Molecular detection and phylogenetic analysis of *Anaplasma marginale* and *Anaplasma centrale* amongst transhumant cattle in north-eastern Uganda

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Abstract

There is little molecular data from *Anaplasma marginale* and *Anaplasma centrale* isolates from cattle in Uganda. Between November 2013 and January 2014, blood was collected from 240 cattle in 20 randomly-selected herds in two districts of the Karamoja Region in north-eastern Uganda. A duplex quantitative real-time polymerase chain reaction (qPCR) assay was used to detect and determine the prevalence of *A*. *marginale* (targeting the *msp1β* gene) and *A*. *centrale* (targeting the g*roEL* gene). The qPCR assay revealed that most cattle (82.9%; 95% confidence interval [CI] 78.2-87.7%) were positive for *A*. *marginale* DNA, while fewer cattle (12.1%; 95% CI 7.9-16.2%) were positive for *A*. *centrale* DNA. A mixed effects logistic regression model showed that the age of cattle was significantly associated with *A*. *centrale* infection, while the prevalence of *A*. *marginale* varied significantly according to locality. The near full-length 16S ribosomal RNA (16S rRNA) gene and the heat shock protein gene, *groEL*, for both *Anaplasma* species were amplified from a selection of samples. The amplicons were cloned and the resulting recombinants sequenced. We found three novel *A*. *marginale* 16S rRNA variants, seven *A*. *marginale groEL* gene sequence variants and two *A*. *centrale groEL* gene sequence variants. Phylogenetic trees were inferred from sequence alignments of the 16S rRNA gene and GroEL amino acid sequences determined here and published sequences using maximum likelihood, Bayesian inference and parsimony methods. Phylogenetic analyses classified the 16S rRNA gene and GroEL amino acid sequences into one clade for *A*. *marginale* and a separate clade for *A*. *centrale*. This study reveals a high prevalence and sequence variability of *A*. *marginale* and *A*. *centrale*, and is the first report on the phylogenetic characterisation of *A*. *marginale* and *A. centrale* from cattle in Uganda using molecular markers. Sequence variation can be attributed to mobile pastoralism, communal grazing and

grazing with wildlife. These data support future epidemiological investigations for bovine anaplasmosis in Uganda.

Keywords: 16S rRNA; *groEL*; *Anaplasma*; Karamoja; sequence analysis

1. Introduction

The main cause of bovine anaplasmosis in cattle is *A*. *marginale*, which is an intra-erythrocytic bacterium, transmitted mechanically through blood sucking flies or blood-contaminated fomites; and biologically by ticks (Battilani et al., 2017). Transplacental transmission from dams to their calves can also occur (Silvestre et al., 2016). The disease is characterised by severe anaemia and death in infected cattle, and is responsible for economic losses due to high morbidity and mortality, reduced weight gains and milk production, abortions, and treatment costs amongst cattle worldwide (Battilani et al., 2017).

Approximately 20 species of ticks of the genera *Rhipicephalus*, *Dermacentor* and *Ixodes* have been incriminated as vectors for *A*. *marginale* worldwide (Battilani et al., 2017). The tick species that have been reported to infest cattle in Karamoja Region in Uganda are *Rhipicephalus appendiculatus*, *R*. *decoloratus*, *R*. *pulchellus*, *R*. *evertsi evertsi*, *Hyalomma truncatum*, *Ambylomma gemma*, *A*. *variegatum* and *A*. *lepidum* (Byaruhanga et al., 2015a). Haematophagous insects, namely *Tabanus*, *Stomoxys*, *Culicoides* and mosquito species were also prevalent in the grazing areas in Karamoja (Byaruhanga et al., 2015b). *Anaplasma centrale* causes a milder form of anaplasmosis, and is used as a live vaccine against *A*. *marginale* in several African, South American and Middle Eastern countries (Bell-Sakyi et al., 2015). Transmission of *A*. *centrale* can occur mechanically, by blood inoculation, or

biologically by tick vectors (Battilani et al., 2017). Only the African tick, *R*. *simus*, has been shown to be a vector of *A*. *centrale* (Potgieter and van Rensburg, 1987), but this tick has not been reported in Uganda. Previous molecular studies have demonstrated the occurrence of *A*. *marginale* in 8.7% (Asiimwe et al., 2013), 3.7% (Muhanguzi et al., 2010) and 16% (Oura et al., 2011b) of cattle in various parts of Uganda, while the occurrence of *A*. *centrale* was found to be 4.3% (Asiimwe et al., 2013) and 4.5% (Muhanguzi et al., 2010). We recently reported the detection of the two *Anaplasma* spp. in cattle from Karamoja Region in north-eastern Uganda (Byaruhanga et al., 2016). In other African countries, varying prevalences of *A*. *marginale* were reported: 6.1% in Sudan (Awad et al., 2011), 25.4% in Tunisia (Belkahia et al., 2015), 89.7% in Madagascar (Pothmann et al., 2016) and 65% to 100% in South Africa (Mutshembele et al., 2014). For *A*. *centrale*, prevalences of 15.1% (Tunisia) (Belkahia et al., 2015) and 42.2% (Algeria) (Rjeibi et al., 2017) have been reported.

Although cattle are well-known hosts of *A*. *marginale* and *A*. *centrale*, the host range of the two parasites comprises a wide range of wild ruminant species (Battilani et al., 2017). African buffalo (*Syncerus caffer*) in particular are reservoirs of infections, and prevalences of 68% (*A*. *marginale*) and 56% (*A*. *centrale*) have been demonstrated in Kidepo Valley National Park in Karamoja Region in Uganda (Oura et al., 2011a), 20% (*A*. *marginale*) and 30% (*A*. *centrale*) in Botswana (Eygelaar et al., 2015), 72.2% (*A*. *marginale*) in Mozambique (Machado et al., 2016) and 17.3% (*A*. *marginale*) and 13.1% (*A*. *centrale*) in South Africa (Sisson et al., 2017).

Comparisons of the sequences of the 16S ribosomal RNA (16S rRNA) gene and the heat shock protein, GroEL, have been used to classify and determine phylogenetic relationships between the organisms in the order Rickettsiales – at family, genus and species levels – in various

countries on different continents (Belkahia et al., 2015; Dumler et al., 2001; Lew et al., 2003; Sisson et al., 2017; Yu et al., 2001). There is little data regarding the phylogenetic status of *A*. *marginale* and *A*. *centrale* in cattle in Uganda. One published study involving the 16S rRNA gene generated only one *Anaplasma* sp. sequence, which was short in length [only 1088 bp] (Ikwap et al., 2010).

The objectives of this study were to determine the prevalence of *A*. *marginale* and *A*. *centrale* infections among transhumant Zebu cattle in the Karamoja Region by quantitative real-time PCR (qPCR), and to establish the phylogenetic relationships between *A*. *marginale* and *A*. *centrale* 16S rRNA and GroEL sequences obtained in this study and published reference sequences. This will contribute to the available molecular epidemiological data on the two *Anaplasma* spp.

2. Materials and Methods

2.1 Ethics approval

The study was approved by the Animal Ethics Committee of the University of Pretoria (V026-14) and the National Agricultural Research Organisation of Uganda (no. 1416). Standard techniques were followed during the collection of blood samples.

2.2 Study area

This cross-sectional study was conducted in Moroto and Kotido Districts of Karamoja Region, north-eastern Uganda (Figure 1) from November 2013 through January 2014. The region covers 27,511 km^2 (about 10% of Uganda) and lies between longitudes 33° 30'E to 35°E and latitudes 1° 30'N to 4°N. It is bordered by South Sudan to the north and Kenya to the east.

Rainfall is low (average 500-600 mm per year) with peak rainfall in April-May and July-September (Anderson and Robinson, 2009). Temperatures range from an average minimum of between 15°C and 18°C to an average maximum of between 28°C and 33°C (Anderson and Robinson, 2009).

Fig. 1. Administrative map of Karamoja Region showing the study sites (shown as red dots). Inset is the map of Uganda showing the location of Karamoja Region and the neighbouring countries

In Karamoja Region, the livelihoods of the communities are highly dependent on livestock (IICD, 2010). Cattle in the region are of the short-horned East African Zebu type (*Bos indicus*), and make up a relatively large percentage (19.8%) of Uganda's cattle population (MAAIF, 2011). Indigenous cattle in Africa, including the Zebu type, have evolved a relatively stable relationship with ticks, and are less affected by clinical anaplasmosis than *Bos taurus* breeds (Rechav and Kostrzewski, 1991). The resistance, however, does not prevent transmission and

infection of cattle with *A. marginale* (Kocan et al., 2010). Cattle in Karamoja are extensively grazed on communal land with natural pastures. Climatic variability in the region results in seasonal pasture and water scarcity; therefore, the livestock keepers practice mobile pastoralism (transhumance) as a coping mechanism to avoid cattle loss and ensure livestock productivity (Anderson and Robinson, 2009; Egeru et al., 2014). In the wet season, cattle graze close to the settlement areas (within 5 km) (Byaruhanga et al., 2015b). During the dry season, animals migrate and share grazing and watering points with communities from neighbouring districts and countries (mainly with Turkana from Kenya) (Byaruhanga et al., 2015b).

2.3 Blood sampling and DNA extraction

Twenty superherds were purposively-selected in a participatory manner with the community leaders and veterinary workers, to represent the different settlement areas. A superherd is a group of herds identified as sharing grazing and watering areas during the wet season, and belonging to the same cattle camp during the dry season. One herd was then selected by simple random sampling in a superherd. Considering the available laboratory logistics, a total of 240 cattle were sampled for blood collection. In each herd (herd size: range of 37 to 42 cattle), 12 cattle were selected by systematic sampling for blood collection. A herd was categorised first by age group (5-12 months, 13-24 months and >24 months), then each age group was sub-grouped by sex (male or female). The number of cattle sampled in a sex sub-group was determined proportionally, by dividing the number in the sub-group by the total in a herd and then multiplying by the required herd sample size (12). At first, one animal was selected at random in a sub-group, then other animals were picked using a sampling interval in an enclosure. The sampling interval was calculated by dividing the number of cattle in a

sub-group by the determined sub-group sample size. An equal number of animals (120) was sampled in the Moroto and Kotido Districts.

Blood was collected from the jugular vein of each animal and spotted on FTA® Classic Card (Whatman®-Whatman International Ltd, Maidstone, England) to stabilise the DNA in the blood and for convenient transportation at room temperature. Genomic DNA was extracted from the blood using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) and stored at -20°C until used.

2.4 Duplex quantitative real-time polymerase chain reaction (qPCR)

A duplex qPCR assay was used for simultaneous detection and quantification of *A*. *marginale* and *A*. *centrale* DNA (Decaro et al., 2008). The PCR was performed on a LightCycler® 2.0 (Roche Diagnostics, Mannheim, Germany) using a LightCycler® FastStart TaqMan® kit (Roche Diagnostics, Mannheim, Germany). The primers and probes used in the duplex qPCR assay were as previously described (Decaro et al., 2008), except that the fluorescent reporter dye LightCycler® Red 610, with blackhole quencher 2 (5'-LC610-ATC ATC ATT CTT CCC CTT TAC CTC GT-BHQ2-3'), was used for the detection of *A*. *centrale* (Chaisi et al., 2017).

2.5 Selection of samples for further analyses

Based on the qPCR results, we selected samples with cycle threshold (Ct) values of 18 to 25 for *A*. *marginale* and/or *A*. *centrale* for amplification, cloning and sequencing of the 16S rRNA and the *groEL* genes.

2.6 PCR amplification of 16S rRNA and *groEL* genes

Primers fD1 and rP2 were used to amplify the near full-length 16S rRNA gene (approximately 1,500 bp) as previously described (Weisburg et al., 1991). The near full-length *groEL* gene (approximately 1,500 bp) was amplified using primers GROEL ACMF (5'-GCG CAT TCT GGA GGC TG-3') and GROEL ACMR (5'-GCG TTT GAC TTG GCT GTG TC-3'), designed in this study using CLC Genomics Workbench version 7.5.1 (CLC Bio, Boston, MA, USA); the primers were designed in regions of the *groEL* gene that are conserved between *A*. *marginale* and *A*. *centrale*. The conserved regions were identified through an alignment of nucleotide sequences from nine strains of *A*. *marginale* (Israel tailed, AF414862; Israel non-tailed, AF414861; Uruguay, AF414864; Australia F12, AF414860; Florida, AF414865; Australia CSIRO, AF414859; South Africa SWA, AF414863; Ishigaki, FJ226455; USA 770, AF165812) and four strains of *A*. *centrale* (Australia vaccine, AF414867; South African *Rhipicephalus simus*, AF414866; Italy CC, EF520691; Italy 8, EF520693) obtained from GenBank. The reaction mixtures for amplification of both the 16S rRNA and *groEL* genes contained 2 µl of genomic DNA, 0.4 µM of each primer, 1x Phusion™ Flash High-Fidelity PCR Master Mix (Thermo Scientific™, Randburg, South Africa) and nuclease-free water to a total volume of 25 µl. Thermocycling conditions were an initial denaturation at 98°C for 10 s, followed by 30 cycles at 98°C for 1 s, annealing at 55°C (16S rRNA) or 64°C (*groEL*) for 5 s, and 72°C for 15 s, and a final extension at 72°C for 1 min. The PCR was replicated four times for each sample to be sequenced. Following PCR, amplicons were examined on 2.0 w/v (%) agarose gels.

2.7 Cloning and sequencing of PCR products

The 16S rRNA gene was amplified from six samples and *groEL* from seven samples. The PCR products from the four replicate reactions were pooled, to minimise the possibility of

obtaining sequence errors caused by priority effects of amplification or variable composition of DNA fragments across individual PCR reactions in the early rounds of PCR. Amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), cloned into the pJET vector (CloneJET® PCR Cloning Kit, Thermo Scientific™, Randburg, South Africa) and transformed into JM 109 High Efficiency Competent cells (Promega, Madison, USA). At least 10 colonies per sample were screened by colony PCR using vector primers pJET 1.2F (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET 1.2R (5'-GAA GAA CAT CGA TTT TCC ATG GCA G-3'). The colony PCR mixture contained 0.2 µM of each primer, 1x DreamTaq Green PCR Master Mix (Thermo Scientific™, Randburg, South Africa), one colony as template and nuclease free water to a total volume of 20 µl. The amplification cycles, following an initial denaturation at 95°C for 3 min, consisted of 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by a final extension step cycle at 72°C for 7 min. Subsequent plasmids with inserts of the correct size (approximately 1,500 bp for 16S rRNA and 1,500 bp for *groEL*) were sequenced at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa), with primers pJET 1.2F and pJET 1.2R.

2.8 Sequence and phylogenetic analyses

Sequences obtained from the 16S rRNA and *groEL* gene clones were processed using CLC Genomics Workbench version 7.5.1 (CLC Bio, Boston, MA, USA). The Basic Local Alignment Search Tool [BLAST] (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for homologous reference sequences, from a range of geographical areas and animal species, using the blastn algorithm. Alignments of near full-length 16S rRNA gene (1,433 bp) and *groEL* gene (1,484 bp) sequences were constructed using the Multiple Alignment using Fast Fourier Transform (MAFFT) [version 7] program (Katoh and Standley, 2013) and manually edited using

BioEdit (version 7.2.5) program (Hall, 1999). Sequence identities (in % and number of nucleotides) were calculated by pairwise comparison using MEGA7 (Kumar et al., 2016).

The *groEL* nucleotide sequences were translated to amino acid sequences using EMBOSS Transeq (EMBL-EBI, 2016). JModelTest version 2.1.3 (Darriba et al., 2012) was used to select the best-fit nucleotide substitution model, GTR + I + G, for the 16S rRNA sequences while ProtTest version 3.0 (Darriba et al., 2011) was used to select a JTT + G + F model of evolution for proteins for the GroEL sequences, both under the Akaike Information Criterion (AIC) as the model selection criterion. Phylogenetic trees for the 16S rRNA gene and *groEL* gene were reconstructed using maximum likelihood (ML) as implemented in PhyML 3.1 (Guindon et al., 2010), parsimony in Phylogenetic Analysis Using Parsimony [PAUP] (Swofford, 2002) and Bayesian inference (BI) using MrBayes version 3.2 (Ronquist et al., 2012). The reliability for the internal branches for ML and parsimony methods was assessed using bootstrapping [1000 bootstrap replicates] (Felsenstein, 1985). Bayesian phylogenetic inference was achieved using Markov chain Monte Carlo (MCMC) analysis to calculate posterior probabilities for 1,000,000 generations, sampled every 1000 generations. Graphical representation and editing of the phylogenetic trees were performed with MEGA7 and Paint Tool for Windows 10.0. The topologies of the trees produced from BI, parsimony and ML analyses were compared.

2.9 Statistical analyses

The qPCR data for *A*. *marginale* and *A*. *centrale* were used to estimate the population prevalences, with 95% confidence intervals using the quantiles formation of the normal distribution (qnorm) with MASS package in R (Venables and Ripley, 2002). Considering the cattle production system in the Karamoja Region, candidate variables selected as predictors

for *A*. *marginale* and *A*. *centrale* infections in this study were age (5-12 months, 13-24 months and >24 months), sex (male and female), district (Moroto and Kotido) and herd. Cattle breed, grazing and health management practices were not included in the analysis. All cattle in the Karamoja Region are of the short-horned East African Zebu type and the management practices including tick control, grazing system, and disease control and treatment are similar among the herds. Since cattle were randomised at herd level (cluster) and individual animals selected in herds, the intracluster correlations (a measure of the relatedness or similarity of clustered data) were tested for infection with *A*. *marginale* and *A*. *centrale* as determined by the qPCR assay. Intracluster correlations were tested to avoid reporting significance where none exists due to clustering. Thereafter, a mixed effects logistic regression model was implemented in which the qPCR binary outcome variable (positive or negative for *A*. *marginale* or *A*. *centrale*) was modelled as a linear combination of both random (herd) and fixed (age category, sex and district) effects. The data were analysed using R Console version 3.2.1 (R Core Team, 2015) at 5% level of significance.

3. Results

3.1 Prevalence of *A*. *marginale* and *A*. *centrale*

Most of the 240 cattle sampled in Karamoja Region were female (77.5%). By age group, 82 (34.2%) of the sampled cattle were 5-12 months, 37 (15.4%) were 13-24 months, and 121 (50.4%) were >24 months. The qPCR assay showed that most cattle were positive for *A*. *marginale* (82.9%; 95% confidence interval [CI] 78.2-87.7%), while fewer were positive for *A*. *centrale* (12.1%; 95% CI 7.9-16.2%). The Ct values for *A*. *marginale* ranged from 16.6 to 29.5 (absolute quantification of 1.2×10^4 to 1.3×10^8 copies of *msp1β*/µl of blood) while those for *A*. *centrale* ranged from 18.3 to 30.4 (4.8×10^2 to 2.3×10^6 copies of *groEL*/µl of blood). The

log ratio test for herd effect showed that the infection of cattle with *A*. *marginale* and *A*. *centrale* as determined by the qPCR assay was clustered within herds (p<0.001); therefore, cattle in the same herd were not independent. If one cattle is positive for *A*. *marginale* or *A*. *centrale* in a herd, the risk could be higher for other cattle in this herd compared to the cattle outside the herd. As a result, logistic regression analysis was done with the cattle randomised at herd level but analysed at an individual level. The mixed effects logistic regression analysis revealed that age was significantly associated with *A*. *centrale* infection (p=0.006), but not with *A*. *marginale* (p=0.21). More cattle were positive for *A*. *centrale* in the age category of 5 to 12 months (23.2% infected) compared to those 13 to 24 months (5.4% infected) and >24 months (6.6% infected) of age. There was a significant difference (p=0.02) between the proportions of cattle that were positive for *A*. *marginale* in Moroto (89.2%) and Kotido (76.7%), but not for *A*. *centrale* between the two districts (p=0.57). Sex of cattle had no significant association with *A*. *marginale* or *A*. *centrale* infections (p=0.45 and p=0.53, respectively). Of the 29 cattle samples that were positive for *A*. *centrale*, 28 showed co-infection (11.7% of sampled cattle) with *A*. *marginale*.

3.2 *A*. *marginale* and *A*. *centrale* 16S rRNA and *groEL* sequence analyses

Table 1 summarises the samples from which the 16S rRNA and *groEL* genes were amplified, together with the clones and sequences obtained. *Anaplasma marginale* and *A*. *centrale* sequences were identified by blastn sequence homology search from 21 clones from six samples for the 16S rRNA gene and 58 clones from seven samples for the *groEL* gene. Sequence analyses revealed the presence of single or mixed infections of *A*. *marginale* and *A*. *centrale* in cattle for both genes (Table 1), confirming the qPCR results. Some cloned

sequences showed variation, even from the same sample, while others were identical (Table 1).

Table 1: A summary of quantitative real-time PCR (qPCR), cloning and sequencing results from blood samples selected for *A*. *marginale* and *A*. *centrale* 16S rRNA and *groEL* sequence and phylogenetic analyses. The samples were collected from cattle in Karamoja Region in north-eastern Uganda.

Gene	Sample ID	qPCR results	No. of sequenced clones	No. of nucleotide differences from reference sequence*	Closest match as determined by BLASTn analysis
16S rRNA	RB069[a]	*A*. *marginale*	7	2-7	*A*. *marginale*
	RB083[a]	*A*. *marginale*	1	2	*A*. *marginale*
	RB086	*A*. *marginale*	1	2	*A*. *marginale*
	RB123[b]	*A*. *marginale*	4	2	*A*. *marginale*
			2	0	*A*. *centrale*
	RE025	*A*. *marginale*	5	1-3	*A*. *marginale*
	RE028[a,c]	*A*. *marginale*	1	2	*A*. *marginale*
		A. *centrale*			
groEL	RB069[a]	*A*. *marginale*	10	8-18	*A*. *marginale*
	RB083[a]	*A*. *marginale*	12	6	*A*. *marginale*
	RE059[b,c]	*A*. *marginale*	1	8	*A*. *marginale*
		A. *centrale*	4	23	*A*. *centrale*
	RE083[b,c]	*A*. *marginale*	4	8	*A*. *marginale*
		A. *centrale*	4	23	*A*. *centrale*
	RE028[a,b,c]	*A*. *marginale*	8	8	*A*. *marginale*
		A. *centrale*	2	36	*A*. *centrale*
	RE084[b,c]	*A*. *marginale*	9	7-8	*A*. *marginale*
		A. *centrale*	1	23	*A*. *centrale*
	RE010[c]	*A*. *centrale*	3	23	*A*. *centrale*
		A. *marginale*			

[a]Samples from which both 16S rRNA and *groEL* sequences were obtained.

[b]Samples from which both *A*. *marginale* and *A*. *centrale* sequences were obtained.

[c]Samples that showed mixed infections of *A*. *marginale* and *A*. *centrale* from qPCR.

*Sequences CP001079 (*A*. *marginale* Florida strain) and CP001759 (*A*. *centrale* Israel strain) were used as references for *A*. *marginale* and *A*. *centrale* 16S rRNA sequence comparison. Sequences CP000030 (*A*. *marginale* St. Maries strain) and CP001759 (*A*. *centrale* Israel strain) were used as references for *A*. *marginale* and *A*. *centrale groEL* gene sequence comparison

We compared the near full-length *A*. *marginale* and *A*. *centrale* 16S rRNA and *groEL* sequences determined in this study with reference sequences in the GenBank to establish the extent of nucleotide variation (Tables 2 and 3). Since some sequences were identical, only one

representative of each unique sequence is shown. Sequence comparison demonstrated three novel *A*. *marginale* 16S rRNA sequences (GenBank Accession nos. KU686789, KU686790, KU686793) (Table 2). The sequences were 99% identical (2 to 7 nucleotide difference, 100% query cover) with published sequences from: cattle from South Africa, black wildebeest from South Africa, African buffalo from South Africa, and a sequence from the genome of *A*. *marginale* Florida strain (CP001079) [Table 2]. The *A*. *centrale* 16S rRNA sequence obtained in this study was 100% homologous (query cover 100%) to the *A*. *centrale* Israel vaccine strain, Italian CC strain and a sequence from the genome of *A*. *centrale* Israel (CP001759), but presented 99% identity (2 nucleotide difference) with a sequence from African buffalo from South Africa (Table 2).

Table 2: Pairwise differences (number of nucleotides) among the different near full-length *A*. *marginale* and *A*. *centrale* 16S rRNA sequences from cattle from Karamoja Region, Uganda and reference sequences from cattle and other animal species from different parts of the world

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	**KU686774 (A.m, Uganda)**																								
2	**KU686789 (A.m, Uganda)**	3																							
3	**KU686790 (A.m, Uganda)**	6	3																						
4	**KU686792 (A.m, Uganda)**	1	2	5																					
5	**KU686793 (A.m, Uganda)**	2	3	6	1																				
6	AF311303 (A.m, USA)	0	3	6	1	2																			
7	AF414873 (A.m, SA)	1	2	5	0	1	1																		
8	CP001079 (A.m, Florida, USA)	1	4	7	2	3	1	2																	
9	AF414874 (A.m, Australia)	0	3	6	1	2	0	1	2																
10	AF414877 (A.m, Uruguay)	0	3	6	1	2	0	1	2	0															
11	HM061603 (A.sp., Uganda)	2	3	3	3	3	2	3	3	2	2														
12	AF414871 (A.m, wildebeest, SA)	1	4	7	2	3	1	2	3	1	1	3													
13	KT264188 (A.m, Thailand)	0	3	6	1	2	0	1	1	1	1	2	2												
14	DQ341369 (A.m, Hongan buffalo, China)	0	3	6	1	2	0	1	12	3	3	2	4	10											
15	KR492643 (A.sp., African buffalo, Mozambique)	3	3	3	3	3	3	3	3	3	3	3	4	3	3										
16	KY287601 (A.m, African buffalo, SA)	2	3	3	3	3	2	3	3	2	2	2	3	2	2	3									
17	AF414872 (A.m, Eland, SA)	4	5	8	3	4	4	3	6	4	4	6	5	5	7	4	6								
18	**KU686784 (A.c, Uganda)**	10	11	14	9	10	10	9	11	10	10	8	11	10	10	3	5	12							
19	AF309869 (A.c vaccine, Israel)	10	11	14	9	10	10	9	11	10	10	8	11	10	10	3	5	12	0						
20	EF520686 (A.c, Italy)	10	11	14	9	10	10	9	11	10	10	8	11	10	10	3	5	12	0	0					
21	AF414869 (A.c, *R*. *simus*, SA)	10	11	14	9	10	10	9	12	10	10	8	11	11	13	3	5	12	0	0	0				
22	AF414868 (A.c, vaccine, Australia)	11	12	15	10	11	11	10	13	11	11	9	12	12	14	3	6	13	1	1	1	1			
23	CP001759 (A.c, Israel)	10	11	14	9	10	10	9	11	11	11	8	12	10	13	3	5	13	0	0	0	1	2		
24	KC189841 (A.c, African buffalo, SA)	9	10	13	8	9	9	8	10	9	9	8	10	9	9	4	7	11	2	2	2	2	3	2	

Unless otherwise indicated, the sequences are from cattle. The sequences obtained in this study are indicated in bold.

A.m, *Anaplasma marginale*; A.c, *Anaplasma centrale*, A.sp., *Anaplasma* sp.

Analysis was conducted in MEGA7. All ambiguous positions were removed for each sequence pair. There were a total of 1,433 positions in the final dataset. There was variation in the 16S rRNA gene between sequences from cattle in this study and sequences from African and Hongan buffalo (2 nucleotides for *A*. *centrale*; ≤ 6 nucleotides for *A*. *marginale*).

Table 3: Pairwise differences (number of nucleotides) among the different near full-length *A*. *marginale* and *A*. *centrale groEL* sequences from cattle from Karamoja Region, Uganda and from cattle and other animal species from different parts of the world

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	**KY522985 (A.m, Uganda)**																								
2	**KY522981 (A.m, Uganda)**	2																							
3	CP000030 (A.m, St. Maries)	8	8																						
4	AF414863 (A.m, SA)	9	7	14																					
5	**KY522984 (A.m, Uganda)**	6	6	6	9																				
6	**KY522987 (A.m, Uganda)**	8	6	8	5	6																			
7	**KY522986 (A.m, Uganda)**	7	5	7	6	5	1																		
8	**KY522982 (A.m, Uganda)**	6	6	8	7	6	4	5																	
9	**KY522983 (A.m, Uganda)**	6	6	18	10	10	6	7	4																
10	AF414862 (A.m, Israel)	5	5	10	8	3	5	4	5	10															
11	AF414864 (A.m, Uruguay)	5	5	10	10	3	5	4	5	9	4														
12	AF414860 (A.m, Australia)	4	4	10	8	2	4	3	4	8	2	2													
13	KR492659 (A.sp., African buffalo, Mozambique)	27	27	28	26	28	26	27	24	23	28	28	28												
14	KY305562 (A.m, African buffalo, SA)	100	100	99	97	96	98	99	94	96	98	99	98	6											
15	**KY522988 (A.c, Uganda)**	44	44	47	44	50	44	45	44	40	50	49	48	23	126										
16	**KY522989 (A.c, Uganda)**	44	44	66	59	46	40	41	40	40	63	62	61	33	124	6									
17	EF520692 (A.c, Italy)	50	50	61	57	56	50	51	50	46	63	61	61	23	130	6	29								
18	EF520691 (A.c, CC Italy)	46	46	57	53	52	46	47	46	42	59	59	57	23	126	2	25	4							
19	AF414867 (A.c, vaccine, Australia)	39	39	50	50	39	39	38	41	45	46	44	44	28	127	23	36	23	27						
20	AF414866 (A.c, R. simus, SA)	38	38	47	43	40	38	37	40	40	47	47	45	26	124	10	27	18	14	13					
21	CP001759 (A.c, Israel)	39	39	51	52	39	39	38	41	50	48	44	46	28	127	23	36	25	29	3	16				
22	KX714585 (A.c, African buffalo, SA)	81	79	81	78	79	77	77	78	80	80	79	79	-	68	55	61	56	56	58	59	58			
23	KY305552 (A.c, African buffalo, SA)	39	39	41	42	39	39	38	41	45	38	36	37	28	127	23	19	21	25	0	13	0	57		
24	KY305551 (A.c, African buffalo, SA)	122	120	121	117	122	118	119	116	118	120	121	120	4	68	95	93	99	95	106	101	106	13	106	

Unless otherwise indicated, the sequences are from cattle. Sequences from this study are indicated in bold.

A.m, *Anaplasma marginale*; A.c, *Anaplasma centrale*; A. sp., *Anaplasma* species

Analysis was conducted in MEGA7. All ambiguous positions were removed for each sequence pair. There were a total of 1,484 positions in the final dataset.

There was high *groEL* sequence variation between cattle in this study and African buffalo (*A*. *marginale,* 84 to 100 nucleotides; *A*. *centrale*, 19 to 93 nucleotides). There was high variation within *groEL* sequences from African buffalo (*A*. *centrale*, 10 to 106 nucleotide differences; *A*. *marginale*, 96 nucleotides).

Seven unique *A*. *marginale groEL* sequences were demonstrated in this study (Table 3). The sequences presented 99% identity (6 to 18 nucleotide differences, query cover 100%) with the reference sequences from cattle from South Africa and the *A*. *marginale* St. Maries strain, but were 93% identical (26 to 100 nucleotides, query cover 23% to 99%) to sequences from African buffalo. Two novel *A*. *centrale groEL* variants were identified herein (Table 3); sequence comparison revealed 99% identity (23 to 36 nucleotide differences, query cover 98%) with the vaccine strain from Australia and the *A*. *centrale* Israel strain. The published *A*. *centrale groEL* sequences from African buffalo showed divergences ranging from 23 to 95 nucleotides (92% to 98% identity) with the newly-generated sequences from cattle in Uganda (query cover 59% to 97%).

3.3 Phylogenetic analyses

The topology of the phylogenetic trees generated employing ML, MP and BI methods were similar (data not shown for MP and BI); hence, only the ML trees for each of the two genes are presented here (Figures 2 and 3). The 16S rRNA and GroEL phylogenetic trees inferred in this study showed congruent tree topologies. The newly-generated *A*. *marginale* and *A*. *centrale* sequences grouped each in one cluster together with previously reported isolates from various geographical areas, including those from African buffalo (Figures 2 and 3).

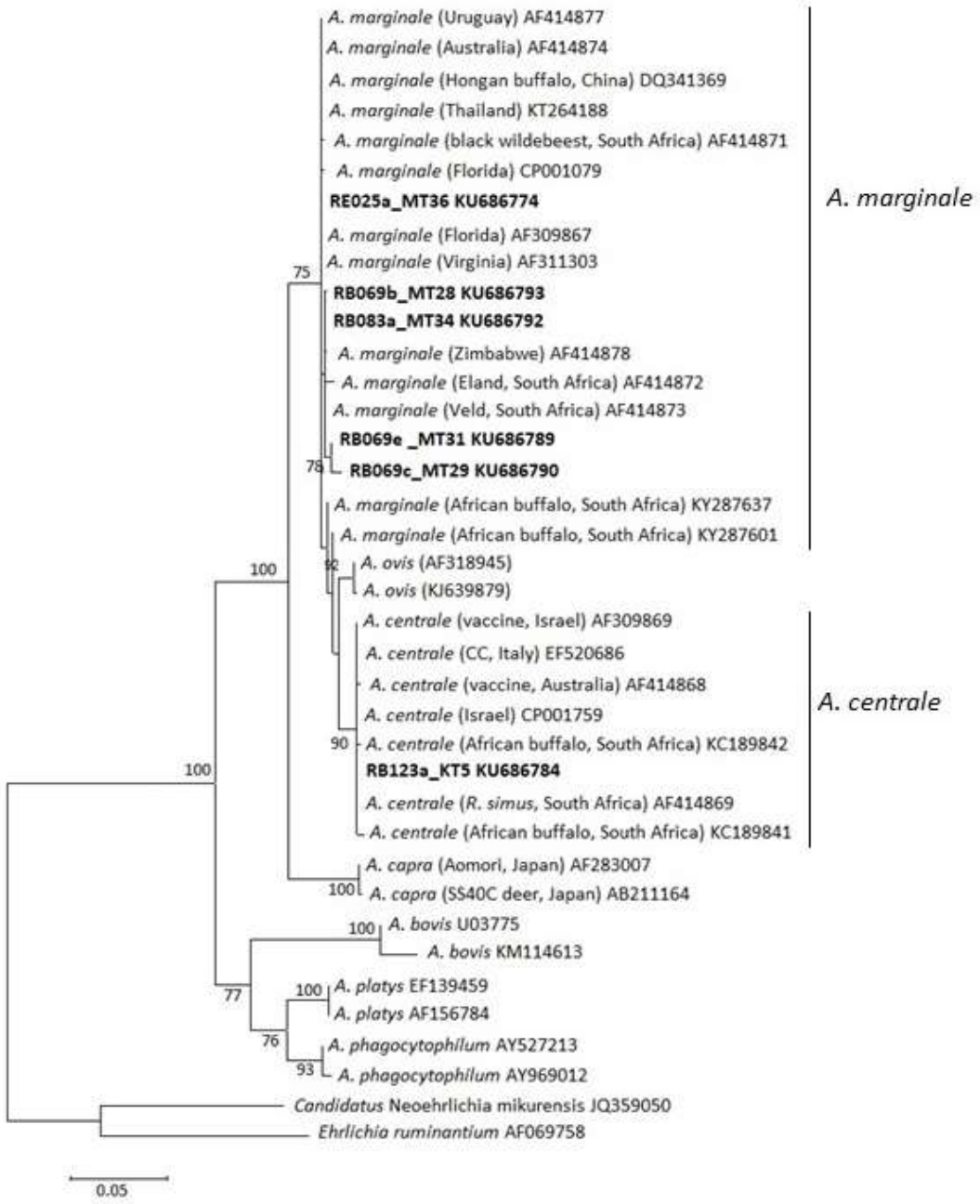

Fig. 2. A maximum likelihood tree showing the phylogenetic relationship between the *A. marginale* and *A. centrale* 16S rRNA sequences, identified in blood samples from cattle in Karamoja Region, Uganda with other *Anaplasma* species. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. Some sequences obtained in this study were identical to each other; therefore, only representatives of sequences are shown (in bold). The tree was rooted using the 16S rRNA gene sequences of *Candidatus* Neoehrlichia mikurensis and *Ehrlichia ruminantium*. The accession number of each sequence is indicated in the sequence name. Branch lengths are proportional to the estimated genetic distance (number of nucleotide substitutions per site over a length of 1,433 bp of the 16S rRNA gene) between the taxa. The newly-generated *A. marginale* 16S rRNA sequences grouped with isolates from cattle from South and North America, Australia, Asia and Africa, and African buffalo, Eland and black wildebeest from South Africa (92% bootstrap support, 0.71 posterior probability). *A. centrale* sequences obtained in this study positioned with those with isolates from Europe, Australia, Asia, Africa, including those from African buffalo (90% bootstrap support, 1.0 posterior probability)

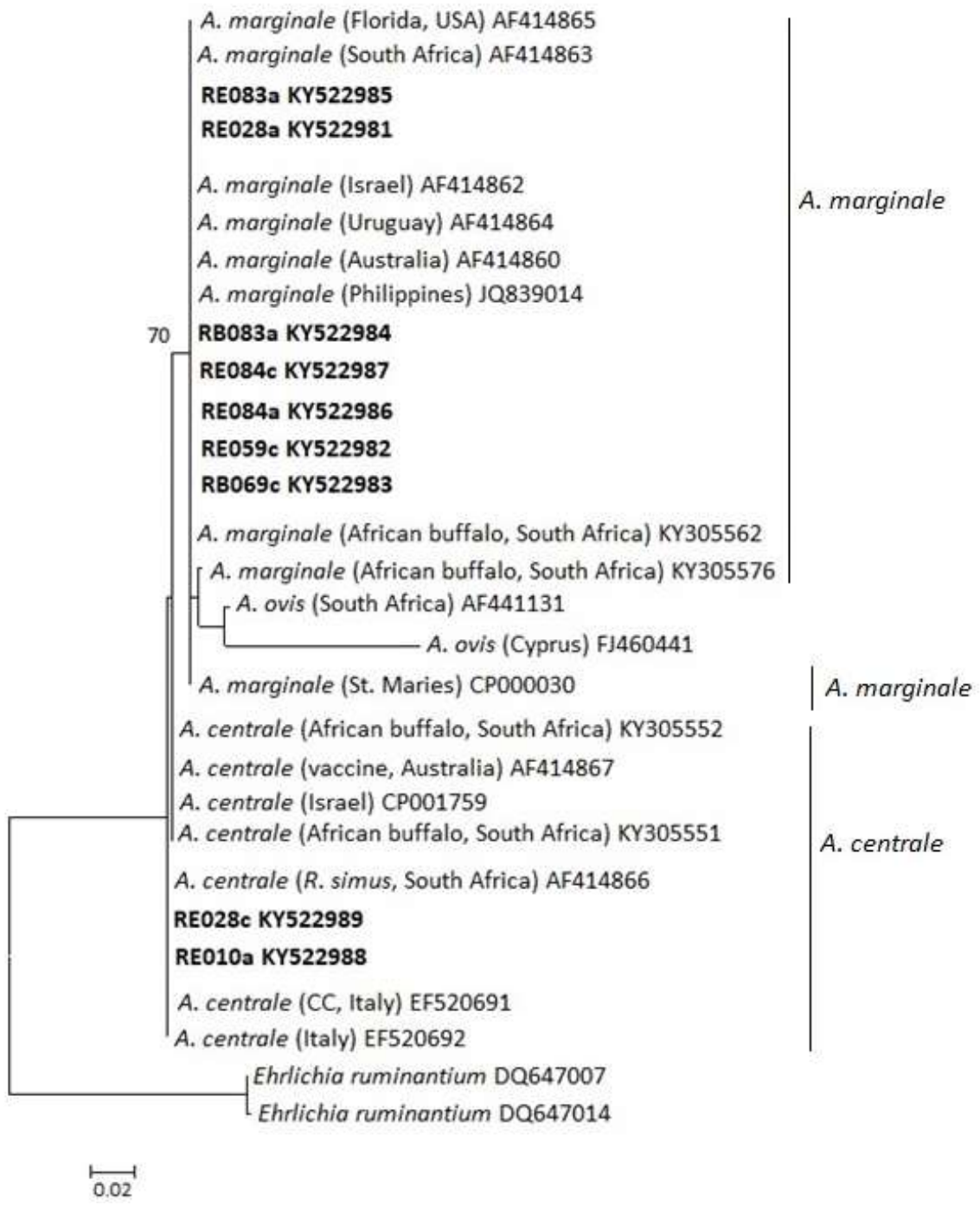

Fig. 3. A maximum likelihood tree showing the phylogenetic relationship between the *A. marginale* and *A. centrale* GROEL variants, identified in blood samples from cattle in Karamoja Region, Uganda with sequences available in GenBank. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. Some sequences obtained in this study are identical to each other; therefore, only representatives of variants are shown (in bold). The tree was rooted using GroEL sequences of *Ehrlichia ruminantium*. The accession number of each sequence is indicated in the sequence name. Branch lengths are proportional to the estimated genetic distance (number of amino acid substitutions per site over a length of 494 amino acids of the *groEL* gene) between the taxa. The newly-generated *A. marginale* GroEL sequences positioned with isolates of different geographical areas (North and South America, Africa, Asia and Australia) with 70% bootstrap support and 0.65 posterior probability.

4. Discussion

Understanding the epidemiology and genetic variation within *Anaplasma* spp. provides important knowledge for the design and implementation of appropriate measures to prevent and control bovine anaplasmosis. In this study, we determined the prevalence and the 16S rRNA and *groEL* gene sequences and phylogenetic relationships between *A*. *marginale* and *A*. *centrale* in cattle kept under a transhumant production system in Karamoja Region, north-eastern Uganda.

The prevalence of *A*. *marginale* (82.9%) in cattle from Karamoja, as determined by the qPCR assay, was 5-22 times higher than previously reported in cattle in other parts of Uganda [using reverse line blot (RLB) hybridisation assay] (Asiimwe et al., 2013; Muhanguzi et al., 2010; Oura et al., 2011b), Sudan [6.1% using *msp4* gene PCR] (Awad et al., 2011) and Tunisia [25.4% using qPCR] (Belkahia et al., 2015), but similar to that reported in Madagascar [89.7% using *msp1β* qPCR] (Pothmann et al., 2016) and seven out of the nine provinces of South Africa [≥65% using *msp1α* PCR] (Mutshembele et al., 2014). Although prevalence of *Anaplasma* spp. can vary according to the diagnostic methods used (Chaisi et al., 2017), large differences in prevalence are most likely to be due to differences in tick control programmes, habitat suitability for ticks, grazing management, infection rate of ticks with pathogens and management of clinical cases. Other factors are climatic, husbandry practices, antigenic/genetic diversity of the pathogens and proximity to wildlife reservoir hosts.

The high prevalence of *A*. *marginale* in this study can be attributed to the relatively high tick species diversity and high tick infestation. We recently observed high tick infestation among cattle in the Karamoja Region, which was attributed to inappropriate or less effective tick

control practices such as daily picking of ticks by hand, irregular hand spraying with insufficient acaricides and the recycling of contaminated fomites (e.g. needles) (Byaruhanga et al., 2015b). In comparison, tick control is more regular in other areas in Uganda where a lower prevalence of *A*. *marginale* in cattle has been noted (Asiimwe et al., 2013; Muhanguzi et al., 2010). Furthermore, we found various tick species, namely *R*. *appendiculatus*, *R*. *decoloratus*, *R*. *evertsi evertsi*, *R*. *pulchellus*, *H*. *truncatum*, *A*. *gemma*, *A*. *variegatum* and *A*. *lepidum* on cattle in Karamoja (Byaruhanga et al., 2015a). The prevalence of *R*. *decoloratus*, the main tick vector of *A*. *marginale* in Uganda, was 53.4% among cattle sampled in Karamoja (Byaruhanga et al., 2015a). Transmission of *A*. *marginale* is effected by around 20 tick species worldwide (Kocan et al., 2010). Some of the ticks found on cattle in Karamoja, therefore, could be infected, or carry *A*. *marginale* in their blood meals, resulting in biological and mechanical transmission of the pathogen. With regard to mechanical transmission, haematophagous insects – *Tabanus*, *Stomoxys*, *Culicoides* and mosquitoes – which are vectors for *A*. *marginale*, were prevalent in the grazing areas in the Karamoja Region (Byaruhanga et al., 2015b).

Given the high prevalence of *A*. *marginale*, endemic stability for anaplasmosis is likely to establish in cattle in Karamoja Region compared to other areas of Uganda and the rest of Africa, where lower prevalences of infection or lower levels of previous exposure among cattle were reported. In this context, calves, which are less susceptible to anaplasmosis than adults (Battilani et al., 2017), are exposed to infection from the environment or acquire the infection transplacentally. This leads to acquired immunity in adulthood. Nevertheless, although a high seroprevalence for *A*. *marginale* (86.6%) was observed from various cattle herds in Karamoja Region (Byaruhanga et al., 2015a), we found cases (on average 2 cases per herd) of anaplasmosis confirmed by clinical and post-mortem examinations, laboratory

diagnosis and interviews with the pastoralists (Byaruhanga et al., 2015b). Cattle are likely to experience clinical cases of anaplasmosis in high prevalence endemic areas, which are characterised by high *A*. *marginale* genetic/antigenic diversity. This is due to limited or lack of cross-protection among pathogenic strains (Palmer and Brayton, 2013). In high prevalence areas, most animals, although persistently infected, develop immunity only to the primary strain of *A*. *marginale*. The clinical cases may therefore be attributed to the few naïve animals and/or new infections as a result of superinfection (Palmer and Brayton, 2013). With regard to the present study, the high *A*. *marginale* rickettsaemia presented by some cattle in Karamoja, as assessed by qPCR absolute quantification (see sub-section 3.1), may also facilitate the occurrence of clinical disease. Notably, communal grazing, seasonal movement of cattle and the wildlife-livestock interface in Karamoja can facilitate exposure or co-infection of cattle with varying genotypes of *A*. *marginale* and *A*. *centrale*. These climate-driven changes to animal host and tick dynamics can expose cattle to ticks with various infection rates, and the emergence and fixation of novel genotypes of the tick-borne pathogens, leading to clinical disease (Ogden and Lindsay, 2016). With regard to seasonality, tick infestations and the risk of anaplasmosis were higher in the wet season and in the communal grazing areas (Byaruhanga et al., 2015b). Furthermore, African buffalo can act as a reservoir of *A*. *marginale* and *A*. *centrale* (Kocan et al., 2010; Oura et al., 2011a; Oura et al., 2011b). We recently observed that cattle in Karamoja Region share grazing grounds with free-ranging wildlife, including African buffalo (Byaruhanga et al., 2015b). The wild reservoirs facilitate transmission of the pathogens to cattle when they are infested with the tick and insect vectors from the wild animals. In this study, cattle in Moroto District were significantly more likely to be infected with *A*. *marginale* than those in Kotido. This variation in infection may be due to the higher tick infestation on cattle in Moroto than Kotido District (Byaruhanga et al., 2015a).

The prevalence of *A*. *centrale* (12.1%) was three times higher than that observed in other parts of Uganda in previous studies (Asiimwe et al., 2013; Muhanguzi et al., 2010), similar to that reported in Tunisia [15.1%] (Belkahia et al., 2015) and lower than that reported in Algeria [42.2% using 16S rRNA PCR] (Rjeibi et al., 2017). However, *R*. *simus*, a tick vector reported for *A*. *centrale* in South Africa (Potgieter and van Rensburg, 1987), was not found among cattle in Karamoja (Byaruhanga et al., 2015a). Possibly, some of the tick species found in Karamoja Region (Byaruhanga et al., 2015a), and not reported in other parts of Uganda, may be responsible for transmission of *A*. *centrale*, resulting in the relatively higher infection rates in this region. Although *A*. *centrale* is considered to be less pathogenic than *A*. *marginale*, an acute case of anaplasmosis attributed to the *A*. *centrale* strain CC (which has a 16S rRNA gene sequence 100% identical to the *A*. *centrale* 16S rRNA sequence obtained in this study) was reported in a dairy cow in Italy (Carelli et al., 2008). Of the 29 cattle that were found to be positive for *A*. *centrale*, 28 were co-infected with *A*. *marginale*. This may imply that the tick species, or other vectors, that transmit *A*. *centrale* in the study area, also transmit *A*. *marginale*. Cattle of less than one year of age had significantly higher *A*. *centrale* infection rates than the yearlings. This can be attributed to transplacental transmission in calves, but also to the presence of other infections, which increases susceptibility in calves. The yearlings and older cattle could then have acquired immunity as they grew.

This is the first phylogenetic study on *A*. *marginale* and *A*. *centrale* in cattle in Uganda using molecular markers. The present study revealed variation within the 16S rRNA and *groEL* genes sequences of *A*. *marginale* and *A*. *centrale*. Comparison with isolates previously reported revealed three *A*. *marginale* 16S rRNA variants, seven *A*. *marginale groEL* variants and two *A*. *centrale groEL* variants. This suggests that cattle in this pastoral area may harbour a complex

of genotypes and/or isolates of *A*. *marginale* and *A*. *centrale*. The heterogeneity may be attributed to cattle movements, which increase the likelihood of infection with distinct *A*. *marginale* strains (Belkahia et al., 2015; de la Fuente et al., 2007). Furthermore, we observed variations of 92% to 98% between *A*. *marginale* and *A*. *centrale groEL* sequences determined here and sequences from African buffalo. A previous study in Mozambique showed that *Anaplasma groEL* sequences obtained from African buffalo were more heterogeneous and the inferred trees could not assign them to any species of *Anaplasma* (Machado et al., 2016). However, the *groEL* sequences obtained by Machado et al. (2016) were much shorter (only 520 bp) than the sequences obtained in this study.

More understanding of the diversity, pathogenicity and origin of different strains of *A*. *marginale* and *A*. *centrale* in Uganda using MSP genes is still required. There is also a need to elaborate on the vector-host-parasite dynamics with respect to *A*. *marginale* and *A*. *centrale* by studying the vector competence of the tick species in Karamoja, the impact of mechanical transmission and the genotypes circulating in wildlife.

Conclusions

This is the first study to elucidate the phylogenetic relationships of *A*. *marginale* and *A*. *centrale* from cattle in Uganda using molecular markers. We detected higher prevalences of *A*. *marginale* and *A*. *centrale* than previously reported in other parts of Uganda, and some areas in Africa. The 16S rRNA and GroEL sequence and phylogenetic analyses of *A*. *marginale* and *A*. *centrale* revealed within species variation and unique variants of the organisms in cattle in Karamoja. The sequences grouped with previously published sequences including those from African buffalo. This molecular epidemiological data raises awareness among

researchers and animal health authorities to the diversity of *A*. *marginale* and *A*. *centrale* present in Karamoja and this could support future epidemiological investigations for bovine anaplasmosis in Uganda.

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