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Molecular characterisation of *Anaplasma* species from African buffalo (*Syncerus caffer*) in Kruger National Park, South Africa

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ABSTRACT

Bovine anaplasmosis is a tick-borne disease, mainly caused by *Anaplasma marginale* and *A. centrale* and is distributed in tropical and sub-tropical areas. This study aimed to characterise *A. marginale* and *A. centrale* from African buffaloes in Kruger National Park (KNP), South Africa, using the DNA sequences of the genes coding for major surface protein (*msp1β*) and heat shock protein (*groEL*), respectively. A total of 747 blood samples were collected from February 2014 to August 2016 from African buffaloes kept in KNP, and DNAs were tested using a molecular-phylogenetic approach. Out of 747 samples tested, 129 (17.3%) and 98 (13.1%) were positive for single infection with *A. marginale* and *A. centrale*, respectively; whereas 113 (15.1%) were positive for both *Anaplasma* spp. Pairwise difference of 1.6–8.5% was observed in *msp1β* sequences of *A. marginale* whereas that was only 0.3–2.4% for *groEL* sequences of *A. centrale*. Separate phylogenetic analyses of *msp1β* and *groEL* sequences of *A. marginale* and *A. centrale*, respectively, revealed that sequences of *Anaplasma* spp. from African buffaloes were unique and they grouped separately when compared with previously published sequences of both species. This is the first study to characterise *A. marginale* and *A. centrale* from African buffalo using species specific molecular markers. This study will pave the way for future studies to assess genetic variation among *Anaplasma* spp. from wild ruminants using molecular markers that are better at differentiating between species and strains than the more commonly used 16S rRNA gene, and help to undertake health and fitness studies and host-parasite dynamics using quantitative molecular tools.

1. Introduction

Anaplasmosis is an important tick-borne disease (TBDs) of domestic and wild animals in tropical and subtropical regions of the world, and is caused by obligate intracellular bacteria of the genus *Anaplasma* (Rickettsiales: Anaplasmataceae). It is one of the four most detrimental tick-borne diseases (TBDs) of bovines in sub-Saharan Africa (others: babesiosis, cowdriosis and theileriosis) (Debeila, 2012), and is estimated to be responsible for 3% of all cattle mortalities in South Africa (Aubry and Geale, 2011; Brown, 2012; De Waal, 2000; Eygelaar et al., 2015). In South Africa, anaplasmosis is mainly transmitted by *Amblyomma* spp. and *Rhipicephalus* spp. (Gallivan et al., 2011; Horak et al., 2007; Horak et al., 2011). Bovine anaplasmosis is mainly caused by *Anaplasma marginale* and, to a lesser extent, by *A. centrale*. The disease is usually transmitted by ticks, but it can also be transmitted transplacentally, or mechanically by biting flies or blood-contaminated fomites (Aubry and Geale, 2011; Potgieter and Stoltz, 2004). Major clinical signs in infected cattle include pyrexia, progressive anaemia, jaundice, anorexia, depression, reduced milk production, abortion in pregnant animals, and death, particularly in exotic breeds (Aubry and Geale, 2011; Debeila, 2012; Kocan et al., 2010; Potgieter and Stoltz, 2004).

While anaplasmosis is well documented in cattle, very little is known about the disease and its impact in wild bovids such as the African buffalo (*Syncerus caffer*). Tick-borne infections are common in buffalo, which serve as a reservoir for bovine theileriosis (*Theileria parva*) (Debeila, 2012; Henrichs et al., 2016). Buffalo appear to be only mildly affected by *Anaplasma* infections (Berggoetz et al., 2014; Debeila, 2012; Kuttler, 1984), which raises the question whether they might also function as reservoirs for this group of parasites. To date, *A. marginale*, *A. centrale*, *A. sp. Omatjenne*, *A. bovis* and *A. phagocytophilum* have been detected from African buffaloes using the 16S rRNA gene, though *A. phagocytophilum* has only been found once in

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one animal (Fyumagwa et al., 2013; Henrichs et al., 2016). African buffalo are a useful wildlife species in which to study *Anaplasma* species dynamics as they are known to be wildlife reservoirs for numerous diseases such as foot-and-mouth disease, bovine tuberculosis and theileriosis, yet their role in maintaining *Anaplasma* species is unknown (Debeila, 2012). Identification of wildlife reservoirs is essential to controlling infection in livestock which may have a direct or indirect contact with the reservoir population (Haydon et al., 2002).

Various methods have been used to diagnose anaplasmosis, including microscopy, serology (complement fixation, rapid card agglutination, indirect immunofluorescent antibody tests, capillary tube agglutination tests, enzyme-linked immunosorbent assays, latex agglutination and radioimmunassays) and molecular methods (Aubry and Geale, 2011; Potgieter and Stoltz, 2004). For molecular methods utilising polymerase chain reaction (PCR), a number of markers such as the 16S rRNA, major surface protein (*msp1α*, *msp1β*, *msp2*, *msp3*, *msp4* and *msp5*), citrate synthase *gltA* and the heat shock protein *groEL* genes have been used for the detection of *Anaplasma* spp. (Carelli et al., 2007; Ceci et al., 2008; de la Fuente et al., 2001; Lew et al., 2002, 2003; Molad et al., 2006); whereas, *msp1α*, *msp1β*, *msp4* and *groEL* have been used for finer scale differentiation of various *Anaplasma* species and strains.

Very little is known about the diversity of *Anaplasma* spp. from African buffaloes and only one marker, the 16S rRNA gene, has been used so far to characterise them (Debeila, 2012; Henrichs et al., 2016). However, the most commonly used markers for characterisation of these species, such as genes for major surface proteins and heat shock protein *groEL*, allow for better resolution which might reveal significant genetic differences among *Anaplasma* spp. found in domestic and wild ruminants, and can be used in quantitative molecular methods. Therefore, the aim of this study was to characterise the two important species of *Anaplasma* (*A. marginale* and *A. centrale*) from African buffaloes in South Africa using DNA sequences from the genes coding for a major surface protein and heat shock protein *groEL*, respectively. This study will allow future research into buffalo as a reservoir host of *Anaplasma* spp.

2. Materials and methods

2.1. Study site, animal characteristics and blood collection

Blood samples were collected as a part of a longitudinal disease study of African buffaloes in Kruger National Park (KNP), an area 360 km long (north-south) and 90 km wide (east-west) in the north-east corner of the Republic of South Africa (Fig. 1). Kruger National Park is in a summer-rainfall area with an average temperature of 22 °C (18–26 °C) and average annual rainfall ranging from 458 to 746 mm (SA Weather Bureau; Zambatis, 2003). From February 2014 until August 2016, approximately 60 buffaloes were sampled every two-to-three months (12 captures in total) from a 900 ha predator-free enclosure near Satara rest camp (S 23°23′52″, E31°46′40″) (Fig. 1) (SANParks, 2016). At any given time depending on births and deaths, the herd consisted of 49–70 buffaloes. As the buffaloes are restricted in grazing grounds by the double-fenced enclosure, supplementary feed, including formulated feed and lucerne hay, is occasionally supplied during the dry season, and a permanent man-made water source is available year-round as natural water sources tend to dry out seasonally.

For this study, blood samples ($n = 747$) collected over a two-year period (2014–2016) were used. Blood samples were taken from the jugular vein into EDTA-coated tubes by experienced, practicing veterinarians and stored at −80 °C. The Animal Care and Use Proposal (ACUP) for this study was approved by the Institutional Animal Care and Use Committee (ACUP 4478) of the Oregon State University.

2.1.1. Molecular characterisation

DNA was extracted from EDTA bloods using DNeasy Blood and Tissue Kit (Qiagen, USA) following manufacturer's protocol and stored at −80 °C.

For the detection of *A. marginale*, the *msp1β* gene was amplified using a nested PCR as previously described by Molad et al. (2006). The external primers (AM456/AM1164) were used to amplify a PCR product of 700 bp while internal primers (AM100/AM101) yielded an amplicon of 246 bp. The PCR was performed in a final reaction volume of 25 μl, the PCR amplification mix contained 5–10 ng of purified genomic DNA as template, 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega), 3.5 mM $MgCl_2$, 6.25 μM of each deoxynucleotide triphosphate (dNTP), 100 pmol of each primer, and 1 U of Go*Taq* polymerase (Promega, USA). PCR cycling conditions were an initial denaturation at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s (61 °C for 10 s for nested PCR) and 72 °C for 30s, and 72 °C for 5 min.

Anaplasma centrale was detected using an amplification of a partial fragment of the *groEL* gene. PCR primers DS_Ac_F1 (forward 5′-GAGAAGATGCTGGTGGAGTT-3′) DS_Ac_R2 (reverse 5′-ACCACCGCATTCAAGGTCAT-3′) were designed to published *groEL* gene sequences (GenBank accession numbers EF520691-EF52069 and AF414866-AF414867 (Ceci et al., 2008; Lew et al., 2003) and used to specifically PCR-amplify part (1,250 bp) of *A. centrale*. The reagents and PCR conditions were optimised in a series of experiments; the final PCR was conducted in a 25 μl volume containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega, USA), 3.5 mM $MgCl_2$, deoxynucleotide triphosphates (dNTPs; 200 μM each), primers (35 pmol each) and 1 U Go*Taq* polymerase (Promega) using the following protocol: 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 15 s at 55 °C and 45 s at 72 °C, followed by a final extension of 5 min at 72 °C.

Negative and positive controls were included in each PCR. Following PCR, an aliquot (5 μl) of each amplicon was examined on 1.5% w/v agarose gels.

About 15% of PCR amplicons positive for each *Anaplasma* spp. were randomly selected for DNA sequencing. Selected PCR amplicons of both *A. marginale* and *A. centrale* were treated with shrimp alkaline phosphatase and exonuclease I (Fermentas Inc., USA) (Werle et al., 1994) and subjected to direct bi-directional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, USA) using the same primers used in PCR. The quality of sequences was assessed using the program Geneious Pro 2.0.10 (Larkin et al., 2007).

2.1.2. Phylogenetic analyses

Nucleotide sequences of the gene *msp1β* for *A. marginale* and *groEL* for *A. centrale* regions determined here were aligned using Mesquite v3.4 (Maddison, 2015) with MuscleAlign (Edgar, 2004), with those of homologous reference sequences for each respective *Anaplasma* spp. in current databases (accessed on the 1st January 2017; GenBank accession numbers AF111195-AF111197, AF348137, AF112479-AF112480, AF348138, AF110808-AF110810, AF221692, EU281852, AY841153 for msp1β sequences of A. marginale; EF520691, AF414867, and AF414866 *groEL* sequences of *A. centrale* and *groEL* sequences of *A. marginale* (JQ839014) and *Anaplasma ovis* (AF441131) were used as outgroup) and classified. Reference sequences for *A. marginale* were chosen based on their high sequence identity to sequences from this study when run through an nBLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and to compare a range of geographical areas. For *A. centrale*, as there were very few sequences available, all were included and those that had 100% sequence identity in the region used for sequence comparison were discarded. Sequence identities (in%) were calculated by pairwise comparison using the program BioEdit v7.2.5 (Hall, 1999). All DNA sequences obtained for the *msp1β* and

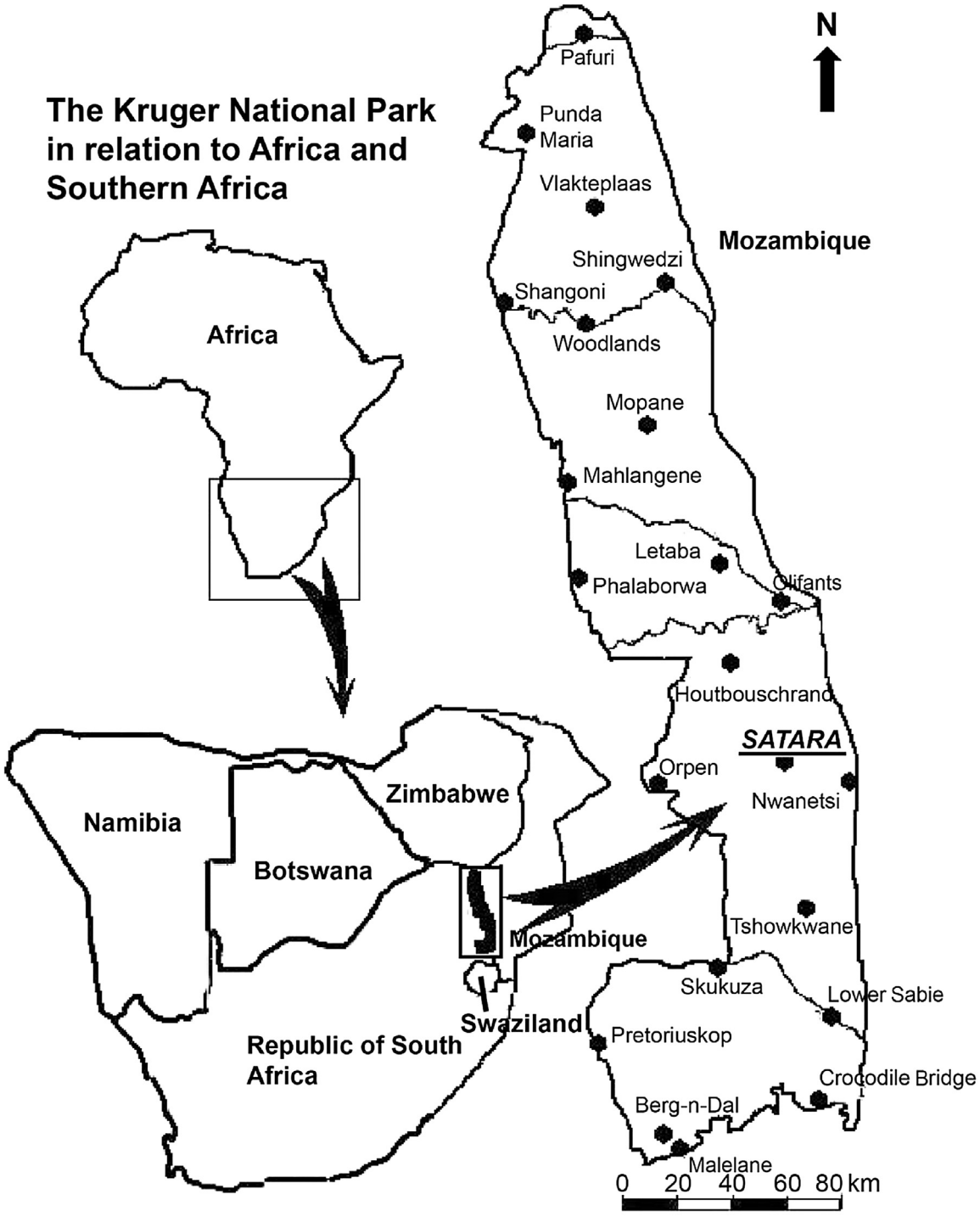

Fig. 1. Map showing the study site (right) in the Kruger National Park, in relation to the rest of South Africa, and the African continent. Rest camps within Kruger National Park are shown as black spots. Buffalo enclosure is located in the Satara rest camp (bold and underlined) (modified from SANParks).

groEL genes were aligned over 345 and 881 nucleotide positions, respectively.

Phylogenetic analyses were performed using Bayesian Inference (BI) and Neighbor Joining (NJ) methods. The BI was conducted, using Monte Carlo Markov Chain (MCMC) analysis in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The likelihood parameters for BI were based on the Akaike Information Criterion (AIC) test in jModeltest v2.1.5 (Darriba et al., 2012; Guindon and Gascuel, 2003). The General Time Reversible with a proportion of invariable sites (GTR + I), and transversion model with gamma distribution and (TVMef + G) were utilised for the analyses of the *A. centrale* and *A. marginale* sequence data, respectively. Four simultaneous tree-building chains were used to calculate posterior probabilities (pp) for 2,000,000 generations, saving every 100th tree produced. Based on the final 75% of trees generated, a consensus tree was constructed. The NJ analyses were performed using the program MEGA 6.0 (Tamura et al., 2013), and the nodes were tested for robustness with 10,000 bootstrap repli-

cates. The phylogenetic trees produced from the BI and NJ analyses were compared for concordance in their topologies.

3. Results

PCR amplicons were of expected size (*msp1β, 700*/246; *groEL*, 1250) when examined using agarose gel electrophoresis. PCR-sequencing analysis revealed the presence of single or mixed infections of *Anaplasma* spp. in African buffaloes. Out of 747 samples tested, 129 (17.3%) and 98 (13.1%) were positive for single infection with *A. marginale* and *A. centrale*, respectively; whereas 113 (15.1%) were positive for both *Anaplasma* spp. Of the 103 individuals sampled at least once throughout the sampling period, only 17 were negative for both *A. marginale* and *A. centrale* infection at all times, with the majority of those being calves (12/17). DNA sequencing of 41 PCR amplicons of *A. marginale* revealed four unique sequences of msp1β (GenBank accession nos. KX714578-KX714581) from African buffaloes while that of 36 PCR amplicons of A. centrale revealed seven unique sequences of groEL (GenBank accession nos. KX714582- KX714588).

Subsequently, we studied sequence variation within the *msp1β* and *groEL* sequences. Within *A. marginale* sequences of *msp1β* (aligned over 345 bp; GC content 52.6-55.6%), a nucleotide variability ranging from 1.6 to 8.5% (Table 1) was found, mainly attributed to transversions (A ↔ G, T ↔ C, G ↔ C) at nucleotide positions 85, 147, 190, 213, 255, 271, 279, 306, and 321, and just one transition (T ↔ G) at nucleotide position 165 (see Supplementary Fig. 1). Two *msp1β* sequences (KX714579 and KX714581) had three insertions at nucleotide positions 154–156, and one (KX714581) of these two sequences had an additional 15 nucleotide differences from positions 241–243 (see Supplementary Fig. 1). Within *groEL* sequences of *A. centrale* (aligned over 881 bp; GC content 49.3-49.9%), a nucleotide difference of 0.3–2.4% was observed (Table 2) due to transitions of either A↔G at nucleotide positions 99, 114, 123, 126, 132, 138, 351, 402 and 429, or T↔C at positions 100, 136, 141, 145, 240, 324, 336, 420, 438, 549, 570 and 807 (see Supplementary Fig. 2). We then compared sequences of *A. marginale* and *A. centrale*, separately, with reference sequences available from GenBank (Tables 1 and 2) to establish the nature and extent of nucleotide variation. All four *msp1β* sequences of *A. marginal*e had the highest similarity (85.9–86.5%) with two reference sequences (GenBank accession no. AY841153, and AF111196 and M59845) from Israel and USA, respectively, whereas they had the lowest similarity with another reference sequence (CP000030) from the USA (Table 1). Similarly, the comparison of seven *groEL* sequences of *A. centrale* determined herein with available reference sequences revealed the highest similarity (93.9%) with a sequence from Italy (EF520691) while lowest with a sequence from Australia (AF414866). All other sequences used for comparisons were from cattle.

Phylogenetic relationships of *A. marginale* and *A. centrale* sequences were explored separately using selected reference sequences (Fig. 2). The topology of the phylogenetic trees generated for both *A. marginale* and *A. centrale* sequences, employing BI and NJ methods were similar (data not shown); hence, the NJ trees for both *Anaplasma* spp. are presented here, with nodal support values given for both methods (Fig. 2). For *A. marginale*, the tree revealed five major clades, where clades 1–3 and 5 contained *msp1β* sequences originated from Brazil, Israel and the USA, whereas clade 4 consisted of four sequences (GenBank accession numbers KX714578-KX714581) determined in this study. However, the nodal support for clade 4 was very weak (Fig. 2A). For *A. centrale*, the tree comprised of two clades where seven *groEL* sequences of *A. centrale* determined here clustered together in clade 1, with strong statistical support (posterior probability value for BI, 1.0; bootstrap value for NJ, 100%), with the exclusion all reference sequences of *A. cen-*

Table 1
Pairwise differences (%) among the different partial major surface protein 1β sequences of Anaplasma marginale from African buffalo.

GenBank accession number (location)	KX714578	KX714579	KX714580	KX714581	AF110808	AF110809	AF110810	AF111195	AF111196	AF111197	AF112479	AF112480	AF221692	AF221693	AF348137	AF348138	AY841153	CP000030	EU281852	M59845
KX714578 (South Africa)																				
KX714579 (South Africa)	3.5																			
KX714580 (South Africa)	1.6	2.5																		
KX714581 (South Africa	8.5	5.0	7.5																	
AF110808 (U.S.A.)	23.4	22.3	22.5	26.1																
AF110809 (U.S.A.)	23.4	22.3	22.5	26.1	0.0															
AF110810 (U.S.A.)	23.4	22.3	22.5	26.1	0.0	0.0														
AF111195 (U.S.A.)	20.2	17.6	19.3	14.1	17.0	17.0	17.0													
AF111196 (U.S.A.)	19.3	16.7	18.5	13.2	14.4	14.4	14.4	4.7												
AF111197 (U.S.A.)	18.8	16.1	17.9	12.6	16.1	16.1	16.1	1.5	3.3											
AF112479 (U.S.A.)	24.8	23.7	23.9	24.6	4.6	4.6	4.6	14.4	14.1	13.5										
AF112480 (U.S.A.)	19.3	16.7	18.5	13.2	15.3	15.3	15.3	3.3	1.5	2.4	14.4									
AF221692 (U.S.A.)	23.4	22.3	22.5	26.1	0.0	0.0	0.0	17.0	14.4	16.1	4.6	15.3								
AF221693 (U.S.A.)	22.8	20.1	21.9	23.9	24.0	24.0	24.0	21.9	18.4	21.0	27.7	19.3	24.0							
AF348137 (U.S.A.)	18.5	15.8	17.6	12.3	15.5	15.5	15.5	2.4	3.0	0.9	13.5	2.1	15.5	20.2						
AF348138 (U.S.A.)	23.9	22.9	23.1	23.7	3.7	3.7	3.7	13.5	13.8	12.6	1.6	14.1	3.7	26.3	12.0					
AY841153 (Israel)	16.1	13.5	15.3	17.3	10.3	10.3	10.3	7.9	5.6	6.5	14.1	6.5	10.3	15.2	6.8	12.6				
CP000030 (U.S.A.)	55.4	54.6	55.4	55.4	54.6	54.6	54.6	54.2	53.4	53.9	54.9	54.2	54.6	56.6	53.4	54.2	52.7			
EU281852 (Brazil)	25.4	24.3	24.5	25.2	7.7	7.7	7.7	15.8	15.5	15.0	5.9	16.4	7.7	28.0	14.4	5.2	15.0	55.1		
M59845 (U.S.A.)	16.1	13.5	15.3	17.3	10.0	10.0	10.0	7.9	5.0	7.1	13.8	5.3	10.0	14.0	6.2	12.3	1.2	52.7	14.7	

Table 2
Pairwise differences (%) among the different partial heat shock protein (groEL) sequences of Anaplasma centrale from African buffalo.

GenBank accession number (location)	KX714582	KX714583	KX714584	KX714585	KX714586	KX714587	KX714588	EF520691	AF414867	AF414866
KX714582 (South Africa)										
KX714583 (South Africa)	0.8									
KX714584 (South Africa)	2.2	2.4								
KX714585 (South Africa)	1.8	2.0	0.6							
KX714586 (South Africa)	0.4	0.6	1.9	1.4						
KX714587 (South Africa)	0.6	1.1	2.0	1.5	0.5					
KX714588 (South Africa)	0.4	0.8	1.9	1.4	0.3	0.3				
EF520691 (Italy)	7.7	7.5	6.1	6.2	7.4	7.4	7.4			
AF414867 (Australia)	7.9	7.8	6.3	6.4	7.7	7.7	7.7	1.2		
AF414866 (Australia)	8.0	7.9	6.4	6.5	7.8	7.8	7.8	0.8	0.4	

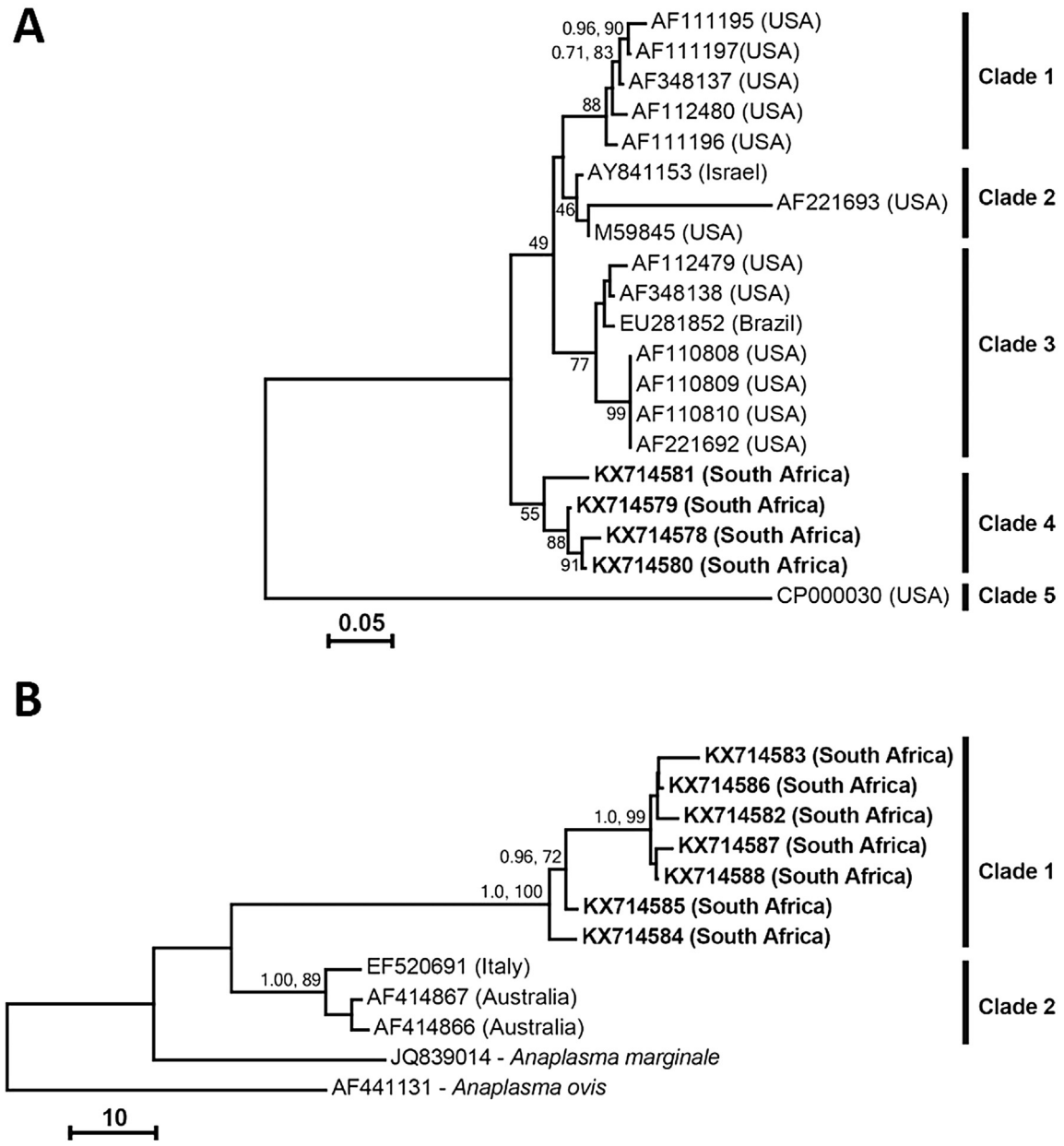

Fig. 2. Genetic relationships of *Anaplasma marginale* (A) and *Anaplasma centrale* (B) detected from African buffalo (*Syncerus caffer*) from Kruger National Park, South Africa (bold) with reference genotypes selected from previous studies. The relationships were inferred based on the phylogenetic analysis of partial sequences of major surface protein 1β (*A. marginale*, A) and heat shock protein (*groEL*) (*A. centrale*, B) using Neighbour Joining (NJ) and Bayesian inference (BI) methods, and *A. ovis* was used as the outgroup for *A. centrale*. Nodal support values are indicated: posterior probability for BI (first), and bootstrap for NJ (second). The scale bar indicates distance.

trale from Italy (EF520691) and Australia (AF414867 and AF414867) grouping in clade 2 (Fig. 2B). The *groEL* sequences of *A. marginale* and *A. ovis* formed separate clades (Fig. 2B).

4. Discussion

This is the first study to characterise *A. marginale* and *A. centrale* from African buffalo using species specific molecular markers, *msp1β* and *groEL*, respectively. Molecular-phylogenetic analyses of *msp1β* and *groEL* sequences of *A. marginale* and *A. centrale*, respectively, revealed that sequences of *Anaplasma* spp. from African buffaloes were unique and they grouped separately when compared with previously published sequences of both species.

In the present study, we found that the overall sample prevalence of *A. marginale* (32.7%; 242/747) was similar to that of *A. centrale* (28.2%; 211/747) in African buffaloes which does appear to differ from findings of previous studies reported from Botswana (Carmichael and Hobday, 1975; Eygelaar et al., 2015) and South Africa (Debeila, 2012; Henrichs et al., 2016), but not statistically significantly. For example, Eygelaar et al. (2015) tested blood samples from African buffaloes located in Chobe National Park and the Okavango Delta from Botswana by employing reverse line blot hybridization analysis (RLB) using 16 s rRNA gene and found that the sample prevalence of *A. centrale* (32.7%; 36/110) was higher than *A. marginale* (21.8%; 24/110). Similarly, using the same RLB method, two recent studies by Debeila (2012) and Henrichs et al. (2016) from South Africa (Kruger National and Hluhluwe-Imfolozi Parks) also found that the sample prevalence of *A. centrale* (49–75% and 14.3–61.2%, respectively) was higher than *A. marginale* (24–42% and 20.9–53.8%, respectively). These differences in the sample prevalence of *Anaplasma* spp. between previous studies and this one could be due to different markers used in PCR as we used *Anaplasma* species specific markers for *A. centrale* (*groEL*) and *A. marginale* (*msp1β*) whereas other studies utilised genus specific marker (16S rRNA gene) followed by species-specific probes. In addition, as *Anaplasma* spp. infection occurs in a cyclical nature, the presence of the parasite has the potential to be missed if only single sampling events occur in an individual host (Aubry and Geale, 2011; Potgieter and Stoltz, 2004). The repeated sampling of buffaloes may increase the probability of identifying *Anaplasma* spp. infection in the host, even if the rickettsia is below the detection level in some sampling periods. These differences in prevalence may also be due to a difference in sampling strategy, uneven representation of individuals in repeat sampling, the vector, individual characteristics and health status, the environment in which these buffalo are contained and co-infection interactions, with all of these factors warranting further investigation.

This study highlights the need to use multiple markers to characterise *Anaplasma* spp. from wild ruminants such as African buffaloes as a 'one gene fits all' approach does not capture the extent of diversity in an organism. To date, only reverse line blot hybridization analysis employing 16S rRNA gene has been used to detect *Anaplasma* spp. (*A. marginale*, *A. centrale*, *A. sp. Omatjenne, A. bovis* and *A. phagocytophilum*) from African buffaloes (Fyumagwa et al., 2013; Henrichs et al., 2016). The full extent of the diversity of *Anaplasma* spp. found in African buffaloes is still not explored as we focussed only on two economically important species (*A. marginale* and *A. centrale*) of bovine anaplasmosis. Future studies should focus on the characterisation of *A. sp. Omatjenne, A. bovis* and A. phagocytophilum species from African buffaloes using multiple species specific molecular markers.KX714581) and seven A. centrale (KX714582- KX714588) sequences found herein were uniquPhylogenetic analyses revealed that four A. marginale (GenBank accession nos. KX714578- e and that within each species analysis, there was clustering of these sequences from South Africa (see Fig. 2A and B), with the exclusion of respective reference sequences. Previously, Debeila (2012) used RLB analysis using 16S rRNA gene to characterise *A. centrale* and *A. marginale* from African buffaloes and found eight novel sequences for *A. centrale* and four for *A. marginale* and this level of variation is very similar to what we observed in *A. centrale* (no. of sequences = 7) and *A. marginale* ($n = 4$). However, Debeila (2012) reported that some (e.g., HIP/A8/b) of 16S rRNA sequences of *A. centrale* found in African buffaloes were 100% identical to previously published sequences (e.g., AF309869) from cattle. Contrarily, in this study, comparison of seven *groEL* sequences of *A. centrale* (KX714582- KX714588) with those of reference sequences (EF520691, AF414867 and AF414867) revealed a pairwise difference of 6.1–8.0% and they grouped together with the exclusion of previously published sequences (Fig. 2B). Similarly, for four 16S rRNA sequences of *A. marginale*, Debeila (2012) observed a high similarity (just two nucleotide differences over 726 bp) with reference sequences, whereas we found a high nucleotide variation (13.2–55.4%) among four *msp1β* sequences of *A. marginale* and previously published sequences ($n = 16$). The uniqueness of *A. centrale* and *A. marginale* sequences from African buffaloes found herein might be due to different molecular markers used in this study (*groEL* and *msp1β*) as compared to that (16 s rRNA) used by Debeila (2012). In addition, a higher pairwise difference in *A. marginale* sequences might be due to shorter sequences used for comparison (See Supplementary Fig. 1) as compared to 726 used by Debeila (2012).

Given that only *msp1β* and *groEL* genes have been used to quantify the burden of *A. marginale* and *A. centrale,* respectively, (Carelli et al., 2007; Decaro et al., 2008) and both of these *Anaplasma* species have been characterised using the two genes in this study, this detailed molecular analysis will allow for the development of a qPCR assay that can be used to quantify burdens and understand consequences of infection to the host.

In conclusion, this is the first study to characterise *A. marginale* and *A. centrale* from African buffalo using species specific molecular markers. Overall, 45.5% blood samples collected from buffaloes kept in KNP, South Africa, were positive for single or multiple infections with *A. marginale* and *A. centrale*. Sequence variation and phylogenetic analyses of *msp1β* and *groEL* sequences of *A. marginale* and *A. centrale*, respectively, revealed that sequences of *Anaplasma* spp. from African buffaloes were unique and they grouped separately when compared with previously published sequences of both species, including those from cattle in sub-Saharan Africa. To our knowledge, no sequences are available from cattle hosts for these *Anaplasma* species in the same location. Sequencing these species in cattle in the same area would allow for more conclusive evidence as to whether African buffalo are a reservoir for anaplasmosis or not. This study will pave the way for future studies to assess genetic variation among *Anaplasma* spp. from wild ruminants using molecular markers that are able to distinguish more fine scale genetic variation, which is useful for molecular epidemiology studies that identify reservoirs and across species transmission, and help to undertake health and fitness studies and host-parasite dynamics using quantitative molecular tools.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ttbdis.2017.01.003.

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