks that inhabit their burrows. Based on the distinctiveness and monophyly of the *Anaplasma* species in warthogs and *Ornithodoros* soft ticks, confirmed through genetic characterisation of three gene regions, we propose that *Candidatus* status be assigned and suggest "*Candidatus* Anaplasma ornithodorii".

1. Introduction

Since its first discovery in South Africa, bacteria of the genus *Anaplasma* (Order: *Rikettsiales*, Family: *Anaplasmataceae*) have been increasingly recognised for the detrimental effect they have on livestock and human health (Theiler, 1910; Aubry and Geale, 2011). Five species of these obligate intracellular alpha-1 proteobacteria are published under the International Code of Nomenclature of Prokaryotes, viz. *Anaplasma caudatum*, *A. centrale*, *A. marginale*, *A. ovis*, and *A. phagocytophilum,* however, several novel species await formal identification (Dumler et al., 2001; Parte et al., 2020). Members of this tick-borne genus are globally distributed, infecting a wide range of mammalian hosts. Infections are often asymptomatic in wildlife reservoirs; however, several species are pathogenic and zoonotic (Nicholson et al., 2010; Rar et al., 2021). *Anaplasma marginale*, the causative agent of bovine anaplasmosis, predominantly infects ruminants and is considered one of the three most economically important tick-borne diseases of cattle (Aubry and Geale, 2011). *Anaplasma phagocytophilum,* which causes human granulocytic anaplasmosis, is a potentially fatal disease of increasing incidence and concern in the United States of America (Dahlgren et al., 2011).

Molecular detection and species characterisation of *Anaplasma* species have primarily been determined utilising the 16S rRNA gene; however, this marker has limited utility as it does not effectively capture the true genetic diversity of the genus (Caudill and Brayton, 2022). Despite this, the bulk of studies continue to rely on this conserved gene region as alternative, more informative gene regions are not readily amplified across different species, and the organism is difficult to culture (Zweygarth et al., 2006; Silaghi et al., 2017).

In suids, estimates of *Anaplasma* prevalence and diversity have

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largely focused on wild and domestic *Sus scrofa* pigs. In Poland, wild boars were identified as natural reservoirs of *A. phagocytophilum* with reported prevalences of 9–20 % (Michalik et al., 2012; Myczka et al., 2021), and in China, *Anaplasma* IgG seropositivity was 8.6 % in 220 domestic pigs (Zhang et al., 2022). The *Anaplasma* status of a wild suid species, the common warthog (*Phacochoerus africanus*), was assessed for the first time using 16S rRNA sequencing (Makgabo et al., 2023). These authors detected *A. centrale* and an unnamed species, previously identified in soft ticks of the *Ornithodoros moubata* species complex (Qiu et al., 2021), in one and seven warthogs, respectively, of the 30 individuals evaluated (Makgabo et al., 2023).

Warthogs are known to be infected with a broad range of viruses and bacteria (Neiffer et al., 2021; Molini et al., 2022) and play a key role as amplification hosts in the African swine fever (ASF) sylvatic cycle that involves virus cycling between warthogs and the *Ornithodoros* soft ticks that infect their burrows (Jori and Bastos, 2009). Given the rapidly expanding distributional range of warthogs in South Africa (Craig et al., 2021) and the ease with which they move between wild and domestic settings (Jori et al., 2011), it is imperative that we understand the broader disease risks associated with these changing dynamics. The study aimed to generate refined estimates of *Anaplasma* prevalence and diversity in the largest free-ranging population of warthogs in the largest wildlife conservancy in South Africa, the Kruger National Park. This was achieved by screening 100 warthog samples with eight published PCR assays targeting three gene regions (16S rRNA, *groEL* and *gltA*), following which all samples were rescreened with a newly developed assay targeting the *groESL* heat-shock operon. In addition, nucleotide sequencing and phylogenetic analysis of three gene regions was undertaken.

2. Methods and materials

2.1. Samples and study site

Veterinary Wildlife Services in the Kruger National Park (KNP) provided 100 EDTA blood samples (40 males and 60 females / 82 adults and 18 juveniles). These samples were opportunistically obtained during routine management activities, including limited culling efforts and the relocation of warthogs from tourist zones, as detailed in Neiffer et al. (2021). The KNP is the largest wildlife conservancy in South Africa, spanning 19,485 km^2 and hosts a significant warthog population. The samples were largely collected in the vicinity of the Skukuza and Satara rest camps from 2007 to 2023 (Fig. 1) and placed in storage at −80°C until retrieved from the SANParks Biobank.

2.2. DNA extraction

DNA was extracted from 200 µl of EDTA blood with Roche High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions following differential lysis of red blood cells. These extractions were performed under BSL2 conditions at the Hans Hoheisen Wildlife Research Station (HHWRS) in compliance with section 20 approval granted for this project.

2.3. Molecular detection and characterisation

To assess *Anaplasma* genome presence, all 100 DNA extracts were screened with the EHR16SD and EHR16SR primers (Parola et al., 2000), which target a ~345 bp region of the 16S rRNA gene (assay A). Positive samples from the first-round screening with assay A were selected for amplification with assay B (pA/27 F + EHR16SR) and assay C (EHR16SD + 1492 R), which target two overlapping fragments of the 16S rRNA gene region, as previously described (Bastos et al., 2015) in order to generate near-complete 16S rRNA sequences. Amplification of the species-informative heat-shock protein (*groEL*) gene was attempted for a subset of positive samples (n = 20) from assay A using a variety of published primers (Assays E – K; Table 1), which amplify regions ranging from ~624 to ~1400 base pairs. All 100 samples were also screened with assay D, which employs the newly designed AnaGroES-F primer, which, when combined with the published AnaGro712R primer (Ybañez et al., 2012), produces a ~923 bp amplicon of the *groESL*

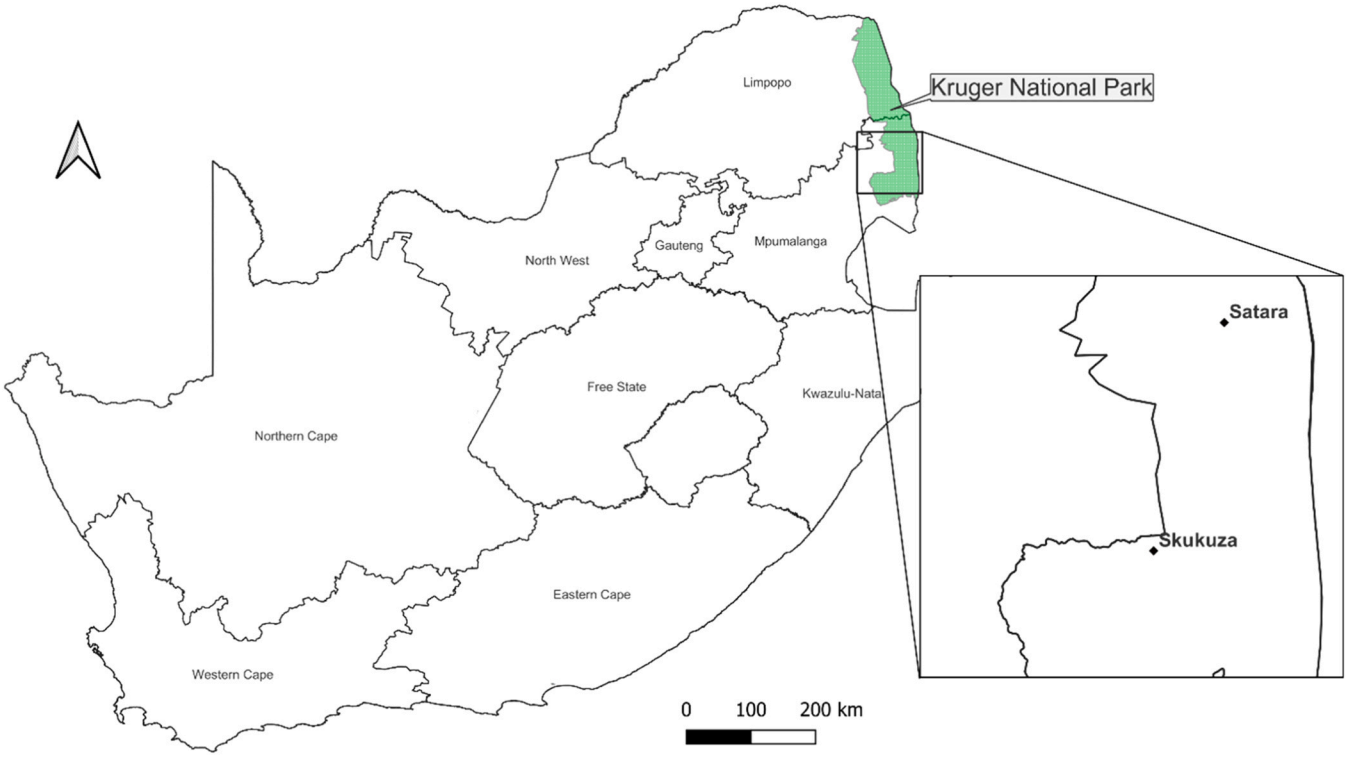

Fig. 1. Map depicting the two sampling localities (Skukuza and Satara rest camps) in the Kruger National Park in north-eastern South Africa.

Table 1
Summary of the twelve PCR assays used to detect and characterise *Anaplasma* in DNA extracts from 100 EDTA blood samples from warthogs (*Phacochoerus africanus*) in the Kruger National Park, South Africa.

PCR assay code	Primer name	Primer sequence (5′-3′)	Gene region targeted	Reference	Expected amplicon size (bp)	Final Ta
A	EHR16SD	GGTACCYACAGAAGAAGTCC	16S rRNA	Parola et al. (2000)	~345	56
	EHR16SR	TAGCACTCATCGTTTACAGC		Parola et al. (2000)		
B	pA (27 F)	AGAGTTTGATCCTGGCTCAG	16S rRNA	Edwards et al. (1989)	~790	56
	EHR16SR	TAGCACTCATCGTTTACAGC		Parola et al. (2000)		
C	EHR16SD	GGTACCYACAGAAGAAGTCC	16S rRNA	Parola et al. (2000)	~1030	56
	1492 R	GGCTACCTTGTTACGACTT		Reysenbach et al. (1992)		
D	AnaGroES	GAATCTAGCTATGTTGCATGATAA	*groESL*	This study	~923	54
	AnaGro712R	CCGCGATCAAACTGCATACC		Ybañez et al. (2012)		
E	AnaPlatF2	GCGTAGTCCGATTCTCCAGT	*groEL*	Bastos et al. (2015)	~650	56
	AnaGro712R	CCGCGATCAAACTGCATACC		Ybañez et al. (2012)		
F*	HS1-F	ATAGTYATGAAGGAGAGTGAT	*groEL*	Sumner et al. (1997)	~1400	52
	HS6-R	CCWCCWGGTACWACACCTTC		Sumner et al. (1997)		
G*	HS3-F	ATAGTYATGAAGGAGAGTGAT	*groEL*	Liz et al. (2000)	~1297	54
	HSV-R	CTCAACAGCAGCTCTAGTA		Liz et al. (2000)		
H*	EhrlCanF3	GACATGGCAAATGTAGTTGTAAC	*groEL*	Bastos et al. (2015)	~1200	50
	HSV-R	TCAACAGCAGCTCTAGTWG		Liz et al. (2000)		
I**	HS1-F	ATAGTYATGAAGGAGAGTGAT	*groEL*	Sumner et al. (1997)	~727	56
	AnaGro712R	CCGCGATCAAACTGCATACC		Ybañez et al. (2012)		
J**	EphplgroEL(569)F	ATGGTATGCAGTTTGATCGC	*groEL*	Alberti et al. (2005)	~573	55
	EphgroEL(1142)R	TTGAGTACAGCAACACCACCGGAA		Alberti et al. (2005)		
K**	EphplgroEL(569)F	ATGGTATGCAGTTTGATCGC	*groEL*	Alberti et al. (2005)	~624	55
	EphplgroEL(1193) R	TCTACTCTGTCTTTGCGTTC		Alberti et al. (2005)		
L	ANA-CS646F	TGCATGCAGATCATGAAC	*gltA*	Inokuma et al. (2005)	~431	54
	ANA-CS1076R	GAGTAAAARTCAACATTBGG		Inokuma et al. (2005)		

* No amplification; **Non-specific amplification; Ta: Annealing temperature

operon. Lastly, all positive samples from assay A and D were rescreened with assay E primers (ANA-CS646F + ANA-CS1076R), which targets a ~431 bp region of the citrate synthase or *gltA* gene (Inokuma et al., 2005), following extensive optimisation.

All genomic amplification reactions were performed in a final reaction volume of 40 μL containing 2–3 μL of template DNA, 1.5 units of DreamTaq™ DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A), 1X buffer (Fermentas, Waltham, Massachusetts, U.S.A), 0.2 μM dNTPs (Fermentas, Waltham, Massachusetts, U.S.A) and 0.4 μM of each primer (Table 1). To minimise cross-contamination, PCR reactions were set up in a UV-decontaminated DNA-free hood. Touchdown PCR reactions were then performed with cycling conditions consisting of an initial denaturation step at 96°C for 12 s, followed by 40 cycles of denaturation at 96°C for 12 s, primer annealing at an optimised primer-specific annealing temperature (Table 1) for 30 s, and elongation at 70°C (time dependent on the size of the targeted amplicon), and a final elongation at 70°C for 60 s. All reactions were run on an Applied Biosystems VeritiPro Thermal Cycler (ThermoFisher Scientific, U.S.A) and included negative (DNA-free) and positive ("*Candidatus* Anaplasma dromedarius"; Bastos et al. (2015)) controls. Amplicons were electrophoresed on a 1.5 % agarose gel stained with GoldView (Geneshun Biotech, Ltd., Guangzhou, China) and sized against a 1 kb molecular weight marker (Fermentas, Waltham, Massachusetts, U.S.A) under ultra-violet light irradiation (Vilber Lourmat, Omni-Science CC, Randburg, South Africa).

PCR products of the expected size were purified directly from the tube using a Roche High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany), according to manufacturer instructions. Uni-directional cycle sequencing was performed using BigDye Terminator chemistry (Applied Biosystems, California, U.S.A), with bidirectional sequencing being performed for a subset of samples representative of all variants. The cycle sequencing products underwent standard sodium acetate DNA precipitation and were submitted to the core Sanger sequencing facility of the University of Pretoria.

2.4. Phylogenetic analyses

Sequences were viewed and edited in Geneious Prime 2023, version 2.1 (https://www.geneious.com). Chromatograms were first trimmed with an error probability limit of 0.05 using the 'trim ends' feature and manually adjusted afterwards. Following this, they were aligned using the MUSCLE v5 algorithm embedded in Geneious. Sequences were then compared to publicly available data on the GenBank database (www.ncbi.nih.gov) using the BLAST (Altschul et al., 1990) nucleotide search function. Similar and relevant reference sequences were added to the dataset, aligned and trimmed to remove primer-binding and end-unaligned regions. Neighbor-joining (NJ) and Maximum likelihood (ML) inferences were conducted in MEGA7 (Kumar et al., 2016) using the best-fit model of sequence evolution identified under the Bayesian information criterion (BIC). Bayesian inference (BI) run in MrBayes 3.2.7a (Ronquist et al., 2012) consisted of 10,000,000 generations, sampled every 1,000th generation, following which 25 % of the run was discarded as burn-in.

2.5. Statistical analyses

Pearson's Chi-squared (χ^2) test, with the Yate's continuity correction, where appropriate, was used to determine if there were statistically significant differences in *Anaplasma* prevalence between age classes, sexes and to test for temporal variation. These tests were conducted with functions in the statistical program R. Only samples for which *Anaplasma* positivity was confirmed through nucleotide sequencing were used in the statistical analyses.

3. Results

3.1. Comparative Anaplasma PCR assay performance

Assay A confirmed tick-borne *Anaplasmataceae* presence in 50 samples based on the amplification of a band of the expected size 345 bp size, of which all were confirmed as *Anaplasma*-positive through nucleotide sequencing and BLAST nucleotide searches. Assay D

identified 45 positive samples, all of which were detected by assay A. Nucleotide sequencing of purified *groESL* amplicons of the expected size (923 bp) confirmed that 44 were positive for *Anaplasma*. The combined results confirm an overall *Anaplasma* prevalence of 50 % based on nucleotide sequencing of at least one of the two assay amplicons (A/D). In contrast, assays E – K, which target various regions of the *groESL* operon, were unsuccessful despite using a diverse combination of published primers and attempting to optimise each of the reactions. Assays F, G and H failed to amplify the expected target, whereas assays I, J and K produced multiple non-specific bands (Table 1).

3.2. Characterisation of the 16S rRNA gene

A subset of all positive samples from assays A and D were amplified and sequenced with assays B and C, allowing the generation of a near-complete 1323 bp contig for the 16S rRNA gene region. Assay B produced seven unambiguous sequences, and assay C produced 36 unambiguous sequences. Nucleotide analysis of sequences generated for each fragment and the contig produced from overlapping fragments revealed no variation between individuals. A BLAST nucleotide search (http://blast.ncbi. nlm.nih.gov/Blast.cgi) confirmed that the 16S rRNA sequence generated in this study was identical to an *Anaplasma* sequence detected in warthogs and lions (*Panthera leo*) in the KNP (Makgabo et al., 2023). Phylogenetic inference confirmed that this well-supported clade (Fig. 2; NJ:99 %, ML:100 % BI:100 %) forms a soft polytomy with a clade containing *Ornithodoros moubata* soft ticks collected from warthog burrows in Zambia and a third clade containing *Anaplasma* from African buffalo (*Syncerus caffer),* African elephant (*Loxodonta africana*) and kudu antelope (*Tragelaphus strepsiceros)* from the KNP (Makgabo et al., 2023). The 16S rRNA variant generated in this study is available in GenBank under accession number PQ683861

3.3. Characterisation of the groEL gene

The *groESL* assay produced 44 sequences, the substantial majority (40; 90.9 %) of which had ambiguities at 9 of the 653 nucleotide sites in the dataset. The remaining four unambiguous sequences were trimmed to just the *groEL* region and used in phylogenetic analyses. These four sequences correspond to three closely related variants (Fig. 3), which cluster within a highly supported clade (NJ:100 %, ML:100 %, BI:100 %) together with two variants from *Ornithodoros moubata* ticks from Zambia. The three generated variants differ at two nucleotide positions in the dataset. Uncorrected pairwise distances (p-distances) revealed that of the formally recognised *Anaplasma* species, *A. ovis* has the highest nucleotide identity to the warthog-tick *Anaplasma* sequences (80.97 %) across the homologous *groESL* gene region characterised. As

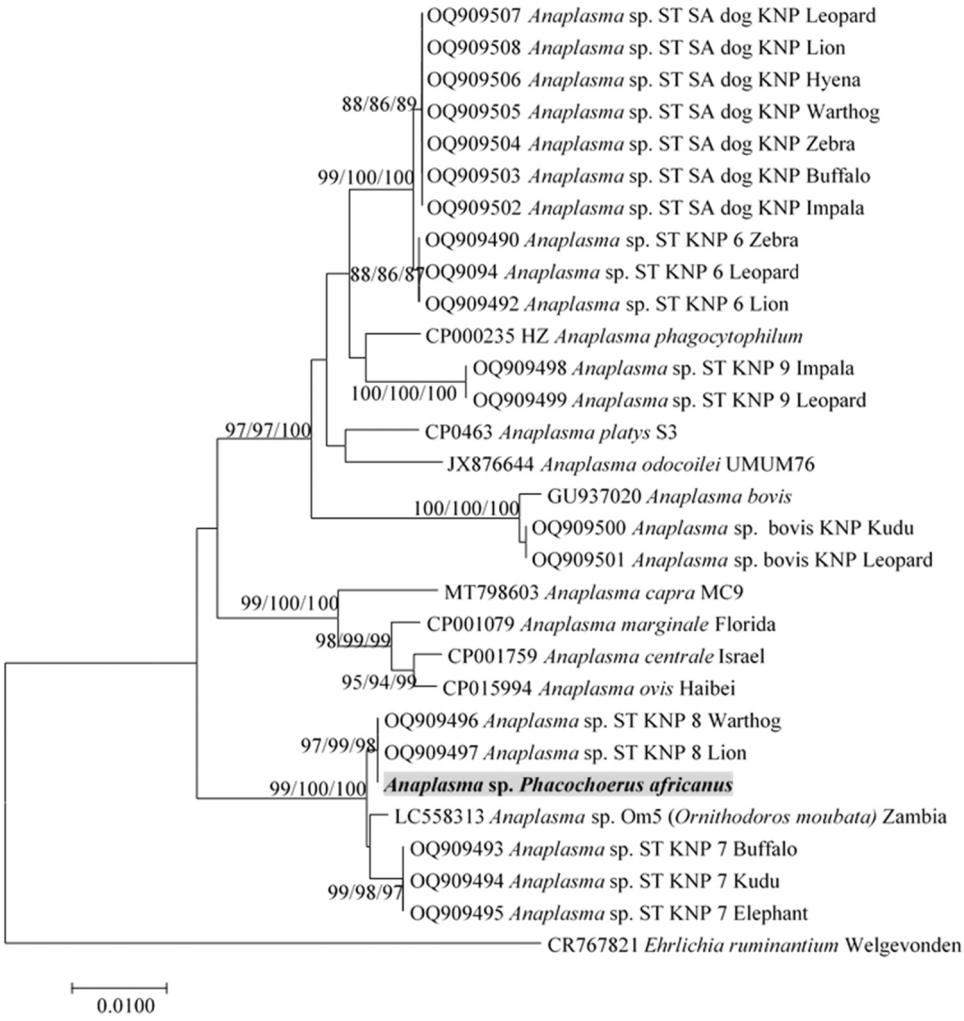

Fig. 2. Neighbor-joining tree depicting the **16S rRNA gene** relationships of *Anaplasma* sequences generated in this study (**bold text**) from common warthogs (*Phacochoerus africanus*) from the Kruger National Park (KNP) in South Africa and relevant reference sequences from Genbank. The phylogeny was inferred using an aligned 1323 bp region of **16S rRNA gene**. Bootstrap values ≥70 % from the neighbor-joining (NJ) and Maximum likelihood (ML) analyses are from 10,000 replicates and posterior probability support values from Bayesian inference (BI: 10 million generations, sampled every 1000 generations, and 25 % burn-in) are indicated NJ/ML/BI on relevant nodes.

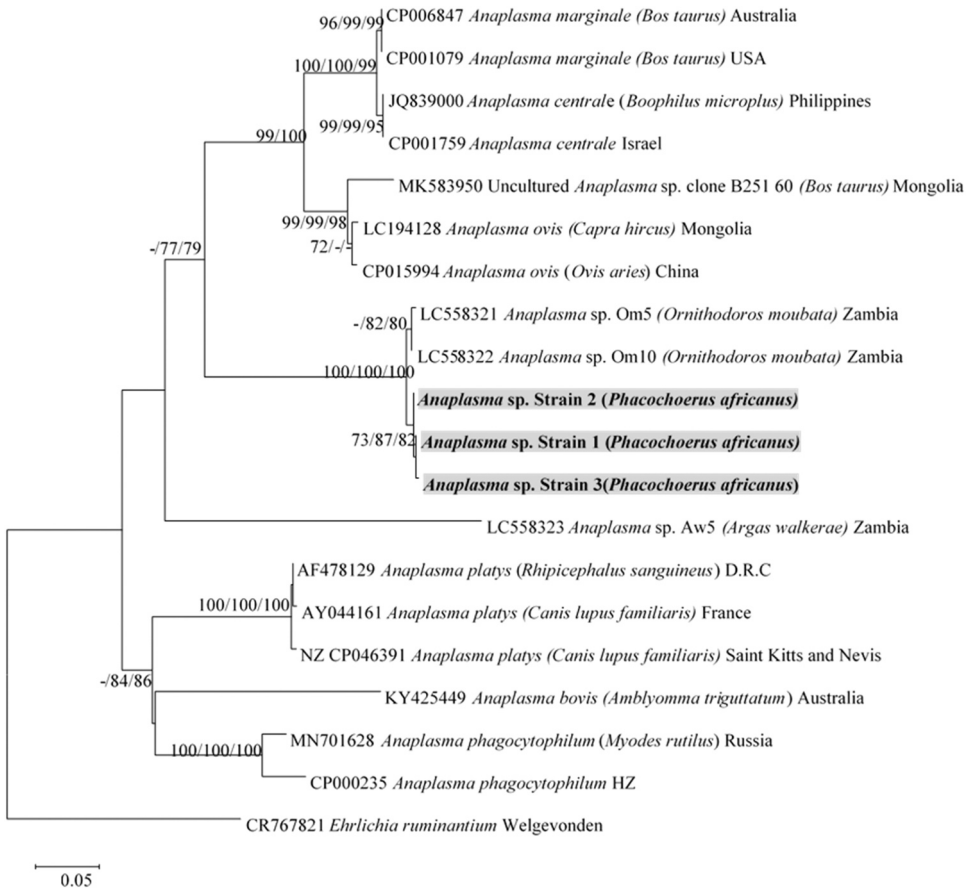

Fig. 3. Neighbor-joining tree depicting the ***groEL*** relationships of *Anaplasma* sequences generated in this study (**bold text**) from common warthogs (*Phacochoerus africanus*) from the Kruger National Park (KNP) in South Africa and relevant reference sequences from Genbank. The phylogeny was inferred using an aligned 631 bp region of ***groEL* gene**. Bootstrap values ≥ 70 % from the neighbor-joining (NJ) and Maximum likelihood (ML) analyses are from 10,000 replicates and posterior probability support values from Bayesian inference (BI: 10 million generations, sampled every 1000 generations, and 25 % burn-in) are indicated NJ/ML/BI on relevant nodes.

the difference is greater than the pairwise differences between other *Anaplasma* congeners, the conclusion of Qiu et al. (2021) that this is a novel species is supported (Supplementary material; Table S1). The *groESL* sequences generated for each of the variants characterised in this study are available on GenBank under accession numbers PQ683502-PQ683504.

3.4. Characterisation of the gltA gene

Despite numerous optimisation attempts, only a single amplicon was produced with the *gltA* assay (E). The resulting sequence, when used in a BLAST search, revealed a 69.81 %% percent identity to a "*Candidatus* Anaplasma boleense" (Accession number: KX987359) detected in *Rhipicephalus microplus* ticks in China (Lu et al., 2017). Phylogenetic analysis was incongruent with 16S rRNA and *groEL* phylogenies, resulting in an unresolved placement of the detected sequence (Fig. 4). The *gltA* sequence generated in this study is available on GenBank under the accession number PQ683501.

3.5. Concatenated gene phylogeny and statistical analyses

The 16S rRNA-*groEL*-*gltA* concatenated phylogeny (Fig. 5) confirmed the placement of the detected species as sister to a clade containing *A. centrale, A. marginale* and *A. ovis* with high levels of nodal support (NJ:100 % ML:99 %;, 10,000 bootstrap replications). A significant difference was found between the age classes (χ^2 = 8.1978, df= 1, $p < 0.05$) with significantly more juveniles (15/18; 83.3 %) testing positive for the *Anaplasma* sp. than adults (35/82; 42,68 %). *Anaplasma*-positivity was identical between sexes, with 30/60 (50 %) females and 20/40 (50 %) males testing positive. No significant relationships in *Anaplasma* positivity between years ($\chi^2 = 9.36$, df= 9, $p > 0.05$) or between wet and dry season ($\chi^2 = 1.69$, df= 1, $p > 0.05$) were found from Chi-square tests for independence.

4. Discussion

In this study, we detected *Anaplasma* DNA in 50 % of all samples, a substantial increase in 16S rRNA prevalence compared to the 26.7 % (8/30) sequence-confirmed positives previously reported by Makgabo et al. (2023). This marked disparity in detected *Anaplasma* prevalence may be due to a few key differences in methodologies between the two studies. These include that Makgabo et al. (2023) screened fewer samples, with a quantitative PCR (StepOnePlus™ Real-Time PCR System; Applied Biosystems, Foster City, CA, USA), and PacBio microbiome sequencing of universal bacterial 16S rRNA primer set amplicons to characterise microbial strains in a wide range of mammals. In contrast, we solely investigated warthogs, with 100 samples initially screened using an *Anaplasmataceae*-specific assay (Assay A, Table 1) to confirm *Anaplasma* infection status, following which the positive samples were screened with multiple assays targeting three genome regions, including the 16S rRNA gene. This expanded sample size also allowed for evaluation of a greater temporal range (2007–2023) compared to the previous study (Makgabo et al., 2023; 2017–2019). The impact of primer specificity is also highlighted in this study, as six assays utilising a diverse

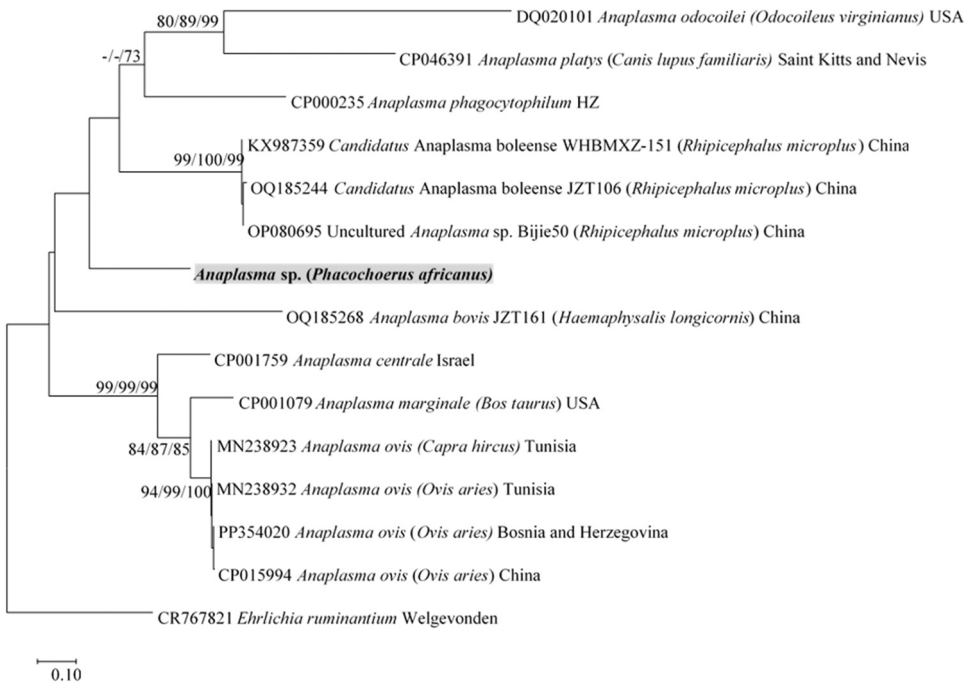

Fig. 4. Neighbor-joining tree depicting the ***gltA* gene** relationships of *Anaplasma* sequences generated in this study (**bold text**) from common warthogs (*Phacochoerus africanus*) from the Kruger National Park (KNP) in South Africa and relevant reference sequences from Genbank. The phylogeny was inferred using an aligned 329 bp region of ***gltA* gene**. Bootstrap values $\geq$ 70 % from the neighbor-joining (NJ) and Maximum likelihood (ML) analyses are from 10,000 replicates and posterior probability support values from Bayesian inference (BI: 10 million generations, sampled every 1000 generations, and 25 % burn-in) are indicated NJ/ML/BI on relevant nodes.

combination of published primers targeting various-sized amplicons of the *groESL* operon were not able to amplify the samples identified as positive with assay A, prompting the design of a novel primer.

Characterisation of the *Anaplasma* strain in warthogs, through 16S rRNA sequencing, revealed that all sequences were identical to the 16S rRNA data previously generated with PacBio sequencing for *Anaplasma* in warthogs (Makgabo et al., 2023). However, despite screening a larger number of samples, we did not detect *Anaplasma centrale* in any of the warthog samples. This is contrary to the findings of Makgabo et al. (2023), who reported the presence of this species in a single warthog. *Anaplasma centrale* is closely related to *A. marginale,* often occurring as a co-infection. These species are responsible for the economically relevant bovine anaplasmosis, and whilst known to occur in KNP, earlier reports revealed these species to be primarily associated with African buffalo (Sisson et al., 2023).

Phylogenetic analysis of both the 16S rRNA (Fig. 2) and the *groEL* (Fig. 3) datasets revealed that the strain detected in 50 % of warthogs in our study is closely related to a species detected by Qiu et al. (2021) in *Ornithodoros moubata* soft ticks collected from warthog burrows in Zambia. This suggests that *Anaplasma* soft tick congeners in the Kruger National Park, whilst not evaluated, are likely to carry the same unnamed novel species. Given that transstadial transmission of *Anaplasma* in ticks is well-established and only limited studies have shown transovarial transmission, the cycling of the bacteria between the warthogs and the ticks likely requires new infections to be established in naïve ticks, which in turn is reliant on the ready availability of bacteraemic vertebrate hosts for maintenance (Dumler et al., 2001; De La Fournière et al., 2023). Given that the eyeless soft tick vector is nidicolous and confined to the warthog burrow, and assuming it is the sole vector of the detected strain, it is unlikely that large terrestrial vertebrates would be exposed to the burrow-dwelling soft ticks.

The noted age class bias, with significantly more juveniles (83.3 %) than adults (42.68 %) testing positive, has been reported in other wildlife species such as African buffalo, *Syncerus caffer* (Sisson et al., 2023). Differences in age classes for tick-borne diseases are driven by both host immunity and the likelihood of exposure (Combrink et al., 2020). Thus, the noted age class bias likely reflects acquired immunity and bacterial clearance in adults, along with early exposure of neonate warthogs to infected ticks in the burrow. No significant temporal variation or trends were found in reported prevalences; however, this was highly constrained by the per-year sample size.

The 16S rRNA phylogeny revealed that the warthog-tick strain is identical to strains previously identified in lion and sister to strains identified in African buffalo, kudu antelope and African elephant with high levels of support (NJ:99 %.ML:100 % BI:100 %). All these sequences group together in a well-supported clade distinct from all recognised *Anaplasma* species (Fig. 2). The closest formally recognised species in terms of *groEL* nucleotide identity is *A. ovis* (80.97 %). As the pairwise nucleotide differences between the warthog-tick strain and *A. ovis* are greater than those for other closely related congeners (Supplementary material Table S1), our results support the findings of Qiu et al. (2021) that this is a novel species. In light of the expanded host and geographical range, supported through multi-locus sequence analysis, we propose that Candidatus status be assigned and suggest "*Candidatus* Anaplasma ornithodorii" in acknowledgement of initial detection by Qiu et al. (2021) in *Ornithodoros moubata* ticks from Zambia.

The data from the Makgabo et al. (2023) study suggest that the *Anaplasma* species identified in warthogs in this study is present in a broad range of host species from disparate taxonomic families, a feature noted in some species of *Anaplasma,* such as the zoonotic *A. phagocytophilum.* Whereas a high degree of host plasticity is associated with this zoonotic pathogen, distinct strains still exhibit a degree of host specificity (Aardema and Von Loewenich, 2015). For most *Anaplasma* species, a narrower host range is the norm, ranging from one to three species (Moraga-Fernández et al., 2023). Additionally, as 16S rRNA sequences alone are often unsuitable for parsing out closely related species (Caudill and Brayton, 2022), the true host range of *Anaplasma* species remains uncertain. A more phylogenetically informative region, like the heat-shock protein (*groEL*), characterised in a

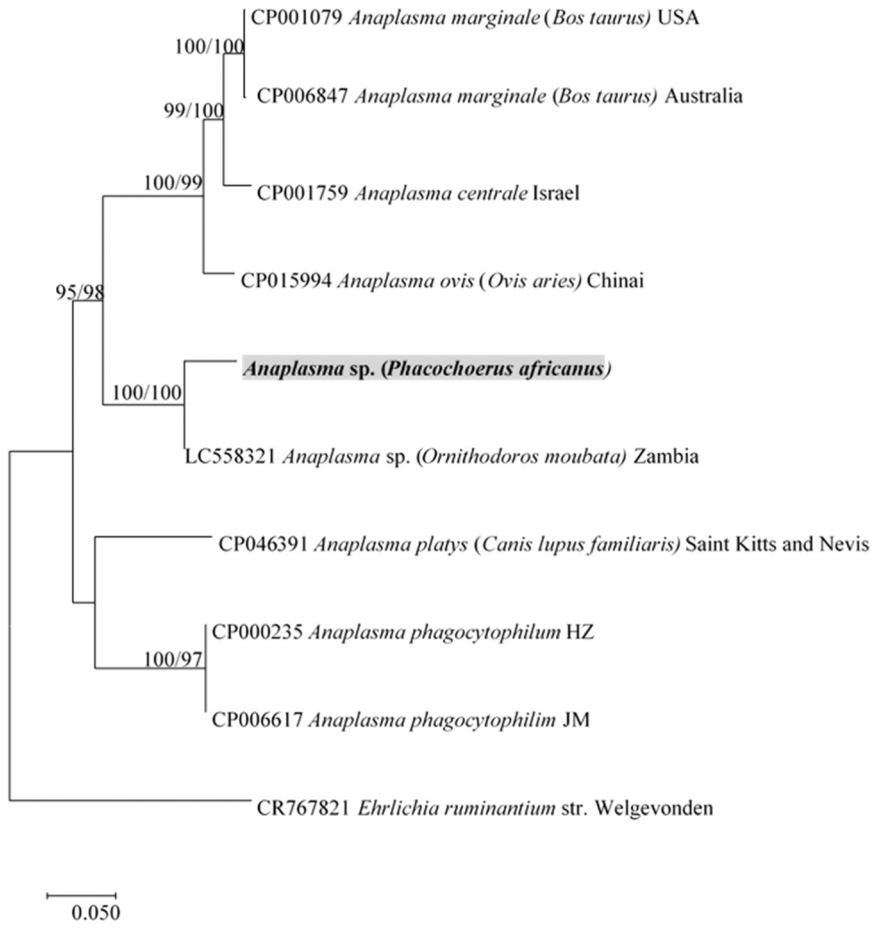

Fig. 5. Neighbor-joining tree depicting the relationships of *Anaplasma* sequences generated in this study (**bold text**) from common warthogs (*Phacochoerus africanus*) from the Kruger National Park (KNP) in South Africa and relevant reference sequences from Genbank. The phylogeny was inferred using **16S rRNA, *groEL*** and ***gltA*** concatenated gene fragment (2181bp). Bootstrap values > 70 % from the neighbor-joining (NJ) and Maximum likelihood (ML) analyses are from 10,000 replicates and are indicated NJ/ML on relevant nodes. Missing data/gaps were deleted in a pairwise manner.

broad range of potential hosts, would improve estimates of host-specificity and assist with accurate *Anaplasma* species delineation.

Sequence data generated from the *groEL* amplicons revealed numerous double peaks throughout the chromatograms; this is likely attributed to the co-infection of these individuals with multiple closely related strains, which are more readily detected with *groEL*. The remaining unambiguous sequences revealed three variants. This increased variability echoes results from Qiu et al. (2021) who also only found a single 16S rRNA variant yet multiple *groEL* variants. This underscores the value of the newly developed assay, which, in addition to generating phylogenetically informative sequences, is sufficiently robust to almost achieve parity in prevalence with the well-established and broadly applied 16S rRNA assay of Parola and co-workers (2000). Additionally, the ubiquity and cost-effective elements of conventional PCR in comparison to qPCR further enhance its value in low-income laboratory settings. Further investigation into the newly-developed primers' ability to amplify other *Anaplasma* species would be valuable in determining the breadth of the species recognition range of this assay.

Analysis of the *gltA* gene region, unlike both the 16S rRNA and *groEL* analysis, could not sufficiently resolve the relationships between the species. Only a single amplicon and sequence were obtained for this gene region, and it was comparatively shorter (329 bp) than all other regions analysed, making it less phylogenetically informative. There are also fewer reference sequences available for this gene region. Despite these limitations, the results highlight the need for a multi-locus approach to accurately characterise species, as the phylogenetic incongruity was resolved when the target regions were concatenated (Fig. 5). Investigations into additional primers with a broader species recognition range for a larger fragment of the phylogenetically informative *gltA* gene target would be valuable.

Further research involving warthogs sampled across a broader geographic range is necessary to establish the distribution of the *Anaplasma* species identified in this study. Given that Qiu et al. (2021) intially detected this species in *Ornithodoros* soft ticks in Zambia, the likelihood exists that other warthog populations and their associated ectoparasites are potential reservoirs for this *Anaplasma* species.

Given the established zoonotic nature of several other *Anaplasma* species, these factors highlight the importance of further studies exploring the transmission dynamics at the human–livestock–wildlife interface. While *Anaplasma* infection is likely asymptomatic in warthogs, it is not known whether this is the case for all potential spillover hosts. Warthogs have an increasing distributional range, are known to move between wild and domestic settings and are infected with a wide range of infectious disease agents (Neiffer et al., 2021). Thus, evaluation of additional infectious agents is important for informing effective wildlife management and limiting spillover, particularly to domestic pigs and to other sympatric wildlife which may share vectors, habitats and resources. Lastly, given that both warthogs and *Ornithodoros* ticks are involved in the ASF sylvatic cycle, the potential for co-infection and modulation/amplification of ASF warrants investigation.

5. Conclusions

This study demonstrates the presence of a single *Anaplasma* species

in 50 % of common warthogs from the Kruger National Park in South Africa at higher levels than previously reported. Multi-locus sequence analysis (16S rRNA, *groEL* and *gltA*) of the warthog *Anaplasma* confirms that it matches the species first identified in *Ornithodoros moubata* soft ticks from warthog burrows in Zambia (Qiu et al., 2021). As this *Anaplasma* species is distinct from all formally recognised species, we propose that Candidatus status be assigned and suggest "*Candidatus* Anaplasma ornithodorii" in acknowledgement of initial detection by Qiu et al. (2021).

CRediT authorship contribution statement

Keaton Rea: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis. **Peter Buss:** Writing – review & editing, Resources. **Armanda Bastos:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Keaton Rea reports financial support was provided by USDA National Institute of Food and Agriculture. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2025.110751.

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