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Unravelling the diversity of *Anaplasma* species circulating in selected African wildlife hosts by targeted 16S microbiome analysis

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ABSTRACT

Organisms in the genus *Anaplasma* are obligate intracellular alphaproteobacteria. Bovine anaplasmosis, predominantly caused by *Anaplasma marginale*, is the most prevalent tick-borne disease (TBD) of cattle worldwide. Other *Anaplasma* species are known to cause disease; these include *A. ovis, A. platys* in dogs, *A. capra* in goats and humans, and *A. phagocytophilum* in humans. The rapid advancement of next-generation sequencing technologies has led to the discovery of many novel sequences ascribed to the genus *Anaplasma*, with over 20 putative new species being proposed since the last formal organization of the genus. Most 16S rRNA gene surveys for *Anaplasma* were conducted on cattle and to a lesser extent on rodents, dogs, and ticks. Little is known about the occurrence, diversity, or impact of *Anaplasma* species circulating in wildlife species. Therefore, we conducted a 16S rRNA gene survey with the goal of identifying *Anaplasma* species in a variety of wildlife species in the Kruger National Park and neighbouring game reserves, using an unbiased 16S rRNA gene microbiome approach. An *Anaplasma/Ehrlichia*-group specific quantitative real-time PCR (qPCR) assay revealed the presence of *Anaplasma* and/or *Ehrlichia* species in 70.0% (21/30) of African buffalo, 86.7% (26/30) of impala, 36.7% (11/30) of greater kudu, 3.2% (1/31) of African wild dog, 40.6% (13/32) of Burchell's zebra, 43.3% (13/30) of warthog, 22.6% (7/31) of spotted hyena, 40.0% (12/30) of leopard, 17.6% (6/34) of lion, 16.7% (5/30) of African elephant and 8.6% (3/35) of white rhinoceros samples. Microbiome sequencing data from the qPCR positive samples revealed four 16S rRNA sequences identical to previously published *Anaplasma* sequences, as well as nine novel *Anaplasma* 16S genotypes. Our results reveal a greater diversity of putative *Anaplasma* species circulating in wildlife than currently classified within the genus. Our findings highlight a potential expansion of the *Anaplasma* host range and the need for more genetic information from other important genes or genome sequencing of putative novel species for correct classification and further assessment of their occurrence in wildlife, livestock and companion animals.

1. Introduction

Bovine anaplasmosis is among the three most important tick-borne diseases (TBDs) of ruminants and results in major economic losses in food animal production globally (Uilenberg, 1995). This disease is mainly caused by the obligate intracellular rickettsial pathogen, *Anaplasma marginale*, which is currently widespread in cattle in South Africa (Hove et al., 2018; Makgabo et al., 2023). Several other species of *Anaplasma* have been reported to infect cattle in South Africa: these include *A. centrale, A. bovis, A. platys* and *Anaplasma* sp. (Omatjenne) (De Kock et al., 1937; Zweygarth et al., 2006; Harrison et al., 2011; Harrison et al., 2013; Khumalo et al., 2016; Kolo et al., 2020). Of the seven species included in the most recent reorganization of the genus *Anaplasma* (Dumler et al., 2001), four species, *A. marginale, A. bovis, A. centrale* and *A. phagocytophilum*, are known to cause anaplasmosis in cattle (Aktas and Özübek, 2017; Hove et al., 2018; Jurković et al., 2020; M'Ghirbi et al., 2016). Of these, *A. marginale* is the most important pathogen in cattle (Kocan et al., 2010). Although *A. bovis, A. centrale*,

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and *A. phagocytophilum* are widely known to cause subclinical disease in cattle, a clinical case of bovine anaplasmosis caused by *A. centrale* was reported in Europe in 2008 (Carelli et al., 2008). *Anaplasma ovis* mainly causes a subclinical disease characterized by fever in sheep and goats (Kuttler, 1984). *Anaplasma phagocytophilum* causes human granulocytic anaplasmosis in humans (Bakken et al., 1994), while *A. capra* infects both goats and humans (Li et al., 2015). *Anaplasma platys* is a pathogen that mostly infects platelets in dogs causing infectious cyclic thrombocytopenia (Abarca et al., 2007).

The past few decades have seen the global occurrence of several new, emerging and re-emerging tick-borne rickettsial pathogens of major public and veterinary health concern (Walker and Dumler, 1996; Dumler et al., 2001; Paddock and Childs, 2003; Li et al., 2015). A significant increase in the wildlife industry in South Africa over the past two decades has resulted in an increase in land use dedicated to wildlife and thus an increase in wildlife species in both game reserves and farming areas, thus resulting in an increase in wildlife-livestock interfaces in many parts of the country (Parker and Bernard, 2005; Smith and Parker, 2010; Jori et al., 2011; Horak et al., 2015). This, in turn, increases potential TBD transmission opportunities between wildlife, livestock and the humans who maintain them, through increased opportunities for ticks to move between them (Yusufmia et al., 2010; Caron et al., 2013; Mbizeni et al., 2013). Very little is known about the role played by wildlife hosts in the distribution and epidemiology of anaplasmosis in domestic animals, livestock and possibly in humans. *Anaplasma marginale, A. centrale* and/or *A. ovis* have been identified in several wild ruminant species in Africa, including African buffalo (*Syncerus caffer*), black wildebeest (*Connochaetes gnou*), blue wildebeest (*Connochaetes taurinus*), blesbok (*Damaliscus pygargus phillipsi*), grey duiker (*Sylvicapra grimmi*), nyala (*Tragelaphus angasii*), eland (*Taurotragus oryx*) and giraffe (*Giraffa camelopardalis*) (Neitz, 1935; Peirce, 1972; Augustyn, 1974; Kuttler, 1984; Smith et al., 1982; Ngeranwa et al., 1998; Potgieter and Stoltsz, 2004; Eygelaar et al., 2015; Khumalo et al., 2016). *Anaplasma bovis* has been identified in rock sengis (*Elephantulus myurus*) (Harrison et al., 2011; Harrison et al., 2013) and nyala (Pfitzer et al., 2011), and a sequence with 99% identity to *A. bovis* was identified in a *Rhipicephalus evertsi* tick collected on a gemsbok from the Sandveld nature reserve (Tonetti et al., 2009). *Anaplasma* sp. (Omatjenne) was identified in 33% of nyalas examined from four game ranches in northern KwaZulu-Natal (Pfitzer et al., 2011). It is clear that African wildlife harbor several *Anaplasma* spp., but the full range of *Anaplasma* spp. present in wildlife hosts is not known, and the importance of wildlife as a disease reservoir is unclear.

The rapid advancement of high-throughput sequencing technologies has enabled a massive increase in molecular, metagenomic, microbiome and taxonomic analyses, which have resulted in the discovery of a plethora of sequences ascribed to the genus *Anaplasma* worldwide. Over 20 putative *Anaplasma* species with unique 16S rRNA sequences have been identified from various hosts since the last formal organization of the genus (Dumler et al., 2001; Caudill and Brayton, 2022). These putative *Anaplasma* spp. have been reported from a variety of hosts including human, livestock and wildlife and/or tick and mosquito vectors from across the world (a list of the putative *Anaplasma* spp. is shown in Table S1, modified from Caudill & Brayton (2022)). Several novel *Anaplasma* 16S rRNA gene sequences have been reported in cattle, including a putative novel *Anaplasma* species from Uganda (Ikwap et al., 2010; Muhanguzi et al., 2010), "*Candidatus* Anaplasma boleense" (Guo et al., 2016; Fernandes et al., 2019; Kolo et al., 2020), *Anaplasma* sp. Saso, *Anaplasma* sp. Hadesa and *Anaplasma* sp. Dedessa (Hailemariam et al., 2017; Kolo et al., 2020), *Anaplasma* sp. Mymensingh (Roy et al., 2018; Kolo et al., 2020), and "*Candidatus* Anaplasma africae" (Dahmani et al., 2019). The phylogenetic relationships of these newly detected agents to known pathogens and their ability to serve as a source of cross-reaction in detection assays have not been well assessed.

The present study was aimed at using next-generation sequencing and bioinformatics to profile *Anaplasma* populations in selected wildlife species, to better understand the range and genetic diversity of *Anaplasma* species with potential for transmission to humans, livestock and companion animals.

2. Materials and methods

2.1. Ethics approval

The study was performed in accordance with the conditions of the Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria (REC 252–19). Research and Material Transfer Agreements were obtained from the Scientific Services Committee of the South African National Parks (SANParks), Kruger National Park (KNP) (BMTA 005/20) and the Hans Hoheisan Wildlife Research Station (HHWRS). Permission to conduct research under Section 20 of the *Animal Disease Act* 35 of 1984 was granted by the Department of Agriculture, Land Reform and Rural Development (12/11/1/1/6 (1734 LH)).

2.2. Field samples

A total of 343 frozen EDTA blood samples collected from 11 free roaming wildlife species in the Kruger National Park and surrounding game reserves including the Timbavati Game Reserve, Klaserie Private Nature Reserve and Manyeleti Game Reserve were made available by the Veterinary Wildlife Services, Kruger National Park (SANParks) and HHWRS biobanks (Table 1). These were collected from African elephant (*Loxodonta africana*), African lion (*Panthera leo*), African wild dog (*Lycaon pictus*), Burchell's zebra (*Equus quagga burchelli*), African buffalo (*Syncerus caffer*), common warthog (*Phacochoerus africanus*), greater kudu (*Tragelaphus strepsiceros*), impala (*Aepyceros melampus*), leopard (*Panthera pardus*), spotted hyena (*Crocuta crocuta*) and white rhinoceros (*Ceratotherium simum*) from 2012 to 2020.

2.3. DNA extractions

DNA was extracted from 1 ml of blood from the SANParks and HHWRS biobanked samples using the Gentra Puregene Kit® (Qiagen) according to the manufacturer's instructions.

2.4. Anaplasma/Ehrlichia group-specific quantitative real-time PCR (qPCR) assay

A multiple sequence alignment of 16S rRNA reference sequences of all known species of *Anaplasma* and closely related species in the genera *Ehrlichia* and *Rickettsia* was created using CLC Genomics Workbench 20 (Qiagen). Primers, Ma16SF: (5′-ACA GAA GTC CCG GCA AA-3′), Ma16SR: (5′-TTG CCC CCT CCG TAT TAC C-3′) (Inqaba Biotech, South Africa) and a TaqMan MGB™ probe, Ma16SP: (FAM-5′-CCG TGC CAG C-3′-MGB) (Thermo Fisher Scientific, South Africa) were designed to target a 64 bp fragment in the V3 hypervariable region that is conserved between *Anaplasma* and *Ehrlichia* species. Reactions, performed in a final volume of 20 µl, contained 2 X TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, South Africa), 0.5 µM of each forward and reverse primer, 0.25 µM of TaqMan MGB™ probe and 2 µl of target DNA. The quantitative real-time PCR (qPCR) assays were performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions included UNG incubation at 50 °C for 2 min, followed by AmpliTaq Gold pre-activation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 20 s and annealing at 60 °C for 1 min. DNA extracted from the *Anaplasma centrale* and *Ehrlichia ruminantium* vaccine strain (Onderstepoort Biological Products, Pretoria, South Africa), *A. marginale* and *A. platys* field samples (confirmed by sequence analysis) collected, respectively, from the Proefplaas dairy farm, University of Pretoria and the Mnisi community, Mpumalanga province, were used as positive controls and molecular grade water as a negative control. Data was analyzed using the StepOnePlus™ software version

Table 1
Origin and number of blood samples collected from wildlife hosts.

Wildlife host	Sample type	Biobank	Origin	Year	Number of samples
Buffalo	EDTA-blood	SANParks	Kruger National Park	2019	30
Impala	EDTA-blood	SANParks	Kruger National Park	2020	30
Kudu	EDTA-blood	SANParks	Kruger National Park	2018/19	30
Wild dog	EDTA-blood	SANParks	Kruger National Park	2017/18	30
	EDTA-blood	HHWRS	Timbavati Game Reserve	2020	1
Zebra	EDTA-blood	SANParks	Kruger National Park	2018/19	30
	EDTA-blood	HHWRS	Private Owner	2020	2
Warthog	EDTA-blood	SANParks	Kruger National Park	2017/18/19	30
Hyena	EDTA-blood	SANParks	Kruger National Park	2019/20	30
	EDTA-blood	HHWRS	Timbavati Game Reserve	2020	1
Leopard	EDTA-blood	SANParks	Kruger National Park	2012–2019	30
Lion	EDTA-blood	SANParks	Kruger National Park	2018/19/20	29
	EDTA-blood	HHWRS	Timbavati Game Reserve	2019	5
Elephant	EDTA-blood	SANParks	Kruger National Park	2019/20	30
Rhinoceros	EDTA-blood	SANParks	Kruger National Park	2020	29
	EDTA-blood	HHWRS	Klaserie Nature Reserve	2020	4
	EDTA-blood	HHWRS	Manyeleti Game Reserve	2020	2
Total					**343**

2.2. The analytical specificity of the qPCR assay was determined by testing DNA samples from tick-borne haemoparasites of ruminants including *Rickettsia africae, Babesia bigemina, Babesia bovis* and *Theileria parva*. All DNA samples extracted from the wildlife blood samples included in this study were screened for the presence of *Anaplasma* (and *Ehrlichia*) species using the *Anaplasma/Ehrlichia* group-specific qPCR assay. Since this assay was developed for screening purposes, no Ct value cut-off for true positives was used.

2.5. 16S rRNA gene amplification and PacBio sequencing

The full-length 16S rRNA gene (V1-V9 variable regions) was amplified in triplicate from all *Anaplasma* and/or *Ehrlichia*-positive wildlife DNA samples using modified barcoded 16S rRNA gene-specific primers, 27F: (5′-AGR GTT YGA TYM TGG CTC AG-3′) and 1492R: (5′-RGY TAC CTT GTT ACG ACT T-3′) as recommended for the PacBio Sequel II platform (Pacific Biosciences, Menlo Park, CA) (Lane, 1991; Turner et al., 1999) (a list of the barcoded primers is shown in Table S2). Reactions were performed in triplicate in a final volume of 25 μl containing 1 X Phusion Flash® High Fidelity Master Mix (Thermo Fisher Scientific, South Africa), 0.15 μM of each forward and reverse primer and 5 μl of target DNA. To prevent contamination, master mixes were prepared in a dedicated master mix preparation laboratory where no DNA or PCR products are allowed. PCRs for each wildlife species were prepared on separate days. DNA extracted from the *A. centrale* vaccine strain (Onderstepoort Biological Products, South Africa) was used as a positive control and molecular grade water as a negative control. Cycling conditions included 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. Amplicons were visualized under UV light after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Amplicons were purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions and submitted to the Genomic Sequencing Core of Washington State University, Pullman, USA for circular consensus sequencing (CCS) on the PacBio (Pacific Biosciences, Menlo Park, CA) platform. Two sequencing libraries were prepared using 500 ng of pooled amplicons in each library and the SMRT Bell library 2.0 express kit. Samples were sequenced following standard annealing and loading conditions detailed in SMRT Link software 8.0 (Pacific Biosciences, Menlo Park, CA).

2.6. 16S Microbiome sequence analysis

The 16S rRNA amplicon sequence data were demultiplexed using SMRT Link software 8.0 according to a minimum barcode score of 70. Sequences were then trimmed and filtered using SMRT Link software 8.0, with the following filtering parameters: QV minimum at 0.9999 (QV40), min read length 500 bp, max read length 3000 bp and 4 passes. Final Fasta and Fastq data sets were analyzed using the Ribosomal Database Project (RDP) 16S classifier (Cole et al., 2009; Gall et al., 2016) for *Anaplasma* genus level classification of the sequences with a 95% confidence interval. Sequences classified in the genus *Anaplasma* were further used in a BLAST search against a local NCBI BLASTn customized database created from all known and published *Anaplasma* spp. sequences downloaded from GenBank using the command line application to establish the correct identity of the sequences. Sequences were further filtered and excluded based on sequence length (minimum of 1275 bp), quality and sequence identity in Microsoft Excel (Gall et al., 2016; Caudill and Brayton, 2022). Since some distinct *Anaplasma* spp. are known to have more than 98.7% shared sequence identity, and *A. platys, Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% 16S rRNA gene sequence identity, it is clear that 16S rRNA gene sequences cannot be used to resolve these organisms to species level (Caudill and Brayton, 2022). Thus, only 16S rRNA sequences that were identical to previously published sequences were classified to species level. The *Anaplasma* species classification was further examined using a newly developed single-nucleotide polymorphism method of identifying and classifying *Anaplasma* spp. (Caudill and Brayton, 2022).

2.7. Terminology

It is difficult to formally name *Anaplasma* species due to their obligate intracellular nature and the requirement to deposit viable cultures in two type collections in different countries, and many of the newly identified putative species have been molecularly detected from samples but not isolated in culture. While the new sequences may well represent novel species, additional sequence data is required for confirmation; we will therefore refer to the newly detected novel 16S rRNA sequences as "sequence type" (ST). Where we refer to the organisms represented by the newly detected sequence types, we will refer to them as putative *Anaplasma* species.

2.8. Sequence and phylogenetic analysis

16S rRNA gene sequences classified as *Anaplasma* were aligned with reference sequences from GenBank and the extent of sequence variation was analysed using CLC Genomics Workbench (Qiagen). Alignments were trimmed using CLC Genomics Workbench. The HKY85 (Hasegawa et al., 1985) evolutionary model (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006; Chevenet et al., 2006) was the best-fit model for the 16S rRNA gene sequences as determined by Jmodel test 1.3 (Darriba et al., 2012). Phylogenetic trees for the 16S rRNA gene sequences were constructed using the maximum likelihood (ML) method in MEGA 7 with a HKY85 substitution model, an estimated proportion of invariant sites and four gamma-distributed rate categories (Kumar et al., 2016).

All of the sequence data generated from this study have been registered in GenBank under the BioProject accession number: PRJNA965916. The raw microbiome sequence reads from *Anaplasma*-positive wildlife hosts are available at the Sequence Read Archive (SRA) under accession numbers SRX20180660 to SRX20180741. The near-full length *Anaplasma* 16S rRNA nucleotide sequences were deposited under GenBank accession numbers OQ909436 to OQ909508.

3. Results

3.1. The presence of Anaplasma/Ehrlichia species in African wildlife hosts

The *Anaplasma/Ehrlichia* group-specific qPCR assay based on the 16S rRNA gene revealed the presence of *Anaplasma/Ehrlichia* spp. in all eleven wildlife species examined (Fig. 1) (qPCR results for individual samples are shown in Table S3).

3.2. 16S rRNA gene amplification and PacBio CCS sequence analysis

Of the samples that tested positive using the *Anaplasma/Ehrlichia* group-specific qPCR assay, a visible 16S rRNA PCR product was obtained from 21 impala, 13 buffalo, 6 kudu, 6 zebra, 8 warthog, 3 spotted hyena, 9 leopard, 6 lion and 2 African elephant samples. No amplicon could be generated from the *Anaplasma/Ehrlichia*-positive wild dog or rhinoceros samples. PacBio CCS sequencing of the 16S rRNA gene amplicons revealed the presence of a total of 40,589 *Anaplasma* 16S nucleotide sequences. Further examination of the genus *Anaplasma* using the RDP 16S classifier and the customized 16S *Anaplasma* NCBI BLASTn databases resulted in the classification of 40,063 of these 16S rRNA nucleotide sequences to the *Anaplasma* species level. A total of 526 sequences were excluded based on sequence quality, length and sequence identity (Table 2).

3.3. Identification of Anaplasma species in wildlife hosts

From the 40,063 16S rRNA nucleotide sequences classified as *Anaplasma*, 13 distinct 16S rRNA sequences distinct were identified. Sequences with 100% identity to the 16S rRNA gene of known species were designated with the formal species name; novel 16S rRNA sequences were designated *Anaplasma* sequence type (ST) KNP-1 to KNP-9. *Anaplasma* spp. identified comprised 11,449 (28.6%) sequences of *Anaplasma* ST KNP-1, followed by 8107 (20.2%) of *Anaplasma* ST SA Dog, 6347 (15.8%) of *A. marginale*, 4361 (10.9%) of *Anaplasma* ST KNP-8, 4163 (10.4%) of *Anaplasma* ST KNP-6, 2597 (6.5%) of *Anaplasma* ST KNP-2, 2482 (6.2%) of *A. centrale*, 271 (0.7%) of *Anaplasma* ST KNP-4,

Table 2
PacBio CCS sequencing data of the 16S rRNA gene of *Anaplasma* spp. from wildlife hosts.

Wildlife species	Number of samples that yielded a visible 16S rRNA amplicon	Number of sequences classified as *Anaplasma*		Excluded sequences
		Genus (16S RDP)	Species (NCBI BLASTn)	
Impala	21	2786	2744	42
Buffalo	13	5458	5393	65
Kudu	6	1351	980	371
Zebra	6	2514	2513	1
Warthog	8	3498	3495	3
Hyena	3	31	31	0
Leopard	9	20,729	20,693	36
Lion	6	3715	3707	8
Elephant	2	507	507	0
Total	**74**	**40,589**	**40,063**	**526**

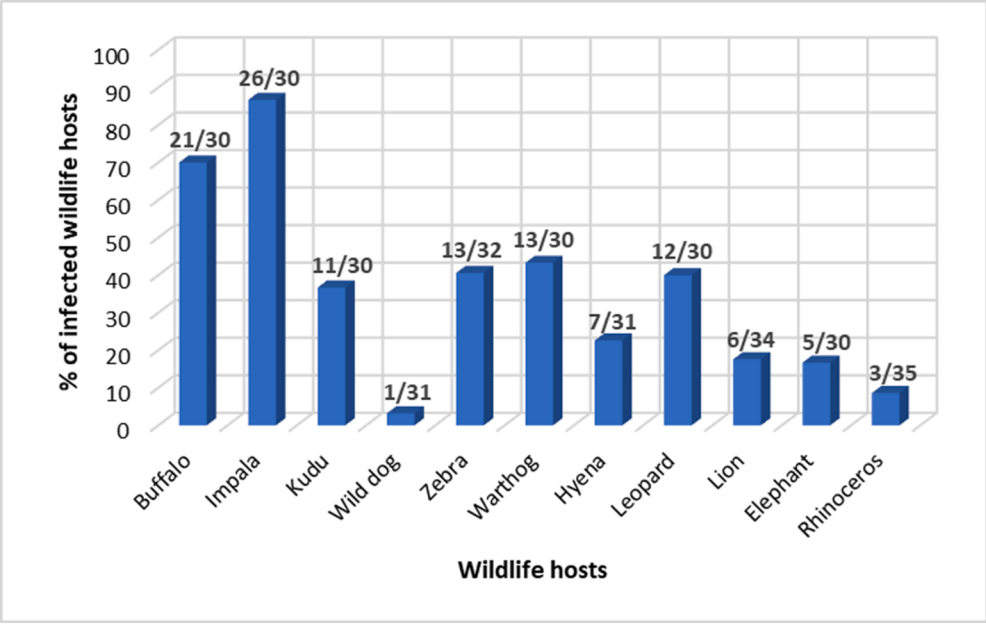

Fig. 1. Percentage of *Anaplasma/Ehrlichia*-positive samples from 11 wildlife species detected using the *Anaplasma/Ehrlichia* group-specific qPCR assay. The numbers above each bar indicate the number of *Anaplasma/Ehrlichia*-positive samples out of the total for each wildlife species included in the study.

206 (0.5%) of *Anaplasma* ST KNP-5, 55 (0.1%) of *Anaplasma* ST KNP-7, and less than 0.1% of *Anaplasma bovis* (14 sequences), *Anaplasma* ST KNP-9 (9 sequences), as well as *Anaplasma* ST KNP-3 (2 sequences).

Table 3 highlights the presence of the 13 *Anaplasma* 16S rRNA sequences in the different wildlife hosts. *Anaplasma* ST KNP-1, *Anaplasma* ST SA Dog and *Anaplasma* ST KNP-2 were detected in seven of the wildlife hosts, followed by *A. marginale* and *A. centrale* detected in four of the wildlife hosts. The remaining *Anaplasma* spp. and STs were detected in either three or two of the wildlife hosts.

3.4. Phylogenetic analyses of 16S rRNA gene sequences from wildlife hosts

The relationships between the near full-length *Anaplasma* 16S rRNA gene sequences identified in the wildlife hosts were revealed by phylogenetic analyses. Maximum likelihood phylogenetic trees of the 16S rRNA gene sequences resulted in two clades (Figs. 2 and 3), as highlighted in previous studies (Kolo et al., 2020; Caudill and Brayton, 2022). The first clade, which will be referred to as clade-1, includes *Anaplasma* spp. known to infect erythrocytes of livestock, such as *A. marginale, A. centrale* and *A. ovis*, while the second clade, which will be referred to as clade-2, includes *Anaplasma* spp. known to infect leukocytes and platelets, namely *A. platys, A. bovis* and *A. phagocytophilum* (Kolo et al., 2020; Caudill and Brayton, 2022).

Only four of the 13 sequences amplified belong to previously identified and described species; these were *A. bovis, A. centrale, A. marginale* and *Anaplasma* ST SA Dog (Fig. 2). *Anaplasma bovis* 16S rRNA gene sequences obtained from kudu and leopard samples were identical to the 16S rRNA gene sequence of *A. bovis* (U03775) from South Africa. *Anaplasma marginale* 16S rRNA gene sequences obtained from buffalo, zebra, leopard and lion samples were conserved and identical to *A. marginale* sequences previously reported from South Africa (AF414873) (Lew et al., 2003) and only varied by one nucleotide from the 16S rRNA gene of *A. marginale* St. Maries strain (CP000030) (Brayton et al., 2005). The *Anaplasma* sp. SA Dog sequences were identical to the *Anaplasma* sp. 16S rRNA sequences previously identified and described in dogs in Zambia and South Africa (LC269823 and MK814441, respectively) (Vlahakis et al., 2018; Kolo et al., 2020). The *A. centrale* sequences obtained from buffalo, zebra, warthog and lion samples were conserved and identical to the *A. centrale* vaccine strain from Israel (CP015994) (Herndon et al., 2010) and only varied by one nucleotide from the 16S rRNA sequence of the *A. centrale* vaccine strain from Australia (AF414868) (Lew et al., 2003).

Phylogenetic relationships between the 16S rRNA sequences of known *Anaplasma* species and the newly detected 16S rRNA sequences are shown in Fig. 3. Except for *Anaplasma* ST KNP-2, which is made up of several similar *A. platys*-like sequences, the unknown *Anaplasma* sequences identified formed monophyletic clades distinct from other validated reference *Anaplasma* spp. (Fig. 3). *Anaplasma* ST KNP-1 and *Anaplasma* ST KNP-3 grouped within clade-1. Two variants of *Anaplasma* ST KNP-1 were identified which formed a sister clade to the *A. ovis* group which includes *Anaplasma* sp. clone Mongolia. *Anaplasma* ST KNP-1a and *Anaplasma* ST KNP-1b had 99.9% sequence identity and had, respectively 99.5% and 99.6% shared sequence identity with *A. ovis* (GenBank accession no: CP015994) and 99.1% and 99.2% identity to *Anaplasma* sp. Mongolia (GenBank accession no: MK575506). *Anaplasma* ST KNP-1 was detected primarily in impala, but was also identified in buffalo, kudu, zebra, leopard, lion and African elephant samples. *Anaplasma* ST KNP-3 was obtained from buffalo and zebra samples and was closely related to *A. centrale*, with 99.6% identity. The two novel sequences, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8, were closely related, with 99.4% identity. The two sequences had, respectively, 99.4% and 99.6% shared sequence identity with *Anaplasma* sp. Om5 (GenBank accession no: LC558313) that was recently detected in *Ornithodoros moubata* ticks collected from African warthog burrows in Zambia (Qiu et al., 2021). *Anaplasma* ST KNP-7, *Anaplasma* ST KNP-8 and *Anaplasma* sp. Om5 and *Anaplasma* sp. Om5 formed a distinct sister group within clade-1 with less than 96.0% sequence identity to a multitude of sequences within the two prominent clades of *Anaplasma. Anaplasma* ST KNP-7 was detected in buffalo, kudu and leopard samples, while *Anaplasma* ST KNP-8 was obtained from warthog and lion samples and could be a variant of the putative novel *Anaplasma* sp. recently identified in *O. moubata* ticks (LC558313).

The remaining unknown *Anaplasma* sequences grouped in clade-2. *Anaplasma* ST KNP-6, identified in zebra, leopard and lion samples, grouped with *Anaplasma* sp. SA dog (GenBank accession no: AY570538) and *Anaplasma* sp. ZAM Dog (GenBank accession no: LC269823) 16S rRNA sequences with 99.6% and 99.8% identity, respectively, suggesting it might be a variant of these. *Anaplasma* ST KNP-9, found only in impala and leopard samples, grouped in a distinct clade with *Anaplasma* sp. boleense (GenBank accession no: KU586025) with 99.0% identity. *Anaplasma* ST KNP-4 and seven variants of *Anaplasma* ST KNP-2 all grouped with *A. platys* (GenBank accession no: CP046391) and have more than 99.5% shared 16S rRNA sequence identity. Similarly, Caudill and Brayton (2022) reported that *Anaplasma* sp. Mymensingh (GenBank accession no: MF576175), "*Candidatus* Anaplasma camelii" (GenBank accession no: 843,824) and *Anaplasma* sp. Omatjenne (GenBank accession no: U54806) all group with *A. platys* with more than 99.5% shared 16S rRNA sequence identity. Although *Anaplasma* ST KNP-5 also groups with *A. platys, Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne, it is less similar with 98.7%–99.1% identity between these sequences. *Anaplasma* ST KNP-4 sequences were obtained from kudu and leopard samples, while *Anaplasma* ST KNP-5 sequences were found in zebra and leopard. *Anaplasma* ST KNP-2 was identified from a variety of wildlife hosts, including impala, buffalo, kudu, zebra, leopard, lion and elephant.

Table 3
Percentage of each *Anaplasma* 16S rRNA sequence type identified in each wildlife host.

Anaplasma 16S rRNA sequence type	Percentage of each *Anaplasma* 16S rRNA sequence type								
	Impala ($n = 21$)	Buffalo ($n = 13$)	Kudu ($n = 6$)	Zebra ($n = 6$)	Warthog ($n = 8$)	Hyena ($n = 3$)	Leopard ($n = 9$)	Lion ($n = 6$)	Elephant ($n = 2$)
A. bovis	0	0	0.71	0	0	0	0.03	0	0
A. centrale	0	24.29	0	0.03	0.37	0	0	31.24	0
A. marginale	0	73.11	0	64.27	0	0	0.03	21.09	0
A. ST SA Dog	3.68	0.06	0	26.70	0.06	100	32.14	17.51	0
A. ST KNP-1	85.94	1.48	0.71	0.32	0	0	43.36	0.22	3.16
A. ST KNP-2	10.20	0.98	93.28	0.28	0	0	3.93	1.21	95.66
A. ST KNP-3	0	0.02	0	0.04	0	0	0	0	0
A. ST KNP-4	0	0	0.61	0	0	0	1.28	0	0
A. ST KNP-5	0	0	0	3.98	0	0	0.51	0	0
A. ST KNP-6	0	0	0	4.38	0	0	18.70	4.96	0
A. ST KNP-7	0	0.06	4.69	0	0	0	0	0	1.18
A. ST KNP-8	0	0	0	0	99.57	0	0	23.77	0
A. ST KNP-9	0.18	0	0	0	0	0	0.02	0	0

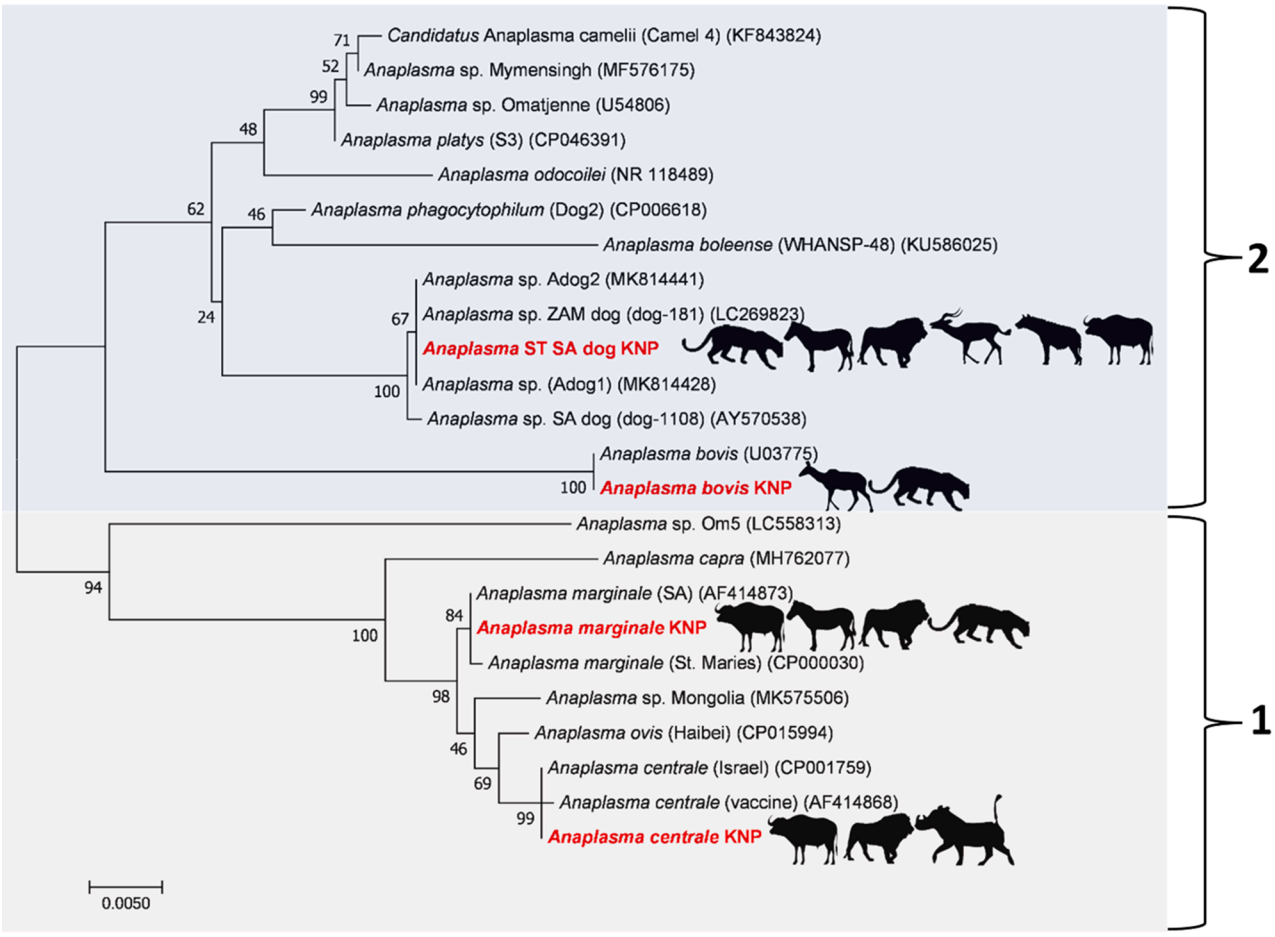

Fig. 2. Maximum likelihood phylogenetic tree based on 16S rRNA sequence data showing the relationships between *Anaplasma* species and highlighting sequences obtained in this study (shown in red) that were identical to known *Anaplasma* species. The tree is separated into the two prominent *Anaplasma* clades; clade-1, shaded in grey and clade 2, shaded in blue. Wildlife silhouettes indicate the hosts in which the sequences were identified. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct the tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model.

3.5. Analysis of Anaplasma 16S rRNA sequences detected in wildlife hosts using an Anaplasma species-discriminating single-nucleotide polymorphism method

The *Anaplasma* species-discriminating bases technique of identifying and classifying *Anaplasma* spp. as proposed by Caudill & Brayton et al. (2022) identified six species-specific bases that differentiate *Anaplasma* species within clade-1. The two 16S rRNA gene sequence variants of *Anaplasma* ST KNP-1 (*Anaplasma* ST KNP-1a and *Anaplasma* ST KNP-1b) varied by a single nucleotide which was not one of the six species-discriminating bases. The *Anaplasma* ST KNP-1 sequences varied from known species in two to four of the species-discriminating bases (Table 4). Similarly, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8 varied from the other *Anaplasma* sequences in clade-1 by two or three species-discriminating bases (Table 4). The six species-discriminating bases of *Anaplasma* ST KNP-3 differed from *A. centrale, A. ovis* and *Anaplasma* sp. Mongolia by one to five bases but were identical to those of *A. marginale*. However, *Anaplasma* ST KNP-3 grouped most closely with *A. centrale* sequences on phylogenetic analysis. Should further analysis of these putative novel species indicate that they are distinct from the known *Anaplasma* species, a new typing scheme will become necessary.

Caudill & Brayton (2022) proposed 14 bases to differentiate between species within clade-2. One of these 14 bases in the *Anaplasma* ST KNP-4 16S rRNA sequence differed from the *A. platys* sequence (Table 5), and there were other nucleotide differences elsewhere in the sequence. The *Anaplasma* ST KNP-5 sequence varied from *A. platys* and closely related species by two to eight *Anaplasma* species-discriminating bases, and *Anaplasma* ST KNP-6 varied from *A. platys* and closely related species by four to seven *Anaplasma* species-discriminating bases. *Anaplasma* ST KNP-9 varied from *A. platys* and closely related species by four to eight *Anaplasma* species-discriminating bases.

Seven variants of *Anaplasma* ST KNP-2 (a-g) 16S rRNA sequences were detected which group in clade-2. Although all of the *Anaplasma* ST KNP-2 variants, except for *Anaplasma* ST KNP-2 g, were identical to *A. platys* according to the single-nucleotide polymorphism method of classifying *Anaplasma* spp. (Table 6), the sequences differed from *A. platys* elsewhere in the full length 16S rRNA sequence. Similarly, three of the *Anaplasma* ST KNP-2 sequences (2b, 2d and 2e) were identical to "*Candidatus* Anaplasma camelii" according to the species-discriminating nucleotides, however, the full length 16S rRNA sequences differed elsewhere.

4. Discussion

This study provides insight into the diversity of *Anaplasma* species circulating in wildlife hosts in the Kruger National Park and surrounding game reserves. The most recent reorganization of the genus *Anaplasma* included seven species: *Anaplasma marginale, A. centrale, A. ovis, A. bovis, A. platys, A. phagocytophilum* and *A. caudatum* (Dumler et al., 2001). *Anaplasma capra* has been effectively published in the literature but not formally recognized (Li et al., 2015; Yang et al., 2017). More than 20

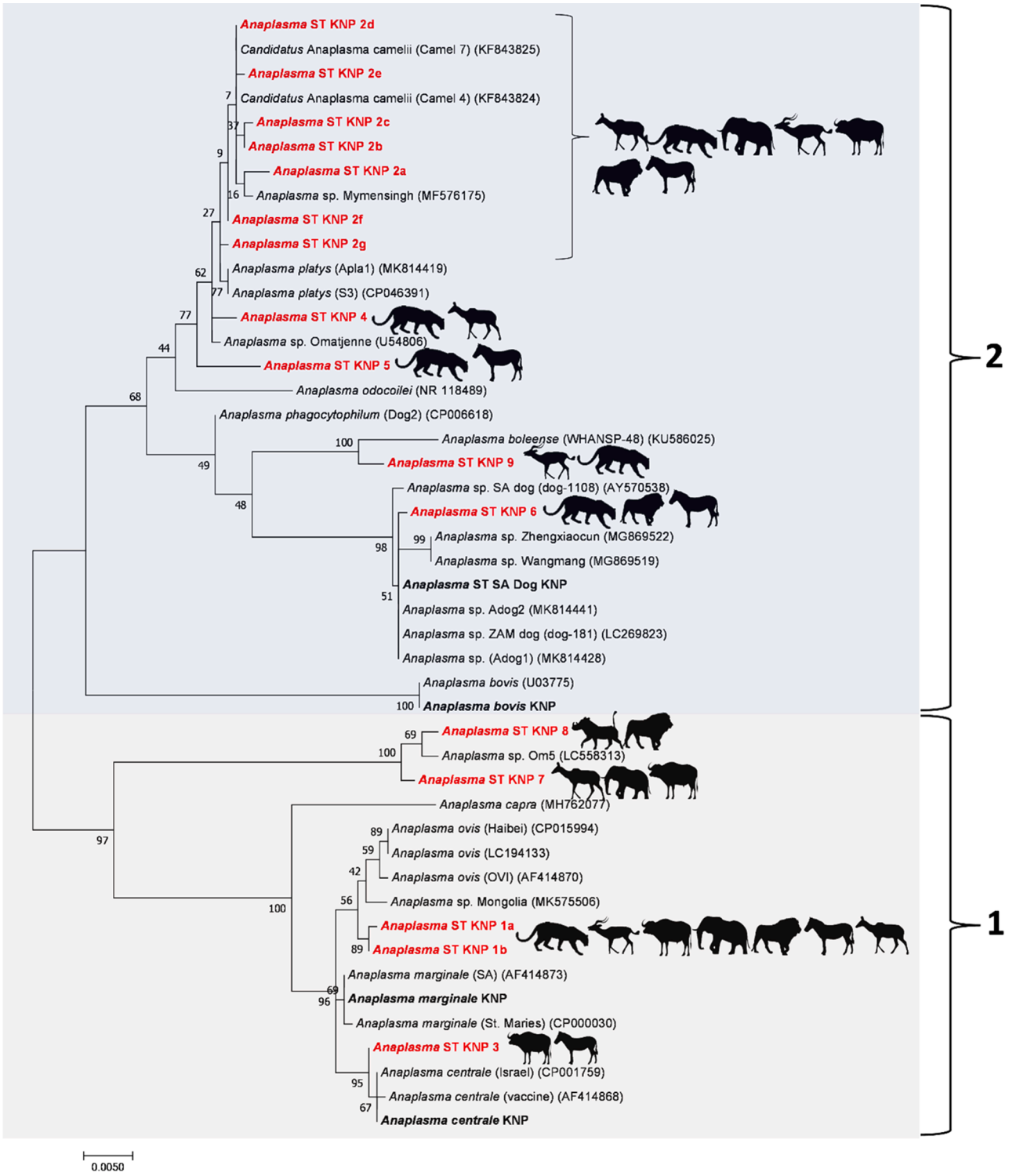

Fig. 3. Maximum likelihood phylogenetic tree based on 16S rRNA sequence data showing the phylogenetic relationships between the novel *Anaplasma* 16S rRNA sequence types identified (shown in red) and previously described *Anaplasma* species. The tree is separated into the two prominent *Anaplasma* clades; clade-1, shaded in grey and clade 2, shaded in blue. Wildlife silhouettes indicate the hosts in which the novel *Anaplasma* 16S rRNA sequences were identified. Near full-length 16S rRNA gene sequences of approximately 1328 bp in length were used to construct the tree. The numbers associated with each node indicate the percentage of 1000 bootstrap replications supporting the node. Phylogenetic analyses were conducted in MEGA7 with an HKY85 evolutionary model.

other putative *Anaplasma* species have been identified, mostly by 16S rRNA gene sequence analysis (Caudill and Brayton, 2022). We identified four previously known and nine novel *Anaplasma* genotypes in nine wildlife hosts, namely, African buffalo, impala, kudu, zebra, warthog, hyena, leopard, lion and elephant. The four known *Anaplasma* sequences identified were *A. marginale, A. centrale, A. bovis* and *Anaplasma* ST SA dog. The nine novel *Anaplasma* genotypes were genetically distinct from but closely related to known *Anaplasma* spp. based on the 16S rRNA gene sequence analysis. Although not much is known about the pathogenicity of *Anaplasma* species in wildlife hosts, subclinical infections of known

Table 4
Species-discriminating bases of clade-1 of the genus *Anaplasma*.

	Base Number*					
	144	156	220	265	274	1250
A. marginale	A	G	T	T	G	T
A. centrale	A	A	T	T	G	T
A. ovis	G	R†	Y†	C	T	T
Anaplasma sp. Mongolia	G	A	C	C	G	C
Anaplasma ST KNP-1a & b£	G	A	C	T	A	T
Anaplasma ST KNP-3	A	G	T	T	G	T
Anaplasma ST KNP-7	G	G	C	T	G	T
Anaplasma ST KNP-8	G	G	C	T	G	T

*Numbering and sequence alignment based on the *Anaplasma marginale* St. Maries 16S rRNA gene sequence. Differences between the six species-discriminating bases in *A. marginale* and the other *Anaplasma* spp. are highlighted by white text on a black background.
£Two variants with identical species-differentiating bases but differ elsewhere in the sequence.
†The degenerate position R denotes either A or G, while Y denotes either C or T.

Table 5
Species-differentiating bases of clade-2 of the genus *Anaplasma*.

	Base Number*													
	213	224	262	289	693	696	878	879	885	886	890	1052	1309	1358
A. platys	A	T	T	T	N†	T	R†	C	G	T	T	R†	Y†	C
A. sp. Mymensingh	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A. sp. Omatjenne	A	C	T	T	C	T	R†	C	G	T	T	G	C	T
"*Candidatus* A. camelii"	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. odocoilei	G	A	G	C	A	A	G	T	A	C	C	G	C	C
A. ST KNP-4	A	T	T	T	C	T	G	T	G	T	T	G	T	C
A. ST KNP-5	A	T	T	T	A	A	G	C	G	C	T	A	C	C
A. ST KNP-6	A	T	T	C	C	T	A	T	A	T	C	G	C	C
A. ST KNP-9	A	T	T	T	A	A	A	T	A	T	T	G	C	T

*Numbering and sequence alignment based on the 16S rRNA gene from the *Anaplasma platys* strain S3 genome sequence. Differences between the 14 species-discriminating bases in *A. platys* and the other *Anaplasma* spp. are highlighted by white text on a black background.
†The degenerate position N denotes any possible nucleotide, while R denotes either A or G and Y denotes either C or T.

Table 6
Species differentiating bases of clade-2 of the genus *Anaplasma*.

	Base Number*													
	213	224	262	289	693	696	878	879	885	886	890	1052	1309	1358
A. platys	A	T	T	T	N†	T	R†	C	G	T	T	R†	Y†	C
A sp. Mymensingh	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A sp. Omatjenne	A	C	T	T	C	T	R†	C	G	T	T	G	C	T
"*Candidatus* A. camelii"	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. odocoilei	G	A	G	C	A	A	G	T	A	C	C	G	C	C
A. ST KNP-2a	A	T	T	T	C	T	A	C	G	T	T	A	C	C
A. ST KNP-2b, d & e£	A	T	T	T	C	T	A	C	G	T	T	A	T	C
A. ST KNP-2c & f£	A	T	T	T	C	T	A	C	G	T	T	G	T	C
A. ST KNP-2g	A	C	T	T	C	T	A	C	G	T	T	G	C	C

*Numbering and sequence alignment based on the 16S rRNA gene from the *Anaplasma platys* strain S3 genome sequence. Differences between the 14 species-discriminating bases in *A. platys* and the other *Anaplasma* spp. are highlighted by white text on a black background.
£Two or three variants with identical species-differentiating bases which but differ elsewhere in the sequence. †The degenerate position N denotes any possible nucleotide, while R denotes either A or G and Y denotes either C or T.

Anaplasma species have been reported and wildlife are thus usually regarded as reservoir hosts (Neitz, 1935; Peirce, 1972; Augustyn, 1974; Kuttler, 1984; Smith et al., 1982; Ngeranwa et al., 1998; Potgieter and Stoltsz, 2004; Eygelaar et al., 2015; Khumalo et al., 2016; Sisson et al., 2023). *Anaplasma marginale* infections in cattle can cause disease with varying levels of severity, from icterus and anemia, to abortions and death, while *A. centrale, A. bovis* and *A. platys* are regarded as non-pathogenic in cattle and usually cause subclinical infection in these animals.

As expected, our results indicate that *A. marginale* and *A. centrale* are widespread in the African buffalo population in the Kruger National Park. These two tick-borne haemoparasites have previously been identified in African buffalo, as well as black wildebeest, blue wildebeest, eland and waterbuck (Henrichs et al., 2016; Khumalo et al., 2016; Sisson et al., 2017; 2023). Interestingly, we detected 16S rRNA sequences identical to *A. marginale* in zebra, leopard and lion, while 16S rRNA sequences identical to *A. centrale* were also found in zebra and warthog. These data may suggest an expansion of the potential host range for *A. marginale* and *A. centrale*, as they are regarded as ruminant-specific *Anaplasma* species. However, it should be noted that low numbers of *A. marginale* and *A. centrale* 16S rRNA sequences were detected in a minority of feline samples. Nevertheless, more work needs to be done in a larger population of felines to determine whether the detection of *A. marginale* and *A. centrale* was incidental or if felines are reservoir hosts.

Anaplasma sp. dog strain was initially detected and identified in three dogs at the Veterinary Teaching Hospital of the Medical University of South Africa (Inokuma et al., 2005). The 16S rRNA gene sequence, designated *Anaplasma* sp. SA dog (GenBank accession no: AY570539 and AY570540), has 99.8% sequence identity to the 16S rRNA sequence from an *Anaplasma* sp. identified in dogs in Zambia, designated *Anaplasma* sp. ZAM Dog (GenBank accession no: LC269823). The *Anaplasma* sp. ZAM Dog 16S rRNA sequence was detected in domestic dogs in Lusaka, Zambia (Vlahakis et al., 2018) and recently in dogs and *Rhipicephalus sanguineus* ticks in the Mnisi community, Mpumalanga, South Africa (Kolo et al., 2020). Kolo et al. (2022) suggested that the *Anaplasma* 16S rRNA sequences identified in dogs represent variants of a single novel organism and proposed that it be referred to as *Anaplasma* sp. SA dog (for *Anaplasma* sp. Southern Africa dog) until type material can be deposited. Little is known about this putative *Anaplasma* species. It groups closely with *A. phagocytophilum* and other *Anaplasma* 16S rRNA sequences identified in dogs in clade-2. In our study, a 16S rRNA gene sequence identical to the *Anaplasma* sp. ZAM Dog 16S rRNA sequence (GenBank accession no: LC269823) was widespread in the wildlife species examined; it was detected mainly in leopard, zebra and lion and to a lesser extent in impala, spotted hyena, buffalo and warthog. Although this *Anaplasma* species has thus far only been detected in dogs and associated ticks, our findings suggest additional wildlife hosts as possible reservoir hosts. Interestingly, *Anaplasma* ST KNP-6, detected in leopard, lion and zebra, also grouped in the *Anaplasma* sp. SA dog clade with 99.6% shared 16S rRNA sequence identity. Our data could therefore suggest that *Anaplasma* ST KNP-6, *Anaplasma* sp. ZAM Dog and *Anaplasma* sp. SA dog represent variants of the same species, however, additional genomic data is required to resolve this question, and, given their close relationship to *A. phagocytophilum* further work is required to determine their zoonotic potential.

We detected 16S rRNA sequences that are 100% identical to the *A. bovis* sequence (GenBank accession no: U03775) in kudu and leopard samples. Although *A. bovis* infection is mainly reported in cattle (Noaman and Shayan, 2010; Belkahia et al., 2015), little is known about the epidemiology of this agent. However, it is closely related to *A. phagocytophilum* and is regarded as a zoonotic agent that infects monocytes, it is usually associated with subclinical infection, and *Hyalomma* spp. are considered to be vectors of the organism (Donatien and Lestoquard, 1936). *Anaplasma bovis* was also detected in a population of eastern rock sengis (*Elephantulus myurus*) in South Africa, suggesting that sengis may be natural reservoir hosts of the organism (Harrison et al., 2013). It is thus possible that other rodent species are reservoir hosts of *A. bovis* and possibly other *Anaplasma* spp.

Of the nine novel *Anaplasma* 16S rRNA sequence types identified, *Anaplasma* ST KNP-1, *Anaplasma* ST KNP-3, *Anaplasma* ST KNP-7 and *Anaplasma* ST KNP-8 are found in clade-1, which is commonly referred to as the livestock clade, while *Anaplasma* ST KNP-2, *Anaplasma* ST KNP-4, *Anaplasma* ST KNP-5, *Anaplasma* ST KNP-6 and *Anaplasma* ST KNP-9 are found in clade-2, commonly referred to as the zoonotic clade. It should be noted that, since similarities above 98.70% occur between 16S rRNA sequences of known *Anaplasma* species (Caudill and Brayton, 2022), it is difficult to distinguish between *Anaplasma* species based on 16S rRNA sequences alone. Furthermore, it has previously been shown that the 16S rRNA gene sequences of *A. platys, Anaplasma* sp. Mymensingh, "*Candidatus* Anaplasma camelii" and *Anaplasma* sp. Omatjenne share more than 99.5% sequence identity and do not resolve these organisms to species level, although a high degree of intraspecies variance is evident based on the single-nucleotide polymorphisms used to distinguish the species within this clade (Caudill and Brayton, 2022). Many of the novel 16S rRNA sequences identified in our study are highly similar to previously reported *Anaplasma* sequences, and it is therefore not clear whether they represent novel species or variants of known or previously reported putative *Anaplasma* species. This will require further investigation, including the sequencing and phylogenetic analysis of other genes, or whole genome sequence analyses. It is evident that there is an urgent need to identify an alternative gene or genes for the discrimination of species within the genus *Anaplasma*.

Although *Anaplasma* species and strains infecting one host species might not necessarily infect and cause disease in other host species, we have noted the presence of some of the novel 16S rRNA sequences in a variety of wildlife hosts, suggesting that they may be able to infect multiple host species. Therefore, the ability of these newly identified agents to infect cattle, other domestic animals and possibly even humans should be assessed. Novel 16S rRNA sequences have already been identified in cattle in South Africa. These include "*Candidatus* Anaplasma boleense", *Anaplasma* sp. Mymensingh and *Anaplasma* sp. SA dog (Kolo et al., 2020) which were identified in cattle in the Mnisi community, which borders on the Manyeleti and Timbavati Game Reserves and the Kruger National Park. If the putative *Anaplasma* species identified in our study are found to infect livestock, they could affect the specificity of existing tests for detection of *A. marginale*. Furthermore, the presence of the novel 16S rRNA sequences in wildlife could impact on the use of existing tests for the detection of known *Anaplasma* species in wildlife.

Seroprevalence studies are often used to determine the prevalence of *A. marginale.* Current serological tests used to diagnose anaplasmosis include the competitive ELISA (cELISA), complement fixation test, card agglutination test (CAT) and IFA test, with only cELISA and CAT recommended for the diagnosis of anaplasmosis (Kocan et al., 1992; De la Fuente et al., 2005). The commercially available *Anaplasma* genus-specific cELISA kit (Knowles et al., 1996) uses recombinant major surface protein 5 (Msp5) as antigen. Since the Msp5 epitope is widely conserved between *Anaplasma* species (Munodzana et al., 1998; Hofmann-Lehmann et al., 2004; Dreher et al., 2005; Strik et al., 2007), the cELISA cannot be used for the specific detection of *A. marginale* if multiple species of *Anaplasma* are known to occur in cattle, frequently as co-infections (Zweygarth et al., 2006; Khumalo et al., 2016; Hove et al., 2018; Makgabo et al., 2023). Indeed, in many parts of the world assumptions are generally made by host species: if the test is positive in cattle, it is likely that it is detecting *A. marginale*, whilst a positive result from sheep or goats should indicate *A. ovis* infection. However, these assumptions can lead people astray (Da Silva et al., 2018), due to the broad cross-reactivity among known *Anaplasma* species from both clades (Munodzana et al., 1998; Hofmann-Lehmann et al., 2004; Dreher et al., 2005; Strik et al., 2007). We suspect the cELISA will also recognise the putative *Anaplasma* species identified in wildlife, although more work will be required to confirm this.

Based on our analysis, several primers and probes from nucleic acid-based assays targeting the 16S rRNA gene, such as the *A. platys* (Inokuma et al., 2001) and *A. phagocytophilum* (Kawahara et al., 2006) specific assays, as well the reverse line blot hybridization (RLB) assay (Georges et al., 2001; Bekker et al., 2002), would cross-react with some of the *Anaplasma* ST detected, ST KNP-2, ST KNP-4, ST KNP-5 as well as other previously described putative *Anaplasma* species, including *Anaplasma* sp. Omatjenne and *Anaplasma* sp. Mymensingh, while the *A. phagocytophilum* assay (Kawahara et al., 2006) would also cross-react with *Anaplasma* ST SA dog and *Anaplasma* ST KNP-6. The RLB *Anaplasma* sp. (Omatjenne) probe (Bekker et al., 2002) would cross-react with *Anaplasma* sp. Mymensingh, *Anaplasma* ST KNP-2 (all variants), *Anaplasma* ST KNP-4, as well as *Anaplasma* ST KNP-5. The *A. centrale*-specific probe (Georges et al., 2001) would cross-react with the two variants of *Anaplasma* ST KNP-1. The use of these assays to determine the presence of known *Anaplasma* species in wildlife should therefore be interpreted with caution. This highlights the necessity for more specific assays to be developed to assess the epidemiology of *Anaplasma* species more accurately.

The widely used duplex real-time assay to detect *A. marginale* and *A. centrale* infections in cattle (Decaro et al., 2008; Byaruhanga et al., 2016; Hove et al., 2018) is not based on the 16S rRNA gene but on the *A. marginale msp1β* gene and the *A. centrale groEL* gene (Decaro et al., 2008; Chaisi et al., 2017). While these assays have been used to detect *A. marginale* and *A. centrale* in wildlife (Khumalo et al., 2016), it remains to be seen whether these assays will cross-react with the putative novel *Anaplasma* species since nothing is known about their gene complement.

5. Conclusion

Our results revealed a greater genetic diversity of *Anaplasma* species circulating in wildlife hosts than currently classified within the genus *Anaplasma* and suggest potential for transmission to livestock or companion animals. Furthermore, these novel genotypes are phylogenetically similar to known *Anaplasma* spp. and may serve as a source of cross-reaction in the current detection assays. Although this data, including that of single-nucleotide polymorphisms used to distinguish between the different *Anaplasma* species within the two clades, may provide sufficient genetic divergence between these organisms to potentially suggest classification as separate species within the clade, there is a need for additional genetic data and genome sequencing of these putative species for correct *Anaplasma* species classification and to further determine their occurrence in livestock and companion animals.

CRediT authorship contribution statement

S. Marcus Makgabo: Methodology, Investigation, Formal analysis, Writing – original draft. **Kelly A. Brayton:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Marinda C. Oosthuizen:** Supervision, Writing – review & editing. **Nicola E. Collins:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All of the sequence data generated from this study have been registered in GenBank under the BioProject accession number: PRJNA965916. *Anaplasma* 16S rRNA sequences were deposited under accession numbers OQ909436 to OQ909508. Additional data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100198.

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