Ticks and Tick-borne Diseases 10 (2019) 651–664

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Diversity of *Anaplasma* species in cattle in Mozambique

Simone de Jesus Fernandes[a], Carlos António Matos[a,b], Carla Roberta Freschi[c], Inalda Angélica de Souza Ramos[a], Rosangela Zacarias Machado[a], Marcos Rogério André[a,*]

[a] Paulista State University (Unesp)Universidade Estadual Paulista (Unesp), Faculty of Agrarian and Veterinary Sciences, Jaboticabal, SP, Brazil
[b] Directorate of Animal Sciences, Maputo, Mozambique
[c] Imunodot Diagnósticos Ltda, Jaboticabal, SP, Brazil

ARTICLE INFO

Keywords:
Anaplasma marginale
Anaplasma phagocytophilum
Anaplasma platys
Anaplasma centrale
Anaplasma ovis
'*Candidatus* Anaplasma boleense'

ABSTRACT

Although species of *Anaplasma* are highly prevalent Rickettsiales agents in domestic and wild ruminants with a wide distribution worldwide, few studies have been conducted so far to detect and/or investigate the diversity of these agentsin cattle in Mozambique. In the present study, serological and molecular assays were used to investigate the occurrence of *Anaplasma* spp. in 219 bovines sampled in the districts of Boane, Magude, Matutuíne, Moamba and Namaacha in Maputo, Mozambique. In the iELISA test for detection ofIgG antibodies to *A. marginale*, 86.3% (189/219) of the samples were positive. In qPCR assays for the gene *msp1β* for *A. marginale* and *msp*2 for *A. phagocytophilum,* 97.3% (213/219) and 2.7% (6/219) of the animals were positive, respectively. Two different cPCR protocols based on the 16S rRNA gene showed that 100% of the samples were positive for *Anaplasma* spp. The DNA sequences obtained were phylogenetically related to *A. platys, A. phagocytophilum, Candidatus* Anaplasma boleense, *A. centrale, A. marginale* and *A. ovis*. Phylogenetic inference based on the *msp4* and *msp5* genes positioned the obtained sequences in the clade of *A. marginale,* with evidence of occurrence of 8 and 5 different haplotypes for each gene, respectively. *Anaplasma* sp. phylogenetically associated with *A. platys* was evidenced in phylogenetic analyzes based on 16S rRNA and *groEL* genes. It is concluded that a high diversity of species of *Anaplasma* spp. occurs in cattle in Mozambique.

1. Introduction

The genus *Anaplasma*, belonging to the Rickettsiales Order and Anaplasmataceae family, comprises Gram-negative obligate intracellular bacteria, which are located in intracytoplasmic vacuoles forming agglomerated structures called morulae (Dumler et al., 2001; Silaghi et al., 2017). *Anaplasma marginale*, *A. ovis*, *A. centrale*, *A. bovis*, *A. platys*, and *A. phagocytophilum* are the species included in this group (Dumler et al., 2001). Recently, the inclusion of the species *A. odocoilei* (Tate et al., 2013) and *A. capra* (Li et al., 2015) has been proposed. These pathogens cause diseases in animals and humans, such as bovine anaplasmosis, canine cyclical thrombocytopenia and granulocytic anaplasmosis. The latter is an emerging zoonotical disease caused by *A. phagocytophilum* in the Northern Hemisphere (Jin et al., 2012; Bakken and Dumler, 2015; Silaghi et al., 2017).

Invertebrate and vertebrate hosts play different roles in the biological cycles of *Anaplasma* spp., in which ticks act as primary carriers for transmitting the agent through saliva during feeding. According to the species of the agent involved, specific tropisms by different blood cells are expressed, such as red blood cells, monocytes, macrophages, granulocytes or platelets (Silaghi et al., 2017).

Regarding bovine anaplasmosis, *A. marginale* is the most important agent due to the high morbidity and mortality that it causes in cattle and other ruminants. About 20 species of ticks are incriminated in the biological transmission. While *Dermacentor* ticksare the main arthropod vectors responsible for transmission of this agent in temperate regions, *Rhipicephalus* ticks act as the main vectors for bovine anaplasmosis in tropical regions (Kocan et al., 2010; Silaghi et al., 2017). Other relevant forms of infection include blood transfusions, mechanical transmission by hematophagous insects, or contaminated fomites (Kocan et al., 2010).

The acute phase of bovine anaplasmosis is characterized by fever, weight loss, decreased milk production, abortion and, in some cases, death. If the animal recovers, it remains infected and becomes a continuous source for biological or mechanical transmission of the pathogen (Kocan et al., 2003).

Mozambique stands out among the countries that suffer from economic losses due to bovine anaplasmosis. The situation worsened in the

---

[*] Corresponding author at: Immunoparasitology Laboratory, Department of Veterinary Pathology, Faculty of Agrarian and Veterinary Sciences Júlio de Mesquita Filho (UNESP), Jaboticabal Campus, Via de Acesso Prof. Paulo Donato Castellane, s / n, Zona Rural, Jaboticabal, CEP: 14884-900, São Paulo, Brazil.

E-mail address: marcos_andre@fcav.unesp.br (M.R. André).

https://doi.org/10.1016/j.ttbdis.2019.02.012
Received 29 May 2018; Received in revised form 12 December 2018; Accepted 25 February 2019

1990s when initiatives were taken to improve livestock production in the region, such as restocking cattle by importing animals from neighboring countries without proper monitoring of the health conditions of these animals.These actions led to the spread of various diseases caused by tick-borne agents, such as anaplasmosis, ehrlichiosis, babesiosis and theileriosis (Simuunza et al., 2011; Tembue et al., 2011).Few studies have been carried out to investigate the occurrence of *Anaplasma* spp. in cattle in Mozambique. In this sense, Alfredo et al. (2005) reported a seroprevalence for *A. marginale* of 63% among a sample of 478 cattle in the district of Tete, using the agglutination card test. Moreover, Tembue et al. (2011) found a seroprevalence of 76.5% (619/809) by iELISA in cattle sampled in southern Mozambique. When it comes to molecular studies, Bekker et al. (2002) and Martins et al. (2010) detected *Anaplasma* sp. DNA by thereverse line blot (RLB) hybridization technique in cattle sampled in several locations in the province of Maputo.

The present study aimed to investigate the occurrence and genetic diversity of *Anaplasma* spp. in cattle sampled in Maputo, Mozambique, using serological and molecular techniques.

2. Materials and methods

2.1. Animals and the study of area

The present study was carried out in the districts of Boane, Magude, Matutuíne, Moamba and Namaacha in Maputo, Mozambique (Fig. 1). The managers of surveyed farms were informed about the study and gave their approval for the sampling of cattle. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Universidade Estadual Paulista (Unesp), Jaboticabal, São Paulo, Brazil (Protocol number #017259/14).

The Boane and Namaacha districts are located in the southeast and southwest region of Maputo.In the first one, which presents a subhumid tropicalclimate, the raising of livestock is a tradition, especially related to family livestock (http://www.portaldogoverno.gov.mz/por/content/download/2965/23877/version/%E2%80%A6/Boane.pdf). Namaacha, in turn, presents a humid tropical climate, changing according to altitude. It has highlands (Complex of the Chain of the Libombos), medium plateaus, slopes and small plains. In this district there is great potential for livestock development, due to the abundance of water and good pastures (http://www.portaldogoverno.gov.mz/por/content/download/2968/23892/%E2%80%A6/Namaacha.pdf). In the northern region of Maputo are the districts of Magude and Moamba. In Magude the climate is drysubtropical, the relief is made up of flat areas with highly fertile red clay soils. In this district there is cattle production in the family and private sectors, as well as pigs, goats, sheep and poultry in the family sector (http://www.portaldogoverno.gov.mz/por/content/download/2963/23867/%E2%80%A6/1/%E2%80%A6/Magude.pdf). Moamba has a dry steppe climate and is situated in the Sudano-Zambezi region between savannas and South African forests. This is the second largest cattle breeding district in the province of Maputo (http://www.portaldogoverno.gov.mz/por/content/download/2962/23862/%E2%80%A6/1/%E2%80%A6/Moamba.pdf). Finally, in Matutuíne, in the extreme south of Maputo, the climate is subtropical. In this area the Maputo Special Reserve is located with an area of 70,000 ha (http://portaldogoverno.gov.mz/por/content/download/2964/23872/%E2%80%A6/Matutuine.pdf).

In June and July 2014, 219 blood and serum samples were collected by convenience from milk cattle (Jersey and Friesians) in a semi-intensive breeding system and from beef cattle (Nguni and Nguni crosses)

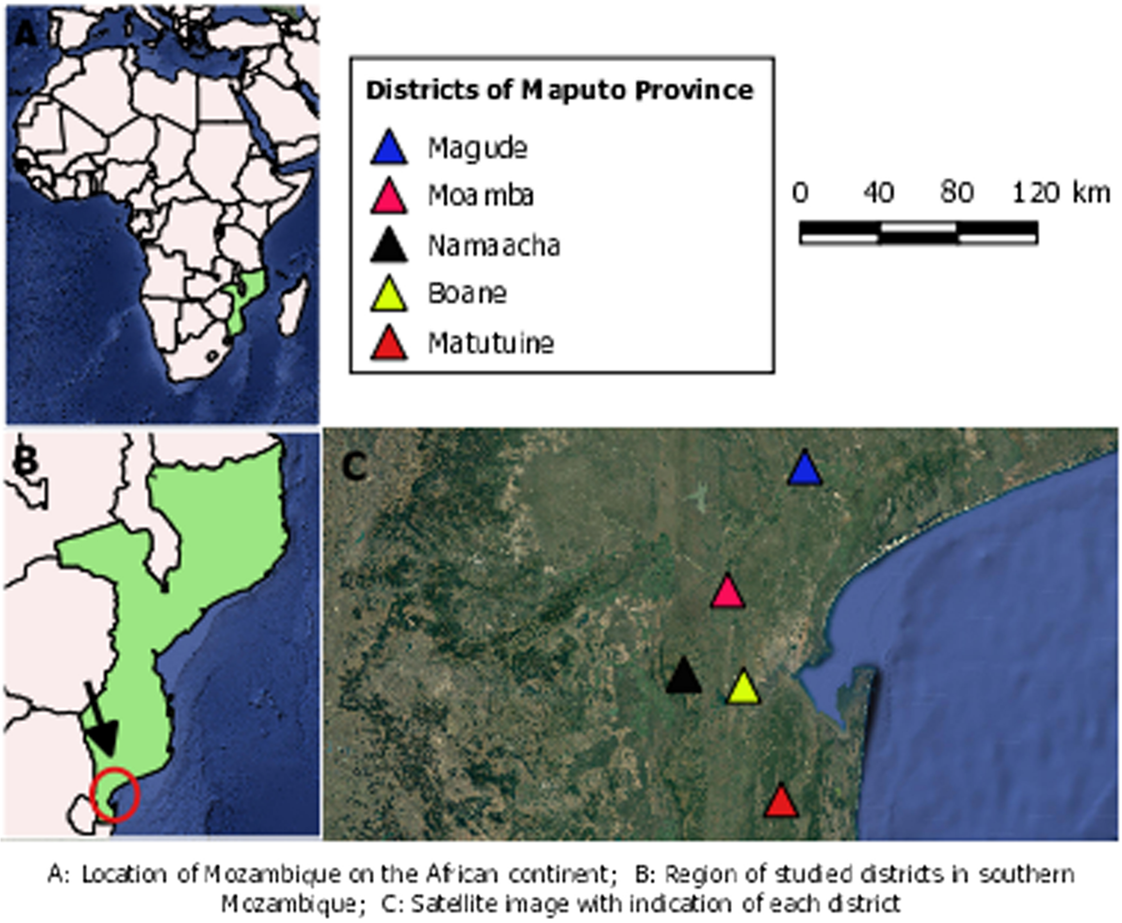

Fig. 1. Map of districts in Maputo Province.

Table 1

Description of oligonucleotides and hydrolysis probes used in the qPCR assays for *Anaplasma marginale* and *Anaplasma phagocytophilum*.

Agent, target gene and size of the gene fragment	Oligonucleotide Sequence	Hydrolysis Probes	Reference
A. marginale (*msp1β* gene) 95 bp	AM F:- 5’-TTGGCAAGGCAGCAGCTT-’3 e AM- R: 5’-TTCCGCGAGCATGTTGCAT-3’	AM-sonda: 6FAM-5’ TCGGTCTAACATCTCCAGGCTTTCAT-3’-BHQ1	Carelli et al., 2007.
A. phagocytophilum *(msp2* gene*)* 122 bp	903f (5`-AGTTTGACTGGAACACACCTGATC-3’) 1024 r (5`-CTCGTAACCAATCTCAAGCTCAAC-3’)	5`[FAM] (939p-TTAAGGACAACATGCTTGTAGCTATGGAAG-GCA) [TAMRA] 3’	Drazenovich et al., 2006.

in an extensive system belonging to the five districts of Maputo mentioned above.Five milliliters of blood were collected from the jugular or caudal vein in tubes with anticoagulant sodium EDTA for molecular analysis. The same amount of blood was collected in tubes without anticoagulant for the indirect enzyme-linked immunosorbent assay (iELISA).

2.2. Indirect enzyme-linked immunosorbent assay (iELISA) for detection of IgG antibodies to A. marginale

The indirect ELISA technique iELISA was used for the detection of IgG antibodies to *A. marginale*, according to protocol previously established by Machado et al. (1997) for *Babesiabovis* and adapted to *A. marginale* by Andrade et al. (2004). The total antigen of *A. marginale* produced showed an optimal concentration of 10 μg/mL, diluted in bicarbonate carbonate buffer (0.05 M, pH 9.6). After 12 h of incubation at 4 °C, the blockade was carried out with PBS Tween 20 (phosphate saline, pH 7.2 and 5% Tween20), added to 6% skimmed milk powder (Molico®, Nestlé, Brasil). Again the plates were incubated for 90 min at 37 °C in a humid chamber,after three washes with PBS-Tween 20 buffer. Positive and negative *A. marginale* bovine control serum samples were added to the ELISA plate (Machado et al., 2015). The serum samples were diluted at 1:400in PBS-Tween 20 solution plus 5% normal rabbit serum. Again, the plates were incubated at 37 °C for 90 min. After three washes with PBS-Tween 20 buffer, the alkaline phosphatase-bound anti-bovine IgG conjugate was added to the ELISA plate at the dilution of 1:30,000 in PBS-Tween 20 plus 5% normal rabbit serum, with subsequent incubation and washing. Finally, the alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma, St. Louis, MO), diluted at 1 mg / mL in diethanolamine buffer (pH 9.8) (Sigma, St. Louis, MO) was added. The plates were sealed with foil and incubated for 30 min at room temperature. The reading was performed in an ELISA reader (B.T.-100; Embrabio, São Paulo, Brazil), with a 405-nm filter. The cutoff point was calculated as 2.5 times the mean absorbance of the control-negative sera (Machado et al., 1997).

3. DNA extraction

DNA was extracted from 200 μL of blood from each sample using the QIAamp DNA Blood Mini kit (Qiagen, Madison, USA), according to the manufacturer's recommendations. The samples had their DNA concentration and 260/280 ratios measured in a NanoDrop spectrophotometer apparatus (ThermoScientific, San Jose, CA, USA). Subsequently, they were stored at -20 °C to perform the PCR assays.

3.1. Control of the PCR by the endogenous gapdh gene

In order to confirm the quality and verify the presence of inhibitors in the DNA samples, a conventional PCR assay was performed for mammalian glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) fragment, as previously described by Birkenheuer et al.(2003). For this purpose, primers *GAPDH*-F (5′ – CCTTCATTGACCTCAACTACAT-3′) and *GAPDH*-R (5′ – CCAAAGTTGTCATGGATGACC-3′) oligonucleotides were used. The amplification reaction was performed using a final total volume of 25 μL, with a mixture containing 5 μL of sample DNA, 0.2 μM of each deoxynucleotide, 0.4 μM of each oligonucleotide primer, 3.0 mM Chloride of Magnesium, 1.25 U of Taq Platinum DNA Polymerase (Invitrogen®, Carlsbad, California, USA), PCR buffer and sterile ultrapure water q.s.p. The thermal sequence and the amplification time were applied with initial denaturation at 95 °C for 5 min, followed by 35 cycles with denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. The final extension was performed at 72 °C for 5 min (Birkenheuer et al., 2003).

3.2. Quantitative real-time PCR (qPCR) assays for A. marginale (msp1β gene) and A. phagocytophilum (msp2 gene)

The positive samples in PCR based on the *gapdh* gene were submitted to quantitative real-time PCR (qPCR) assays for *A. marginale* (Carelli et al., 2007), with modifications for the *msp1β* gene, and for *A. phagocytophilum* based on the *msp2*gene (Drazenovich et al., 2006). All samples were tested in duplicates with final total volume of 10 μL. The mixture contained 1 μL of DNA sample, 0.2 μM of each oligonucleotide primer and hydrolysis probe (Table 1), 5 μL of PCR buffer (GoTaq Probe qPCR Master Mix, Promega, Madison, Wisconsin, USA) and sterilized ultra-pure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) q.s.p. 10 μL. The amplification reactions were conducted on low-profile Multiplate plates (BioRad™, Hercules, California, USA) in a CFX96 Thermal Cycler thermal cycler (BioRad®, Hercules, California, USA). Serial dilutions were done in order to construct standards with different concentrations of plasmid DNA, containing the target sequence (2.0×10^7 copies/μL at $2.0 \times 10^{\circ}$ copies/μL). Plasmid copy number was determined according to the formula: (Xg / μL DNA / [plasmid (pb) size x 660]) x 6.022×10^{23} x copies of the plasmid / μL) (da Silva et al., 2016).

3.3. Conventional PCR assays (cPCR) for Anaplasma spp. based on 16S rRNA, msp4, msp5, and groEL

Initially, all 219 samples were submitted to cPCR based on the 16S rRNA gene (Massung et al., 1998). Among the positive samples for this assay, 10 representatives of each studied area were selected, totalizing 50 samples. These selected samples were submitted to additional cPCR assays based on the following genes: *msp4*, for the detection of *A. marginale, A. centrale* and *A. ovis* (protocol taken from de la Fuente et al., 2004); *msp5*, which detects for *A. marginale* (protocol taken from Echaide et al., 1998 and Singh et al., 2012); 16S rRNA for *Anaplasma* spp. (Zobba et al., 2014); and *groEL* aiming at detecting *A. phagocytophilum / A. platys* (protocol taken from Sumner et al., 1997, Lotric-Furlan et al., 1998 and Nicholson et al., 1999).The cycling conditions and oligonucleotidesused in the abovementioned cPCR assays are described in Table 2. Ultrapure Water (Promega®) was used as a negative control for all reactions. *Anaplasma phagocytophilum* control-positive DNA was kindly provided by Prof. John Stephen Dumler (Uniformed Services University of the Health Sciences, Bethesda, MD, USA). DNA-

Table 2
Description of oligonucleotide sequences and cycling conditions used in the conventional PCR assays for *Anaplasma* spp.

Gene target and Amplicon size (pb)	Agents	Oligonucleotide sequences (5’-3’)	cycling conditions	References
(16S rRNA gene) 1st reaction 932 bp, 2nd reaction 546 bp.	*A.phagocytophilum* *A. bovis* *A. platys*	gE3a: (CACATGCAAGTCGAACGGATTATTC) gE10R: (TTCCGTTAAGAAGGATCTAATCTCC) gE2: (GGCAGTATTAAAAGCAGCTCCAGG) gE9f: (AACGGATTATTCTTTATAGCTTGCT) AACGGATTATTCTTTATAGCTTGCT	95 °C for 2 min, 40 cycles of: 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C. Extension of 72 °C for 5 min. In the second reaction number of cycles: 30.	(Massung et al., 1998)
(*msp4 gene*) 842 bp	*A. marginale* *A. centrale,* *A. ovis*	MSP45:(GGGAGCTCCTATGAATTACAGAGAATTGTTTAC) MSP43:(CCGGATCCTTAGCTGAACAGGAATCTTGC)	94 °C for 30 s, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min.	(de la Fuente et al., 2004)
(*msp5 gene*) 458 bp	*A. marginale*	Amar msp5 eF: (GCA TAG CCT CCG CGT CTT TC) Amar msp5 eR: (TCC TCG CCT TGG CCC TCA GA) Amar msp5 iF: (TAC ACG TGC CCT ACC GAG TTA)	94 °C for 5 min, 34 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, followed by a final extension of 72 °C for 10 min.	(Echaide et al., 1998; Singh et al., 2012)
(*16S rRNA gene*) ˜800 bp	*Anaplasma* spp.	AnaplsppF: (AGAAGAAGTCCCGGCAAACT) AnaplR3: (GAGACGACTTTTACGGATTAGCTC)	94 °C for 3 min, 30 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, followed by a final extension of 72 °C for 10 min.	(Zobba et al., 2014)
(*groEL gene*) 1st reaction 1300 -1450 bp, 2nd reaction 1297 bp.	*A.phagocytophilum* *A. platys*	HS1a: AITGGGCTGGTAITGAAAT HS6a: CCICCIGGIACIAIACCTTC HS43: AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC HSVR: CTCAACAGCAGCTCTAGTAGC	3 initial cycles at 94 °C for 1 min, 48 °C for 2 min, 72 °C for 90 s. 37 cycles at 94 °C for 1 min, 48 °C for 2 min, 72 °C for 90 s, followed by 72 °C for 5 min. In the second reaction, annealing temperature: 55 °C.	(Sumner et al.,1997; Lotric-Furlan et al.,1998; Nicholson et al., 1999)

positive control of *A. marginale* was obtained from naturally infected buffaloes (Machado et al., 2016).

PCR products were submitted to horizontal electrophoresis in 1% agarose gel stained with ethidium bromide in TEB buffer pH 8.0 (44.58 M Tris-base, 44 M boric acid, 12.49 mM EDTA). For the visualization of the results we used a molecular weight marker of 100 base pairs (Thermo Scientific, Sam Jose, CA, United States) and a transilluminator coupled to a computer program of image analysis (ChemiDoc Imaging System, BioRad®).

3.4. Purification and sequencing of amplified products

The amplified products were purified with Silica Bead DNA Gel Extraction Kit (Thermo Scientific, San Jose, CA, USA) according to the manufacturer's recommendations. Quantification of the purified DNA was performed in a NanoDrop 2000c spectrophotometer apparatus (Thermo Scientific, San Jose, CA, USA).

Sequencing was performed according to the dideoxynucleotide chain termination method (Sanger et al., 1977) in the ABI PRISM 3700 DNA Analyzer sequencer (Applied Biosystem, Foster City, CA, USA) in the Department of Technology of the Faculty of Agrarian and Veterinary Sciences (FCAV / UNESP), Center for Biological Resources and Genomic Biology (CREBIO).

3.5. Phylogenetic analysis

The electropherograms generated after sequencing were evaluated by observing the quality of the peaks corresponding to each sequenced base, through the programs FinchTV 1.4.0 and Phred / Phrap / Consed (Gordon et al., 1998; Ewing and Green, 1998; Ewing et al., 1998). The latter was also employed to obtain the consensus sequences. In addition, the Bioedit v. 7.0.5.3 (Hall, 1999) and BLAST (Altschul et al., 1990) were used to compare the sequences obtained with others previously deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank).

The alignment of the sequences saved in "FASTA" mode, Clustal / W software (Thompson et al., 1994) was performed using the Bioedit programs in version 7.0.5.3 (Hall, 1999) and MAFFT (Multiple Alignment using Fast Fourier Transform) (Katoh et al., 2002). For the conversion of the alignment file in Nexus format, the Alignment Transformation Environment site was used (Glez-Peña et al., 2010).

The phylogenetic signal of the different gene fragments (16S rRNA, *msp4, msp5,* and *groEL*) used in phylogenetic inferences was measured using the TREE-PUZZLE program package (version 5.3.rc16) (Schmidt et al., 2002).

The evolutionary model "Best of It" was found for each matrix of data generated after the alignments using the jModelTest 2 software (Darriba et al., 2012; Guindon and Gascuel, 2003), via CIPRES portal (Miller et al., 2010). The maximum likelihood (ML) analyzes were performed by the IQ-TREE web server platform (Trifinopoulos et al., 2016), while clade support indexes were evaluated through bootstrap analyzes (Felsenstein, 1985) of 1000 repetitions. The Bayesian analysis was carried out using the portal CIPRES (Miller et al., 2010) with 10^8 generations, whose numbers of substitution classes varied according to the evolutionary model found for each set of data. Phylogenetic tree editing was performed using Treegraph software 2.0.56-381 beta (Stover and Muller, 2010).

The sequence alignments of the *msp4* and *msp5* genes of *A. marginale* were used to calculate the nucleotide diversity (Pi), polymorphism level (haplotype diversity [hd], haplotype number [h], and mean nucleotide differences [k]) using the DnaSP v6 software (Librado and Rozas, 2009).

4. Results

4.1. Serological test (iELISA) for detection of IgG antibodies to A. marginale

In the iELISA test for IgG antibodies to *A. marginale*, 86.3% (189/219) of the samples showed to be positive, from the calculated cut-off point of 0.249 nm. Six samples (2.7%) were negative in qPCR but seropositive to *A. marginale*. Thirty samples (13.7%) were seronegative but positive on qPCR assay for *A. marginale*.

5. Molecular tests

5.1. qPCR assays for A. marginale (msp1β gene) and A. phagocytophilum (msp2 gene)

All 219 samples were PCR-positive for the endogenous *gapdh* gene. Six samples (2.7%) were positive in qPCR for *A. phagocytophilum* based on the *msp2* gene, with a mean Cq of 34.0 and a standard deviation of 9.92. In the qPCR targeting the *msp1β* gene of *A. marginale*, 97.3% (213/219) were positive, with a mean Cq of 26.45 and a standard deviation of 5.14. The parameters of the qPCR assays (efficiency, slope, coefficient of determination and y-intercept) are shown in Table 3.

Table 3
Parameters of qPCR assays for *A. marginale* (*msp1β*) and *A. phagocytophilum* (*msp2*).

A. marginale (*msp1β gene*)	E	R^2	Slope	Y-intercept	Cq	SQ
Mean	101,4	0,992	−3,290	38,588	26,45	$1{,}627 \times 10^4$
Maximum	105,0	0,999	−3,208	40,297	38,49	$1{,}845 \times 10^5$
Minimum	94,0	0,977	−3,475	38,015	21,26	$7{,}118 \times 10^{-1}$
***A. phagocytophilum* (*msp2 gene*)**						
Méan	91,1	0,970	−3,419	42,507	34,0	$9{,}531 \times 10^4$
Maximum	92,7	0,998	−3,011	44,467	45,69	$3{,}749 \times 10^5$
Minimum	90,0	0,904	−3,587	40,506	22,47	$1{,}969 \times 10^{-1}$

E: Amplification efficiency; R^2: coefficient of determination; Cq: quantification cycle; SQ: (Starting Quantity – Quantity of initial DNA).

5.2. cPCR assays for Anaplasma spp., BLAST analysis and phylogenetic inferences

In conventional PCR assays, 87.2% (191/219) of the samples were positive in the nested PCR for *Anaplasma* spp. based on a fragment of the 16S rRNA gene (protocol taken from Massung et al., 1998). According to BLAST analysis, the fifty DNA sequences based on the 16S rRNA gene showed identity with different species of *Anaplasma* spp. Thirty-six sequences obtained by the protocol taken fromMassung et al. (1998) presented percentage of identity between 99–100% for *A. platys*. Five samples (MH169142, MH169143, MH169145, MH169155, MH169174) showed 99% identity to *A. phagocytophilum*, one sample (MH169156) showed 100% identity to '*Candidatus* Anaplasma boleense' and eight samples (MH169146, MH169152, MH169165, MH169168, MH169171, MH169172, MH169173, MH169184) showed 99%–100% identity with sequences of *Anaplasma* sp. from cattle from Ethiopia (KY924884, KY924886) and gray brockets (*Mazama gouazoubira*) from Brazil (KF020570, KF020572, KF020578, JF952891). Three of the six-qPCR positive samples for *A. phagocytophilum* were also positive for *Anaplasma* sp. according to the cPCR protocol described by Massung et al. (1998). After sequencing, these samples showed 100% identity to *A. platys by* BLAST analysis.

The phylogenetic signals of the target genes used in the present study and obtained by the TREE-PUZZLE program ranged from 73.9% to 89.4% (Table 4).

With respect to phylogenetic inference, the trees constructed by ML (Fig. 2) and BI (Fig. 3) methodologies presented divergent topologies. In the ML analysis, 24 sequences were allocated to the upper portion of the tree in a clade near *Anaplasma* sp. detected in buffalo from Mozambique. Thirteen sequences were allocated to *A. platys*, while eleven were distributed near clades occupied by *A. bovis*, *Anaplasma* sp., and *A. phagocytophilum*. In the tree generated by the BI method, most of the sequences generated in this study were allocated to a clade occupied by sequences of *A. platys* previously detected in Brazil and in Malaysia. The sequence MH169152 was grouped in a clade occupied by *A. bovis*, while MH169180 remained isolated from the others, between sequences of *A. odocoilei* and *Anaplasma* sp. detected in buffalo from Mozambique. Finally, and showing concordant positioning between the ML and BI trees with high support index and clades, the sequences MH169156 were allocated together with the '*Candidatus* Anaplasma boleense' clade, in agreement with the result obtained by BLAST analysis. The sequence MH169173 remained isolated from the others and was positioned close to the outgroup sequences.

Furthermore, 100% (50/50) of the selected samples were shown to be positive in cPCR for *Anaplasma* spp. targeting the 16S rRNA gene, according to theprotocol taken from Zobba et al. (2014). Out of the 10 amplicons submitted to sequencing, 9 presented high sequencing quality and were used in the construction of phylogenetic trees by ML (Fig. 4) and BI (Fig. 5). One sample (MH165337) showed 100% identity to *Anaplasma* sp. detected in buffalo in South Africa. Four sequences (MH165330, MH165331, MH165334, MH165335) showed 100% identity to *A. marginale*, and two sequences (MH165329, MH165333) showed 100% identity to *A. centrale*. Two other sequences (MH165332 and MH165336) showed, respectively, 100% identity for *A. platys* and *A. ovis*. In the phylogenetic analysis by the ML method, the sequences were positioned in the clades corresponding to *A. ovis* (sequences from Nigeria, Iran and Italy), *A. marginale* (sequences from Italy, Philippines and Zimbabwe), *A. platys* (sequence from Cuba) and *Anaplasma* sp. (sequences obtained from African buffaloes in Mozambique). On the other hand, in the BI analysis, most sequences were positioned close to a sequence of *A. marginale* detected in ruminants from Italy, while a single sequence was isolated from the others, next to the clade of "*Candidatus* Anaplasma boleense".

For PCR based on a fragment of the *msp4* gene from *Anaplasma* spp. (de la Fuente, 2004), 94% of the samples (47/50) were positive; of these, 15 (30%) were submitted to sequencing. However, after

Table 4
Phylogenetic signal values for the different genes used in the study.

Gene target	Quartet Statistics			
	Analysed quartets	Fully resolved quartets	Partly resolved quartets	Unresolved quartets
(16S rRNA *gene*) (Massung et al., 1998)	1663740	1411695 (84.9%)	94,784 (5.7%)	157,261 (9.5%)
(*msp4 gene*) (de la Fuente et al., 2004)	73815	54580 (73.9%)	4283 (5.8%)	14,952 (20.3%)
(*msp5 gene*) (Echaide et al., 1998; Singh et al., 2012)	*194580*	*161487 (83.0%)*	14,984 *(7.7%)*	18,109 *(9.3%)*
(16S rRNA *gene*) (Zobba et al., 2014)	91,390	68,320 (74.8%)	15,008 (16.4%)	8062 (8.8%)
(*groEL gene*) (Sumner et al.,1997; Lotric-Furlan et al.,1998; Nicholson et al., 1999)	*8855*	*7918 (89.4%)*	*835 (9.4%)*	*102 (1.2%)*

Table 5
.Analysis of haplotypes for the *msp4* and *msp5* genes of *Anaplasma marginale*.

Gene target	(bp)	N	VS	GC %	h	hd (mean ± SD)	π (mean ± SD)	K
msp4 *(A. marginale)*	841	10	24	50.2	8	0,933 ± 0.077	0.009 ± 0.0022	7.244
msp5 *(A. marginale)*	442	7	37	43.9	5	0.857 ± 0.137	0.05351 ± 0.02461	11.238

N, number of sequences analyzed; VS, number of variable sites; GC, G + C content; h, number of haplotypes; hd, diversity of haplotypes; SD, standard deviation; p, nucleotide diversity (per site = PI); K, mean number of nucleotide difference.

evaluation of the sequencing results, only 10 (20%) DNA sequences could be used in molecular phylogeny (Fig. 6) and haplotype analysis (Table 5). All 10 sequences obtained showed 100% identity to *A. marginale* sequences detected in India. Phylogenetic inferences by both ML and BI methods presented results, positioning all the sequences in the clade of *A. marginale*. The BI tree chosen to represent this gene grouped the sequences obtained in the clade of *A. marginale* represented by sequences from Brazil, Puerto Rico, Australia, Mexico, China, Venezuela and South Africa (Fig. 6), with high bootstrap values (86% to 100%). In the analysis of polymorphism and diversity of haplotypes, only one haplotype was detected among the ten samples analyzed for this gene (Table 5).

All selected samples (50/50) were positive in the nested PCR targeting the *msp5* gene of *A. marginale* (protocol taken from Echaide et al., 1998; Singh et al., 2012). Out of these, 7 samples were submitted to sequencing, but only 4 showed enough quality and were used in the construction of the phylogenetic tree (Fig. 7). All sequences showed 100% identity with *A. marginale*previously detected in Egypt (KU042083.1). In phylogenetic inference by the ML method, all samples were grouped into a single clade containing sequences of *A. marginale*, with high bootstrap values (82% to 90%) in the external branches and ranging from 39% to 100% in the internal branches. Our sequences shared the clade with others from the Philippines, China, Mozambique, Cuba, Egypt and the United States (Fig. 7).Regarding the analysis of haplotypes represented in Table 5, two different haplotypes were found among the 4 sequences analyzed for this gene.

Finally, 22% (11/50) of the selected samples were positive in the nested PCR assay based on the *groEL* gene (protocol taken from Sumner et al., 1997; Lotric-Furlan et al., 1998; Nicholson et al., 1999). Although 7 samples were submitted to sequencing, only 1 DNA sequence (MG763748) could be employed in phylogenetic analysis (Fig. 8), which showed 96% identity for *A. platys*. As for the phylogenetic inference, there was a homogeneous distribution among the species of *Anaplasma*, so that our sequence was placed in the clade of *A. platys* with others previously detected in Portugal, Argentina and the Philippines. *Anaplasma odocoilei* (JX8766421), detected in white-tailed deer (*Odocoileus virginianus*) in the United States, was closely positioned to these sequences (Fig. 8).

6. Discussion

Anaplasma spp. phylogenetically associated with *A. marginale*, *A. centrale*, *A. platys*, *A. phagocytophilum*, *A. ovis*, and “*Candidatus* Anaplasma booleense” were detected in cattle sampled in Maputo, Mozambique. On the other hand, using a similar methodology, Machado et al. (2016) did not detect *Anaplasma* spp. phylogenetically associated with *A. ovis* and '*Candidatus* A. booleense' in African buffaloes in Mozambique. These findings emphasize the need to use different PCR protocols based on distinct gene regions to explore the diversity of genotypes and species in a given biological sample. Different oligonucleotide primers show distinct sensitivity and specificity for species of *Anaplasma* spp.

The results obtained in the serological and molecular tests showed high bovine exposure to *Anaplasma* spp. in the province of Maputo, Mozambique. The seroprevalence for *A. marginale* (86.3%) found in the present study was similar to that one (76.5%) reported among cattle in the southern region of Mozambique (Tembue et al., 2011) using a recombinant-MSP5 ELISA. In the northern region of the country, Alfredo et al. (2005), using agglutination test for *A. marginale*, reported seroprevalence of 63% for *A. marginale* among the population of sampled cattle. However, we cannot rule out the occurrence of serological cross-reactions between the used antigen in the present study (crude antigen) and the antibodies directed to other members of *Anaplasma* genus. Nevertheless, the high exposure to *A. marginale* indicated by the serological assay is confirmed by the results obtained in qPCR assay, in which 97.3% of the samples showed positive results.

A total of 36 samples presented divergent results between the iELISA and qPCR techniques for *A. marginale*. The positivity at qPCR assays and negativity in iELISA verified for some animals, probably was due to the acute phase of infection when the antibody level was below the detection threshold of the ELISA technique. On the other hand, seropositive but qPCR-negative animals may harbour persistent infections, in whichthelow number of circulating bacteria was below the detection threshold of the used molecular assay; in addition, animals that were exposed to the pathogen in question and eradicated the infection would also have residual antibodies for a long period (da Silva et al., 2016). These findings reinforce the relevance of the use of different pathogen detection methods to better assist in the conclusion of a diagnosis and the conduction of epidemiological surveys.

In the present study, 97.3% of the samples were positive in the qPCR assaytargeting *msp1β* gene of *A. marginale*. Other studies in Africa also reported high rates of positivity for *A. marginale* using the same qPCR protocol. For instance, Machado et al. (2016) found 72.2% (70/97) of positivity for *A. marginale* among a population of wild buffalo (*Syncerus caffer*) from the Marromeu Reserve in Mozambique. Elelu et al. (2016) reported 75.9% (192/253) of posivity for *A. marginale* in cattle from Nigeria. Finally, Pothmann et al. (2016), using the same protocol, reported an occurrence of 89.7% (192/214) for *A. marginale* among cattle sampled in Madagascar.

In our study, 2.7% of the samples were positive in qPCR for *A. phagocytophilum* targeting the *msp2* gene. Machado et al. (2016) reported that all buffalo sampled in the region of Marromeu, Mozambique, were negative in qPCR for *A. phagocytophilum*. Although the frequency of *A. phagocytophilum* was low among the sampled cattle, the risk of dissemination of this agent in bovine herds in Mozambique should not be ruled out. In addition to the zoonotic potential presented by this bacterium, *A. phagocytophilum* can also cause cattle grazing fever and tick-borne fever in goats and sheep (Woldehiwet, 2006; Battilani et al., 2017). Animals infected with *A. phagocytophilum*, even in low bacteremia, may act as reservoirs, facilitating the transmission of this agent to other cattle or other ruminants (Woldehiwet, 2010). In addition, *A. phagocytophilum* was associated with a predisposition to secondary infections due to the damage caused by this agent in the activity of granulocytes (Andersson et al., 2017). It is noteworthy that various

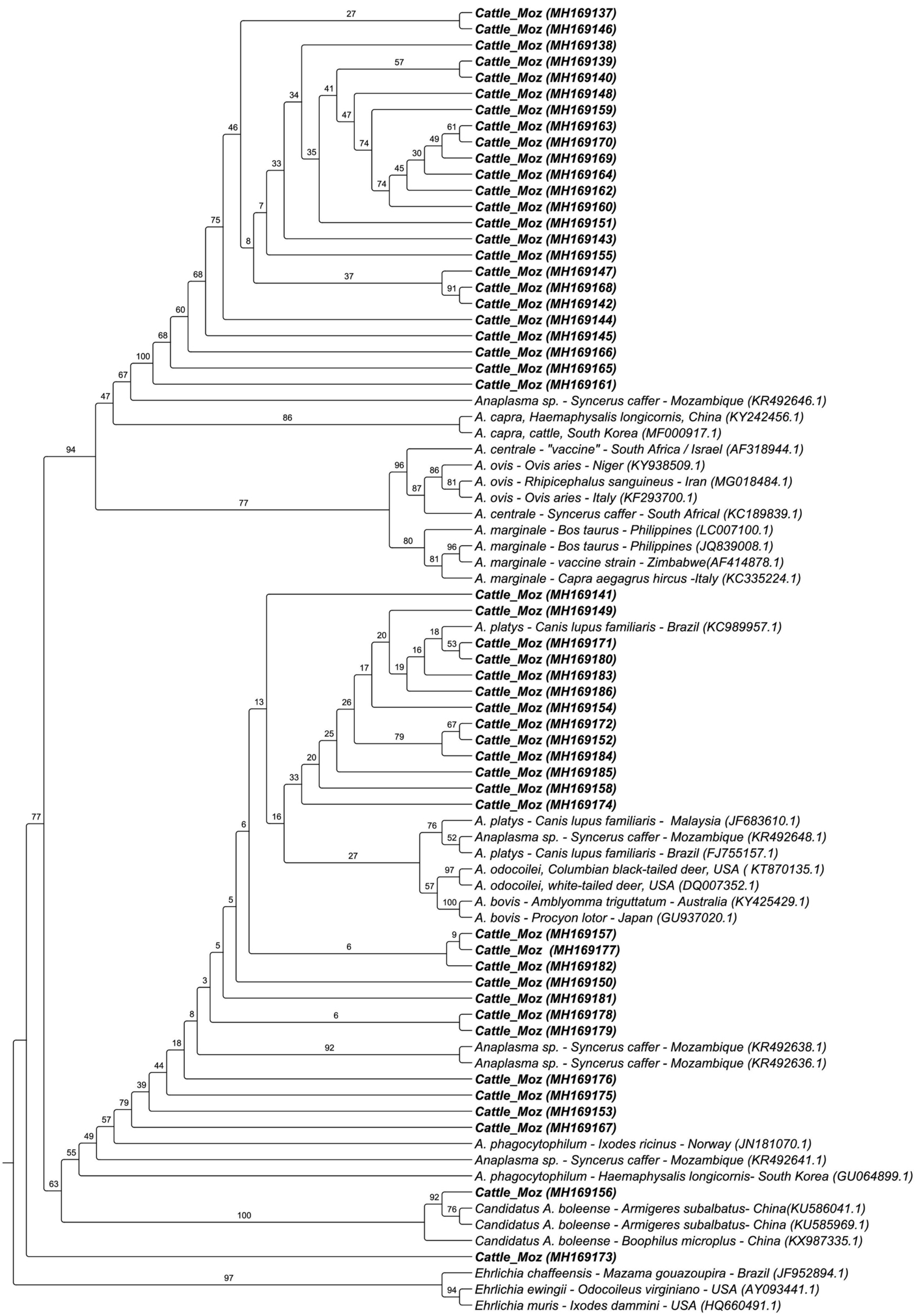

Fig. 2. Phylogenetic position of *Anaplasma* sp. detected in cattle in the province of Maputo, Mozambique; based on the 16SrRNA gene. The tree was constructed using the Maxima Likelihood analysis method using the evolutionary model GTR + F + G4. The numbers in the tree indicate bootstrap values for the branch nodes. The sequences obtained in the study are highlighted in bold. Sequences of *Ehrlichia* sp. were used as outgroup.

strains/variants of *A. phagocytophilum* occur, with different reservoir hosts and pathogenicity. In the USA, for instance, while p-Ap genospecies showed pathogenic *A. phagocytophilum* strains detected in dogs, horses and humans, the apparently non-pathogenic DU1 genospecies have been reported in woodrats (*Neotoma fuscipes*) and bearsin California; finally, the Ap-Variant 1 is found in deer and goats in the USA (Stephenson et al., 2015). Therefore, future studies aiming at genotyping *A. phagocytophilum* strains circulating in wild and domestic

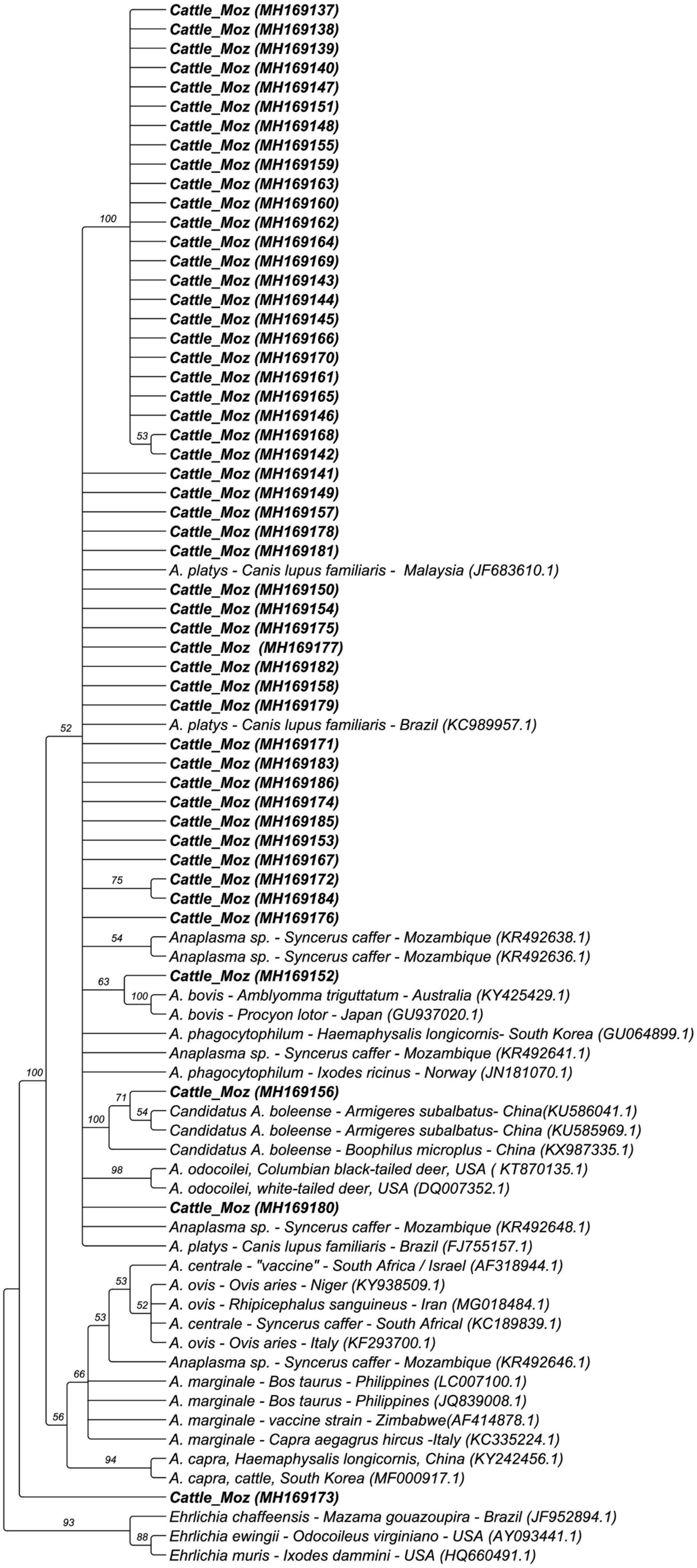

Fig. 3. Phylogenetic position of sequences of *Anaplasma* sp. detected in cattle in the province of Maputo, Mozambique; based on the 16SrRNA gene. The tree was constructed using the Bayesian method using the evolutionary model GTR + F+G4. The numbers in the tree indicate posterior probability values for the branching nodes. The sequences obtained in the study are highlighted in bold. Sequences of *Ehrlichia* sp. were used as outgroup.

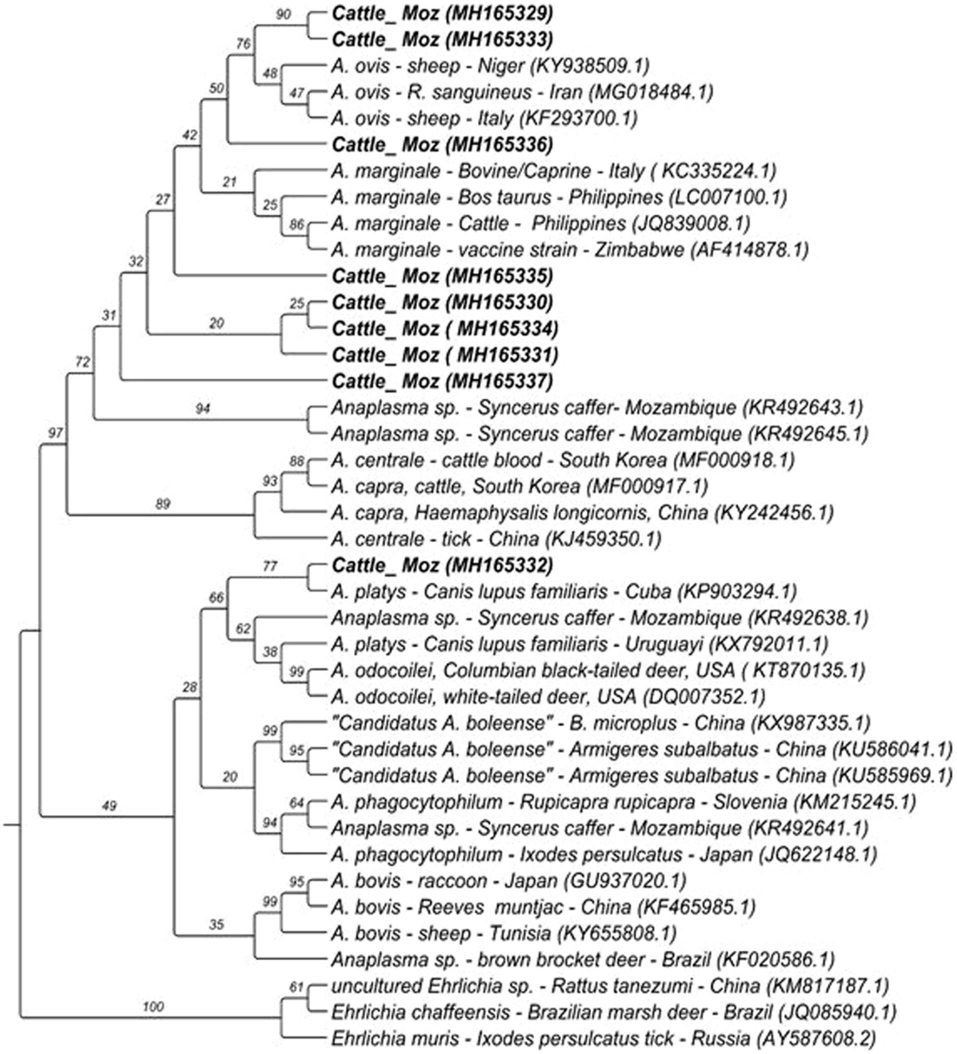

Fig. 4. Phylogenetic position of *Anaplasma* sp. detected in cattle in the province of Maputo, Mozambique; based on the 16SrRNA gene. The tree was constructed using the Maxima Likelihood analysis method using the evolutionary model GTR + F + G4. The numbers in the tree indicate bootstrap values for the branch nodes. The sequences obtained in the study are highlighted in bold. Sequences of *Ehrlichia* sp. were used as outgroup.

ruminants in Africa using different genetic markers (16S rRNA, *groEL, ankA* and 23S^{-5}S rRNA intergenic spacer) (Rejmanek et al., 2012) are much needed in order to the contribute to understanding the role of ruminants in the epidemiology of *A. phagocytophilum* in Mozambique. Additional studies in Mozambique are needed to evaluate seasonal variations, areas of occurrence and vector ticks of this agent between domestic and wild ruminants. Also, the occurrence of *A. phagocytophilum* in other potential reservoirs, such as rodents, should be better investigated in the country.

With regard to the detection of *Anaplasma* spp. using cPCR protocols based on the 16S rRNA gene, positivity rates of 87.2% (191/219) and 100% (50/50) were found according to the protocols of Massung et al. (1998) and Zobba et al. (2014), respectively. For the first protocol, the samples showed identity with previously described sequences of *A. platys*, *A. phagocytophilum*, *Anaplasma* sp. detected in cattle from Ethiopia and white-tailed deer in Brazil, as well as '*Candidatus* Anaplasma boleense'. This last agent, reported for the first time in *Hyalomma asiaticum* ticks in the Bole region of China (Kang et al., 2014), was later detected in the same country by Guo et al. (2016) in specimens of *Aedes albopictus*, *Anopheles sinensis*, *Armigeres subalbatus*, and *Culex quinquefasciatus*. Interestingly, a sequence detected in the present study presented high identity (100%) to '*Candidatus* A. boleense' in BLAST analysis, and was positioned in the same clade as this new *Candidatus* of *Anaplasma*. Thus, future studies aiming to investigate the vector capacity of mosquitoes in the transmission of these new *Anaplasma* genotypes in Mozambique are necessary. On the other hand, the sequences obtained by the protocol of Zobba et al. (2014) showed identity with *Anaplasma centrale*, *A. marginale*, *Anaplasma* sp. (detected in buffalo in South Africa), *A. platys* and *A. ovis*. Although *A. marginale* was detected in qPCR for *msp1β* with very high frequency, its occurrence was little observed in cPCR assays based on the 16S rRNA gene.

A 16S rRNA sequence (MH165336) obtained by the protocol of Zobba et al. (2014) showed 100% identity to *A. ovis* by BLAST analysis. However, a sequence of *A. marginale* (KC335224) was pooled together by the BI analysis, whereas by the ML method it was positioned among sequences of *A. ovis* and *A. marginale*. The conserved characteristic of the 16S rRNA gene did not allow for confidently stating the true

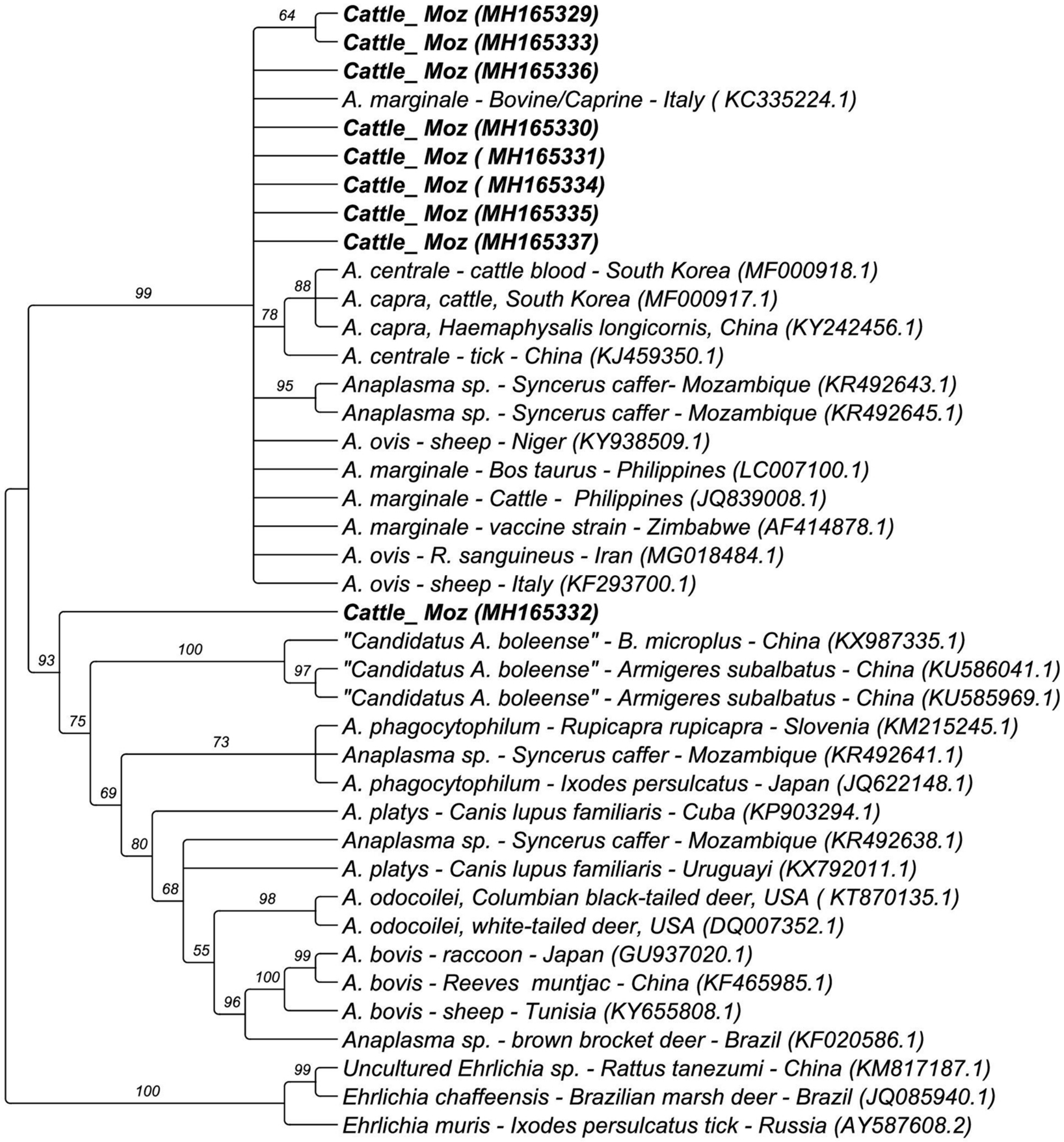

Fig. 5. Phylogenetic position of sequences of *Anaplasma* sp. detected in cattle in the province of Maputo, Mozambique; based on the 16SrRNA gene. The tree was constructed using the Bayesian method using the evolutionary model GTR + F+G4. The numbers in the tree indicate posterior probability values for the branching nodes. The sequences obtained in the study are highlighted in bold. Sequences of *Ehrlichia* sp. were used as outgroup.

identity of this circulating genotype in cattle in Mozambique. *Anaplasma ovis* has already been molecularly detected by Matsimbe et al. (2017) in 6.7% (1/15) of *Rhipicephalus microplus* specimens collected from cattle in Mozambique. Future studies should be conducted in order to investigate the vector capacity of this species of tick in the transmission of *A. ovis*. Studies with the aim of detecting Anaplasmataceae agents in ticks collected from ruminants from Mozambique are still scarce and would help to understand the distribution and prevalence of these pathogens in the region. In Mongolia, Ochirkhuu et al. (2017) detected *A. ovis* in different ruminant species by means of PCR assays based on the *groEL* and *msp4* genes. Phylogenetic inference based on the *groEL* gene positioned the *A. ovis* sequences in two clades, one of which was mainly occupied by sheep and goat sequences and the other by bovine sequences, revealing two divergent varieties of *A. ovis* (Ochirkhuu et al., 2017).

The occurrence of polytomy in the clades occupied by sequences of *A. platys*, *A. phagocytophilum* and *A. marginale* in the BI tree constructed with 16S rRNA sequences, obtained by the protocol taken from Massung et al. (1998), were possibly generated by high conservation of this target gene. Although the samples (MH169140, MH169169, MH169177) were shown to be positive in qPCR for *A. phagocytophilum* based on the *msp2* gene, 16S rRNA sequences of *Anaplasma* sp. obtained from these same samples were closely positioned to *A. platys* in the phylogenetic analysis by the BI method. In addition, considering that the Cqs obtained in the qPCR for *A. phagocytophilum* from these samples were high (42.48, 39.96, 24.27 - values for MH169169, MH169140, and MH169177, respectively), possibly, the conventional PCR based on the 16S rRNA gene was not capable of amplifying DNA from this agent in the samples. These animals were possibly coinfected with a genotype phylogenetically associated with *A. platys*, whose 16S rRNA gene was

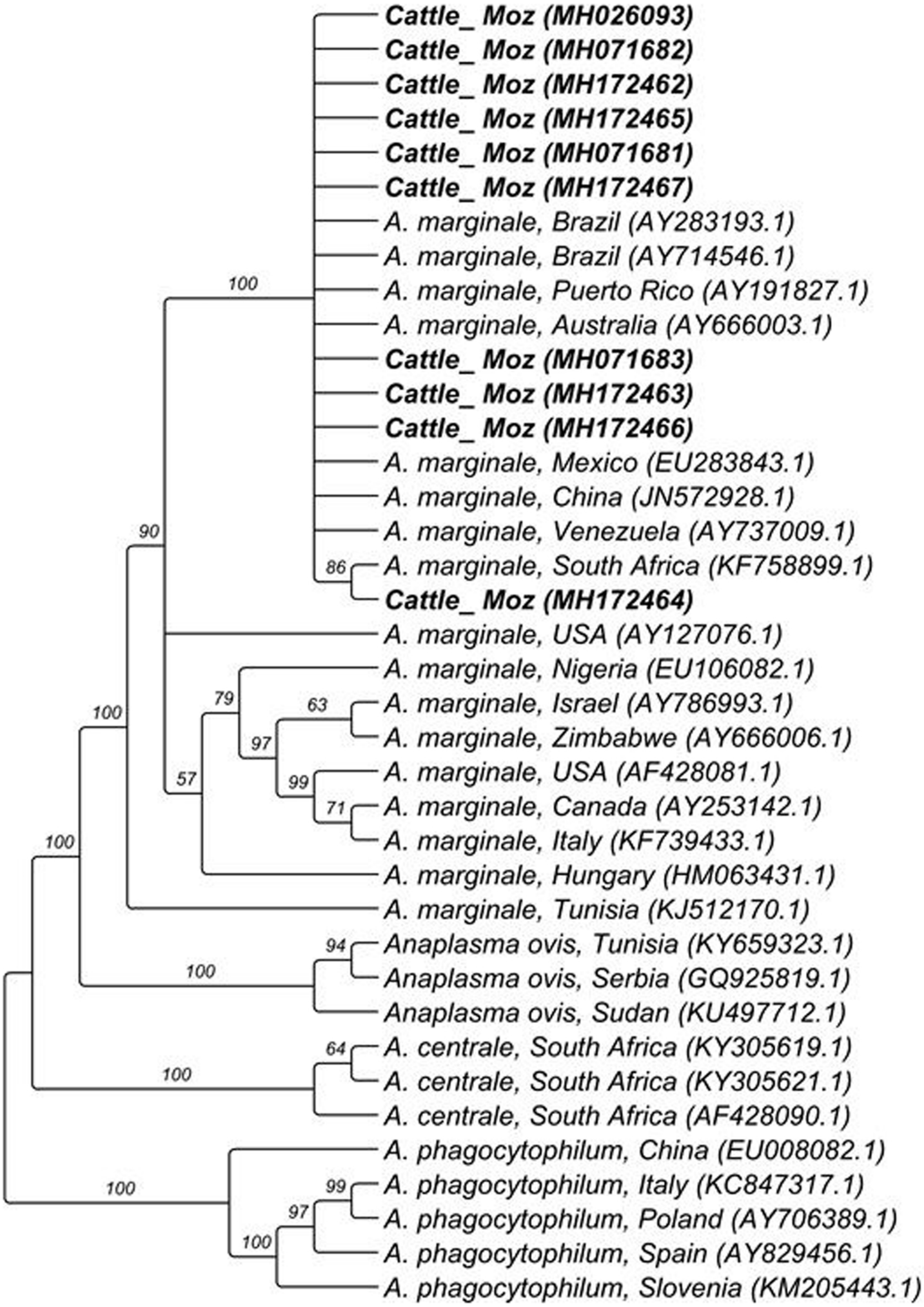

Fig. 6. Phylogenetic position of *Anaplasma* sp. detected in cattle in the province of Maputo, Mozambique; based on the *msp4* gene. The tree was constructed using the Bayesian method using the evolutionary model K2P + G4. The numbers in the tree indicate posterior probability values for the branching nodes. The sequences obtained in the study are highlighted in bold. Sequences of *A. phagocytophilum* were used as outgroup.

amplified in the cPCR assays employed. Thus, although we find genotypes phylogenetically more associated with *A. platys* in the sampled cattle, additional phylogenetic analyzes based on other less conserved gene regions are necessary in order to infer with greater confidence the phylogenetic positioning of the new genotypes found. While employing a qPCR based on 23S rRNA gene in order to detect Anaplasmataceae agentes, Dahmani et al. (2015) identified three genetic variants of *A. phagocytophilum*, *A. platys* and*Anaplasma* sp. in blood samples from cattle in Algeria. Furthermore, *Anaplasma* sp. genotypes phylogenetically associated to *A. platys* were also detected in marsh deer (*Blastocerus dichotomus*) sampled in Brazil (Sacchi et al., 2012) and in wild buffalo from Mozambique (Machado et al., 2016). Future studies aiming to investigate the spillover mechanisms of the genotype phylogenetically associated with *A. platys* from wild ruminants to cattle are necessary, as well as the species of tick involved in the transmission.

The only obtained *groEL* sequence (MG763748) was shown to be phylogenetically related to *A. platys*. On the other hand, while submitting african buffalo (*Syncerus caffer*) blood samples to PCR for *Anaplasma* sp. based on the same gene, Machado et al. (2016) foundsequences phylogenetically close to *A. centrale* and *A. marginale*.

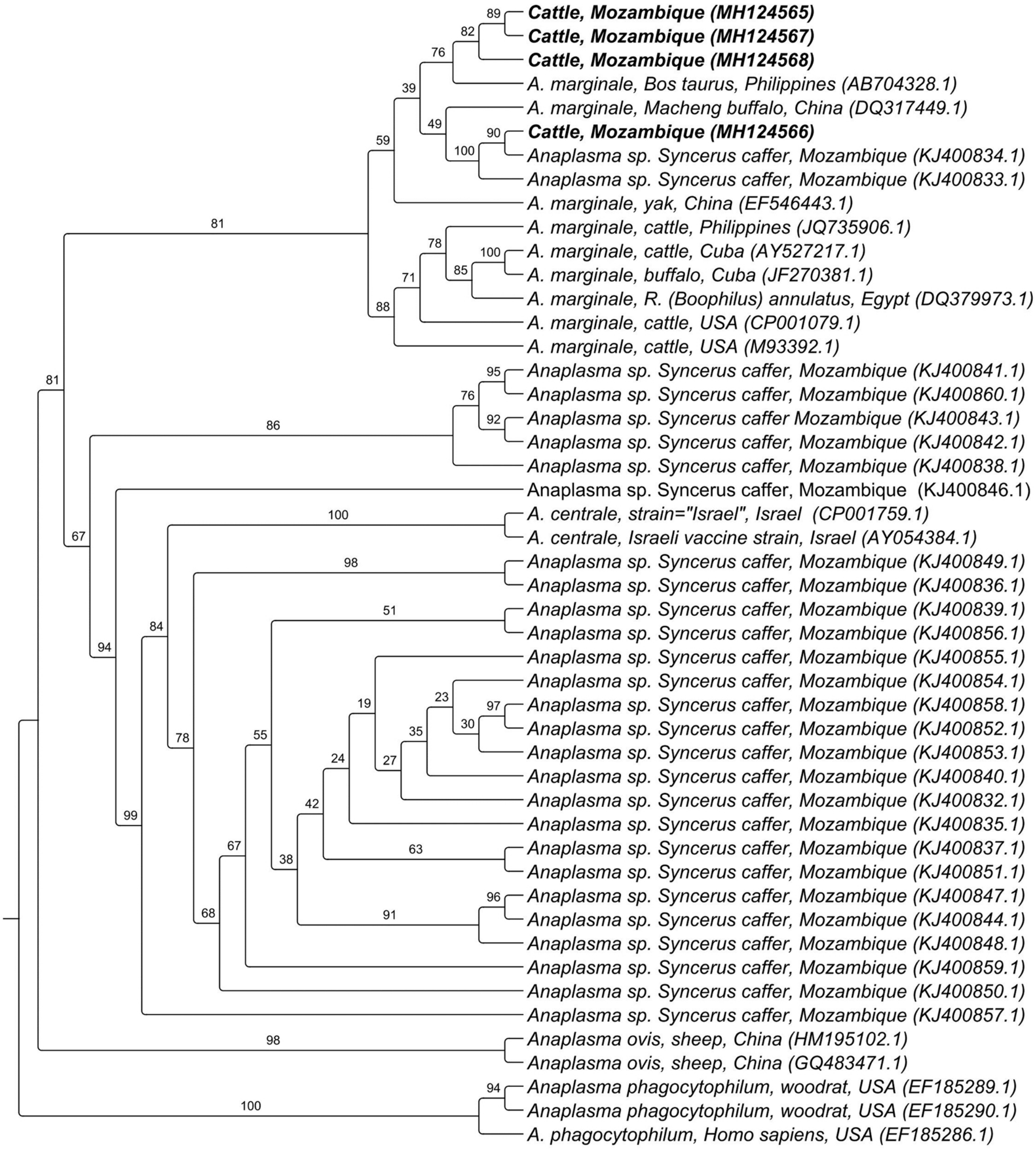

Fig. 7. Phylogenetic position of *Anaplasma* sp. detected in cattle in the province of Maputo, Mozambique; based on the *msp5* gene. The tree was constructed using the Maxima Likelihood analysis method using the TIM3 + G evolution model. The numbers in the tree indicate bootstrap values for the branch nodes. The sequences obtained in the study are highlighted in bold. Sequences of *A. phagocytophilum* were used as outgroup.

Although politomies were found in the phylogenetic tree of *msp4* of *Anaplasma* sp. by the BI method, our sequences were positioned in the clade occupied only by *A. marginale*, so that the results obtained by the BLAST analysis and phylogenetic positioning were congruent. A single *A. marginale* haplotype was detected among the ten sequences evaluated, possibly due to the high similarity between the sequences evaluated. Only one sequence (MH172464) remained isolated from the others, and close to the sequence KF758899, detected in South Africa. Unfortunately, in the study conducted by Machado et al. (2016) regarding the diversity of *Anaplasma* spp. in African buffaloes, this target gene was not used in phylogenetic inferences, preventing us from comparing our *msp4* sequences from *A. marginale* with those circulating in this group of wild ruminants in Mozambique.

While in this study the obtained*msp5* sequences of *Anaplasma* sp. were allocated only in the clade of *A. marginale*, those detected by Machado et al. (2016) in buffaloes in Mozambique were distributed among the clades occupied by *A. centrale* and *A. marginale*. Two different haplotypes were detected among the four sequences evaluated. The MH124565, MH124567 and MH124568 sequences positioned at the top of the tree belonged to the same haplotype. Future studies aimed at genotyping the isolates of *A. marginale* by means of analyses of the *msp1α* gene are necessary in order to better investigate the diversity of this agent in ruminants in Mozambique.

The different PCR protocols based on different gene regions allowed the detection of the presence of several species and/or genotypes of *Anaplasma* sp. circulating among cattle sampled in Maputo, Mozambique: *A. marginale*, *A. centrale*, *A. platys*, *A. phagocytophilum*, *A. ovis*, and '*Candidatus* Anaplasma boleense'.

Although the economic loss caused by *A. marginale* infection in cattle herds is already wellknown worldwide, the pathogenic potential

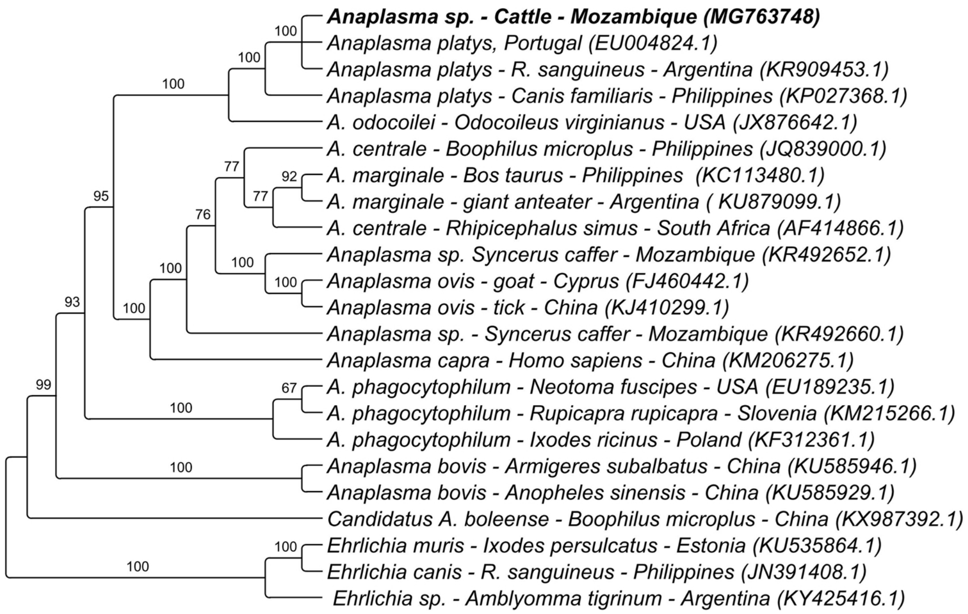

Fig. 8. Phylogenetic position of the sequence of *Anaplasma* sp. detected in cattle in the province of Maputo, Mozambique; based on the *groEL* gene. The tree was constructed using the Bayesian method using the evolutionary model GTR + I + G. The numbers in the tree indicate posterior probability values for the branching nodes. The sequence obtained in the study is highlighted in bold. Sequences of *Ehrlichia* sp. were used as outgroup.

of the other genotypes and/or species reported in our study are still unknown. In addition, the effects generated by coinfection of multiple genotypes/species of *Anaplasma* spp. and other hemoparasites (*Babesia* spp., *Theileria* spp., *Trypanosoma* spp.) are unknown. Also, in view of the diversity of *Anaplasma* spp. observed, including the occurrence of *A. phagocytophilum* in the herds studied, future studies aiming at the genotypic analysis of this last agent in the country are necessary in order to estimate the real risk to human health in the country.

7. Conclusion

The present study, using molecular techniques based on different gene targets (*msp1β*, *msp2*, 16S rRNA, *msp4*, *msp5* and *groEL*), showed the occurrenceof different species/genotypes of *Anaplasma* spp. in cattle sampled in Mozambique: *A. marginale* (agent of bovine anaplasmosis), *A. phagocytophilum* (agent of human granulocytic anaplasmosis), *A. platys*, *A. centrale*, *A. ovis*, and '*Candidatus* Anaplasma boleense'.

Acknowledgements

The authors would like to thank the Graduate Program in Agricultural Microbiology (Unesp University, São Paulo University, Unesp, Campus Jaboticabal), the Coordination of Improvement of Higher Education Personnel (CAPES) and FAPESP (Foundation for Research Support of the State of São Paulo - Process 2015/14896-1) for the financial support and support to carry out this research. MRA is a fellowship researcher of “Conselho Nacional de Desenvolvimento Científico e Tecnólogico” (CNPq Process number # 302420/2017-7). Finally, we are thankful to all the staff of the farms and Direçcão de Ciências Animal, Maputo, Mozambique, for helping in animal sampling.

References

Alfredo, A.A., Jonsson, N.N., Finch, T.M., Neves, L., Molloy, J.B., Jorgensen, W.K., 2005. Serological survey of *Babesia bovis* and *Anaplasma marginale* in cattle in Tete Province, Mozambique. Trop. Anim. Health Prod. 37, 121–131.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Andersson, M.O., Víchová, B., Tolf, C., Krzyzanowska, S., Waldenström, J., Karlsson, M.E., 2017. Co-infection with *Babesia divergens* and *Anaplasma phagocytophilum* in cattle (*Bos taurus*), Sweden. Ticks Tick Borne Dis. 8, 933–935.

Andrade, G.M., Machado, R.Z., Vidotto, M.C., Vidotto, O., 2004. Immunization of bovines using a DNA vaccine (pcDNA3.1/MSP1b) prepared from the Jaboticabal strain of *Anaplasma marginale*. Ann. N. Y. Acad. Sci. 1026, 257–266.

Bakken, J.S., Dumler, J.S., 2015. Human granulocytic anaplasmosis. Infect. Dis. Clin. North Am. 29, 341–355.

Battilani, M., de Arcangeli, S., Balboni, A., Dondi, F., 2017. Genetic diversity and molecular epidemiology of Anaplasma. Infect. Genet. Evol. 49, 195–211.

Bekker, C.P., de Vos, S., Taoufik, A., Sparagano, O.A., Jongejan, F., 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Vet. Microbiol. 89, 223–238.

Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. J. Clin. Microbiol. 41, 4172–4177.

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L., Buonavoglia, C., 2007. Detection and quantification of *Anaplasma marginale* DNA in blood sample of cattle by real, time PCR. Vet. Microbiol. 124, 107–114.

da Silva, J.B., André, M.R., Machado, R.Z., 2016. Low genetic diversity of *Anaplasma marginale* in calves in an endemic area for bovine anaplasmosis in the state of São Paulo, Brazil. Ticks Tick Borne Dis. 7, 20–25.

Dahmani, M., Davoust, B., Benterki, M.S., Fenollar, F., Raoult, D., Mediannikov, O., 2015. Development of a new PCR-based assay to detect Anaplasmataceae and the first report of *Anaplasma phagocytophilum* and *Anaplasma platys* in cattle from Algeria.COmp. Immunol. Microbiol. Infect. Dis. 39, 39–45.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772.

Drazenovich, N., Foley, J.E., Brown, R.N., 2006. Use of real-time quantitative PCR targeting the msp2 protein gene to identify cryptic *Anaplasma phagocytophilum* infections in wildlife and domestic animals. Vector Borne Zoonotic Dis. 6, 83–90.

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Stuart, C.R., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six news species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. 51, 2145–2165.

Echaide, S.T., Knowles, D.P., Mcguire, T.C., Palmer, G.H., Suarez, C.E., Mcelwain, T.F., 1998. Detection of cattle naturally infected with *Anaplasma marginale*in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. J. Clin. Microbiol. 36, 777–782.

Elelu, N., Ferrolho, J., Couto, J., Domingos, A., Eisler, M.C., 2016. Molecular diagnosis of the tick-borne pathogen *Anaplasma marginale* in cattle blood samples from Nigeria using qPCR. Exp. Appl. Acarol. 70, 501–510.

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using Phred II. Error probabilities. Genome Res. 8, 186–194.

Ewing, B., Hillier, L., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces using Phred. I. Acuracy Assessement Genome Res. 8, 175–1985.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fuente, J., Vicente, J., Hofle, U., Ruiz-Fons, E.R., Mera, I.G.F., Van den Bussche, R.A., Kocan, K.M., Gortazar, C., 2004. *Anaplasma* infection in free-ranging Iberian red deer in the region of Castella- La Mancha, Spain. Vet. Microbiol. 100, 163–173.

Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., Posada, D., 2010. Alter: program-oriented conversion of DNA and protein alignments. Nucleic Acids Res. 38, 14–18.

Gordon, D., Abajian, C., Green, P., 1998. Consed: a graphical tool for sequence finishing. Genome Res. 8, 195–202.

Guindon, S., Gascuel, O., 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst. Biol. 52, 696–704.

Guo, W.P., Tian, J.H., Lin, X.D., Ni, X.B., Chen, X.P., Liao, Y., Yang, S.Y., Dumler, J.S., Holmes, E.C., Zhang, Y.Z., 2016. Extensive genetic diversity of Rickettsiales bacteria in multiple mosquito species. Sci. Rep. 6, 1–11.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analyses program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Jin, H., Wei, F., Liu, Q., Qian, J., 2012. Epidemiology and control of human granulocytic anaplasmosis: a systematic review. Vector Borne Zoonotic Dis. 12 (269), 274.

Kang, Y.J., Diao, X.N., Zhao, G.Y., Chen, M.H., Xiong, Y., Shi, M., Fu, W.M., Guo, Y.J., Pan, B., Chen, X.P., Holmes, E.C., Gillespie, J.J., Dumler, S.J., Zhang, Y.Z., 2014. Extensive diversity of Rickettsiales bacteria in two species of ticks from China and the evolution of the Rickettsiales. BMC Evol. Biol. 14, 167.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.

Kocan, K.M., de la Fuente, J., Guglielmone, A.A., Melendez, R.D., 2003. Antigens and alternatives for control of *Anaplasma marginale* infeccion in cattle. Clin. Microbiol. Rev. 16, 698–712.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., Ewing, S.A., 2010. The natural history of *Anaplasma marginale*. Vet. Parasitol. 167, 95–107.

Li, H., Zheng, Y.C., Ma, L., Jia, N., Jiang, B.G., Jiang, R.R., Huo, Q.B., Wang, Y.W., Liu, H.B., Chu, Y.L., Song, Y.D., Yao, N.N., Sun, T., Zeng, F.Y., Dumler, J.S., Jiang, J.F., Cao, W.C., 2015. Human infection with a novel tick-borne *Anaplasma* species in China: a surveillance study. Lancet Infect. Dis. 15, 663–670.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Lotric-Furlan, S., Petrovec, M., Zupanc, T.A., Nicholson, W.L., Sumner, J.W., Childs, J.E., Strle, F., 1998. Human granulocytic ehrlichiosis in Europe: clinical and laboratory findings for four patients from Slovenia. Clin. Infect. Dis. 27, 424–428.

Machado, R.Z., Montassier, H.J., Pinto, A.A., Lemos, E.G., Machado, M.R.F., Valadão, I.F.F., Barci, L.G., Malheiros, E.B., 1997. An enzyme, linked immunosorbent assay (ELISA) for the detection on antibodies against *Babesia bovis* in cattle. Vet. Parasitol. 71, 17–26.

Machado, R.Z., da Silva, J.B., André, M.R., Gonçalves, L.R., Matos, C.A., Obregón, D., 2015. Outbreak of anaplasmosis associated with the presence of different *Anaplasma marginale* strains in dairy cattle in the states of São Paulo and Goiás, Brazil. Rev. Bras. Parasitol. Vet. 24, 438–446.

Machado, R.Z., Teixeira, M.M.G., Rodrigues, A.C., André, M.R., Gonçalves, L.R., Da Silva, J.B., Pereira, C.L., 2016. Molecular diagnosis and genetic diversity of tick-borne Anaplasmataceae agentes infecting the African buffalo *Syncerus caffer* from Marromeu Reserve in Mozambique. Parasit. Vectors 9, 454–462.

Martins, T.M., Neves, L., Pedro, O.C., Fafetine, J.M., do Rosário, V.E., Domingos, A., 2010. Molecular detection of *Babesia* spp. and other haemoparasitic infections of cattle in Maputo Province, Mozambique. Parasitology 137, 1–8.

Massung, R.F., Slater, K., Owens, J.H., Nicholson, W.L., Mather, T.N., Solberg, V.B., Olson, J.G., 1998. Nested PCR assay for detection of granulocytic ehrlichiae. J. Clin. Microbiol. 36, 1090–1095.

Matsimbe, A.M., Magaia, V., Sanches, G.S., Neves, L., Noormahomed, E., Antunes, S., Domingos, A., 2017. Molecular detection of pathogens in ticks infesting cattle in Nampula province, Mozambique. Exp. Appl. Acarol. 73, 91–102.

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. GATEWAY COMPUTING ENVIRONMENTS WORKSHOP (GCE). pp. 1–8.

Nicholson, W.L., Castro, M.B., Kramer, V.L., Sumner, J.W., Childs, J.E., 1999. Dusky-footed wood rats (*Neotoma fuscipes*) as reservoirs of granulocytic ehrlichiae (*Rickettsiales: Ehrlichieae*) in Northern California. J. Clin. Microbiol. 37, 3323–3327.

Ochirkhuu, N., Konnai, S., Odbileg, R., Murata, S., Ohashi, K., 2017. Molecular epidemiological survey and genetic characterization of *Anaplasma* species in mongolian livestock. Vector Borne Zoonotic Dis. 17, 539–549.

Pothmann, D., Poppert, S., Rakotozandrindrainy, R., Hogan, B., Mastropaolo, M., Thiel, C., Silaghi, C., 2016. Prevalence and genetic characterization of *Anaplasma marginale* in zebu cattle (*Bos indicus*) and their ticks (*Amblyomma variegatum, Rhipicephalus microplus*) from Madagascar. Ticks Tick Dis. 7, 1116–1123.

Rejmanek, D., Bradburd, G., Foley, J., 2012. Molecular characterization reveals distinct genospecies of *Anaplasma phagocytophilum* from diverse North American hosts. J. Med. Microbiol. 61, 204–212.

Sacchi, A.B., Duarte, J.M., André, M.R., Machado, R.Z., 2012. Prevalence and molecular characterization of Anaplasmataceae agents in free-ranging Brazilian marsh deer (*Blastocerus dichotomus*. Comp. Immunol. Microbiol. Infect. Dis. 35, 325–334.

Sanger, F., Niklen, S., Coulson, A.R., 1977. DNA Sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5163–5169.

Schmidt, H.A., Strimmer, K., Vingron, M., von Haeseler, A., 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18, 502–504.

Silaghi, C., Santos, A.S., Gomes, J., Christova, I., MateI, I.A., Walder, G., Domingos, A., Bell-Sakyi, L., Sprong, H., Von Loewenich, F.D., Oteo, J.A., de la Fuente, J., Dumler, J.S., 2017. Guidelines for the direct detection of *Anaplasma* spp. in diagnosis and epidemiological studies. Vector Borne Zoonotic Dis. 17, 12–22.

Simuunza, M., Weira, W., Courcierc, E., Taita, A., Shiels, B., 2011. Epidemiological analysis of tick-borne diseases in Zambia. Vet. Parasitol. 175, 331–342.

Singh, H., Jyoti, Haque, M., Singh, N.K., Rath, S.S., 2012. Molecular detection of *Anaplasma marginale* infection in carrier cattle. Ticks Tick Dis. 3, 55–58.

Stephenson, N., Hodzic, E., Mapes, S., Rejmanek, D., Foley, J., 2015. A real-time PCR assay for differentiating pathogenic *Anaplasma phagocytophilum* from an apathogenic, woodrat-adapted genospecies from North America. Ticks Tick Dis. 6, 774–778.

Stover, B.C., Muller, K.F., 2010. TreeGraph2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinform. 11, 1–9.

Sumner, J.W., Nicholson, W.L., Massung, R.F., 1997. PCR amplification and comparison of nucleotide sequences from the groELSL heat shock operon of *Ehrlichia* species. J. Clin. Microbiol. 35, 2087–2092.

Tate, C.M., Howerth, E.W., Mead, D.G., Dugan, V.G., Luttrell, M.P., Sahora, A.I., Munderloh, U.G., Davidson, W.R., Yabsley, M.J., 2013. *Anaplasma odocoilei* sp. Nov. (family Anaplasmataceae) from white-tailed deer (*Odocoileus virginianus*). Ticks Tick Dis. 4, 110–119.

Tembue, A.A.M., Da Silva, J.B., Da Silva, F.J.M., Pires, M.S., Baldani, C.D., Soares, C.O., Massard, C.L., Da Fonseca, A.H., 2011. Seroprevalence of IgG antibodies against *Anaplasma marginale* in cattle from South Mozambique. Rev. Bras. Parasitol. Vet. 20, 318–324.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity search tool. Nucleic Acids Res. 215, 403–410.

Trifinopoulos, J., Nguyen, L.T., Von, Haeseler. A., Minh, B.Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 44 (W1), W232–W235.

Woldehiwet, Z., 2006. *Anaplasma phagocytophilum* in ruminants in Europe. Ann. N. Y. Acad. Sci. 1078, 446–460.

Woldehiwet, Z., 2010. The natural history of *Anaplasma phagocytophilum*. Vet. Parasitol. 167, 108–122.

Zobba, R., Anfossi, A.G., Pinna, P.M.L., Dore, G.M., Chessa, B., Spezzigu, A., Rocca, S., Visco, S., Pittau, M., Alberti, A., 2014. Molecular investigation and phylogeny of *Anaplasma* spp. in Mediterranean ruminants reveal the presence of neutrophil-tropic strains closely related to *A. platys*. Appl. Environ. Microbiol. 80, 271–280.