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Original article

Genetic diversity of *Anaplasma marginale* in beef cattle in the Brazilian Pantanal

Inalda Angélica de Souza Ramos[a,c], Heitor Miraglia Herrera[b], Simone de Jesus Fernandes[a], Renan Bressianini do Amaral[a], Diego Carlos de Souza Zanatto[a], Thiago Merighi Viera da Silva[a], Bruna Le Senechal Horta[a], João Bosco Vilela Campos[b], João Vitor Almeida Alves[b], Gabriel Carvalho de Macedo[b], Rosangela Zacarias Machado[a], Marcos Rogério André[a,*]

[a] Universidade Estadual Paulista (Unesp), Faculty of Agrarian and Veterinary Sciences, Jaboticabal, SP, Brazil
[b] Don Bosco Catholic University (UCDB), Campo Grande, MS, Brazil
[c] "Graduate Program in Veterinary Medicine, Animal Pathology, Host-Parasite Relationship, State University of São Paulo (UNESP), Faculty of Agrarian and Veterinary Sciences, UNESP Jaboticabal", Brazil

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ABSTRACT

There are few studies on the genetic diversity of *Anaplasma marginale* in Brazilian cattle herds, especially about beef cattle. The objective of this study was to evaluate the genetic diversity of *A. marginale*, based on the *msp1α* gene in *Bos taurus indicus* sampled from the Brazilian Pantanal. Aliquots of blood with and without EDTA were taken from 400 cattle (200 cows and 200 calves) across five extensive farms. The samples were submitted to the indirect immunoenzymatic assay (iELISA), quantitative real-time PCR (qPCR) for the *msp1β* gene and to the semi-nested (sn) PCR for the *msp1α* gene. Positive samples were sequenced by the Sanger method and subjected to diversity analysis using the RepeatAnalyser software. The percentage of positive animals by iELISA, qPCR and (sn) PCR was 72.2% (289/400), 56.7% (227/400) and 23% (52/227), respectively. Cows (154/200) showed to be significantly more seropositive than calves (135/200). In qPCR, the number of calves and average quantification value (138/200; 1.3×10^6) *A. marginale msp1α* copies per µL proved to be higher when compared to that found for the cows (89/200; 3.9×10^4). The microsatellite analysis of the 26 sequences obtained from the *msp1α* gene revealed the presence of E (77%), C (15.4%) and B (7.7%) genotypes. Fourteen *A. marginale* strains were identified in the studied region, with eight that have never before been described in the literature (τ-10-13 – 13-18; τ-27-18; EV8-EV8-17; α-β-β-β-100; EV7-11 – 10-15; τ-11-11 – 27-18; τ-11-10-15; τ-27-13-18). Beef cattle are highly exposed to *A. marginale* in the Brazilian Pantanal. Moreover, a high genetic diversity of *A. marginale,* with eight new strains, was found in the studied region. While cows may act as chronic carriers, perpetuating the pathogen within the herd, male beef calves sold to other regions may disperse these strains.

1. Introduction

Bovine anaplasmosis, caused by *Anaplasma marginale* (Rickettsiales, Anaplasmataceae), contributes to considerable economic loss in the cattle industry worldwide (Bekker et al., 2002). In addition to bovines, ruminants such as buffalo and deer are susceptible to the pathogen (Zaugg et al., 1996; Machado and Müller, 2006; Kocan et al., 2010; Machado et al., 2017).

The biological cycle of this pathogen is closely related to the hematophagy of ticks, which are the biological vectors. Around 20 species of ticks contribute to the biological transmission of this pathogen, including those belonging to the genus *Dermacentor* in temperate regions, and *Rhipicephalus* in tropical regions (Kocan et al., 1992, 2010; Silaghi et al., 2017). The transmission of this pathogen may also occur mechanically by hematophagous flies (*Stomoxys calcitrans* and *Haematobia irritans*), horse-flies (*Tabanus* spp.), fomites contaminated with blood, and by transplacental exchange (Palmer et al., 2009; Kocan et al., 2004; Aubry and Geale, 2011).

After the vertebrate hosts are infected, the parasitized erythrocytes are removed by cells of the mononuclear phagocytic system, resulting in the development of immune-mediated hemolytic anemia and fever, weight loss, miscarriage, lethargy and jaundice, causing a drop in

* Corresponding author at: Immunoparasitology Laboratory, Department of Veterinary Pathology, Faculty of Agrarian and Veterinary Sciences Júlio de Mesquita Filho (UNESP), Jaboticabal Campus, Path of Access Prof. Paulo Donato Castellane, s / n, Zona Rural, CEP: 14884-900, Jaboticabal, São Paulo, Brazil.
E-mail address: mr.andre@unesp.br (M.R. André).

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productivity and leading to death. Animals that survive acute infection remain persistently infected, characterized by low levels of cyclical rickettsemia, allowing the transmission of the pathogen to other ticks (Ristic, 1977; Kieser et al., 1990; Kocan et al., 2010).

The Pantanal biome is the largest wetland in the world, with an estimated area of 138,183 km^2 (larger than Portugal or Bulgaria), which occupies areas in Brazil, Paraguay and Bolivia (Alho, 2005). The region's main economic activity is the extensive production of beef cattle, with an estimated four million (Abreu et al., 2010). Corumbá, a municipality within the Pantanal, has the second largest amount of cattle in Brazil, with 1.9 million. Still, the Pantanal region has rich biological diversity, with some species of ticks infesting both wild and domestic animals (Campos-Pereira et al., 2000; Ramos et al., 2016). In the Pantanal region of Nhecolândia, within the municipality of Corumbá, cattle were found to carry the *Rhipicephalus. microplus* (55.7%), *Amblyomma sculptum* (38%) and *Amblyomma parvum* (4.1%) ticks (Ramos et al., 2016).

Currently, there are limitations in terms of controlling and preventing *A. marginale*, since the vaccines developed to date are not highly effective. In addition, antibiotic treatment has not been effective on various farms, as treated animals are often reinfected. Also, antibiotics also present a higher cost to the farmer (Silva et al., 2016).

Studies based on the analysis of tandem repeats of *msp1α* in *A. marginale*, aiming to know the genetic diversity of the parasite, have already been carried out on dairy cattle sampled from some states of Brazil, such as São Paulo (Machado et al., 2015; Silva et al., 2016), Minas Gerais (Pohl et al., 2013), Goiás (Machado et al., 2015), Paraná (Vidotto et al., 2006) and Rio de Janeiro (Baêta et al., 2015; Silva et al., 2015), proving the existence of different strains of the parasite circulating in these herds. Previous studies conducted in Brazil have suggested that the strains τ-10-15 and α-β^3-Γ are associated with the occurrence of clinical anaplasmosis and mortality in lactating heifers, heifers and cows (Machado et al., 2015). In addition, studies on the genetic diversity of *A. marginale* in buffalo were conducted in the states of Pará (Silva et al., 2014a) and Rio de Janeiro (Silva et al., 2014b).

The present study aimed to identify and characterize the genetic diversity of circulating strains of *A. marginale* in beef cattle (*Bos taurus indicus*) in the Brazilian Pantanal, with the long-term purpose of contributing to the development of ways to control this pathogen in this region and, consequently, in Brazil.

2. Material and methods

2.1. Ethical statement

This study was approved by the National Council for Animal Experimentation Control (CONCEA) and the Ethics Committee on Animal Use (CEUA, FCAV, UNESP, Protocol No. 12375/15).

2.2. Animals and area of study

Blood samples from beef cattle (*Bos taurus indicus*) were collected from five breeding and rearing farms (A, S, P, N and C) in the Central Region of the Pantanal sul Matogrossense, Sub-region of Nhecolândia (18° 59′15′ 'S, 56° 37′ 03′) (Fig. 1). The farms were selected for their convenience as the distance between them ranged from 30 to 50 km (Table 1).

Considering the lack of data on the prevalence of *A. marginale* in cattle in the State of Mato Grosso do Sul, an expected prevalence of 50% was assumed, according to Stevenson (2008). Sample size was determined using the systematic random sampling method, where the minimum number of animals had an absolute precision of 5% and a 95% confidence interval as indicated by the formula below:

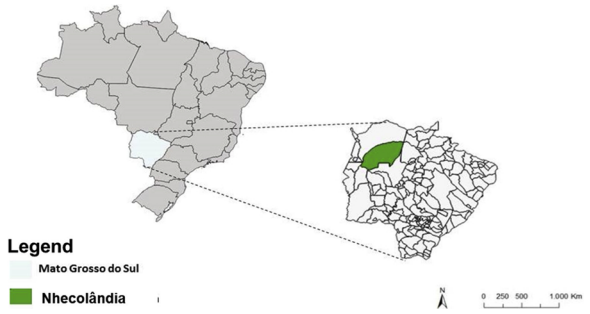

Fig. 1. Location and geographical distribution of the Brazilian Pantanal in the state of Mato Grosso do Sul, with emphasis on the study area, Nhecolândia subregion (in green). (Source: Q-GIS Program v.2.8). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
Location in geographic coordinates of the studied properties and number (n) and animal (cows/calves) sampled by property in the Brazilian Pantanal (August 2016 and April 2017).

Property	Latitude	Longitude	Cows (n)	Calves (n)	Total (n)
*Farm A	19° 08' 34" S	56° 47' 35"W	45	53	98
*Farm S	19° 16' 27" S	56° 38' 16" W	42	41	83
*Farm P	18° 54' 26" S	56° 31' 23" W	39	41	80
**Farm N	19° 15' 08" S	57° 03' 44" W	38	34	72
**Farm C	19° 09' 19" S	57° 50' 42" W	36	31	67
Total			**200**	**200**	**400**

* First sampling (August / 2016).
** Second sampling (April / 2017).

$$n = \frac{z^2(1\text{-}Py)\times Py}{d^2}$$

z = confidence coefficient (z = 1.96)
n = sample size
Py = expected prevalence (50%)
d^2 = Absolute precision (5%)
(Stevenson, 2008).

Blood samples were collected from 400 animals, cows (≥ 6 years) and calves (7–12 months). Two sampling procedures were performed in a cross-sectional study, the first one in August 2016 and the second in April 2017. Whole blood samples, collected directly from the caudal vein, were packed in an EDTA anticoagulant (Ethylenediamine Tetra - Sodium Acetic Acid 300 mmol / L) and used for DNA extraction and subsequent Polymerase Chain Reaction (PCR). Blood serum samples obtained with non-anticoagulant tubes were used to perform the Indirect Immunoenzymatic Assay (iELISA) for the detection of anti-*A. marginale* IgG antibodies.

The animals were kept on an extensive breeding and rearing regime, and were fed two species of grasses, *Axonopus purpusii* and *Reimarochloa brasiliensis*, on all the farms where the study took place. Calves, ranging in age from 8 to 12 months, were separated from their mothers, and later sold or auctioned to farms in other regions of Mato Grosso do Sul and São Paulo state. Farm C also auctioned its calves to properties located in Bolivia. The cows that were used for breeding and that showed good clinical condition throughout their breeding lives were slaughtered upon reaching an age of between 13 and 15 years. All farms presented animals infested with *Rhipicephalus microplus* and *Amblyomma sculptum* ticks, which were identified using previously described taxonomic keys (Martins et al., 2003). Co-infestation by the two tick species was observed in some animals. Macrocyclic lactones

(avermectins) were widely used for the control of ticks. Hematophagous diptera, such as hornflies (*Haematobia irritans*) and horse-flies (*Tabanus* spp.) were observed on almost all of the farms under study. Needles were reused on the farms but not during the experiments and accurate physical examinations were not performed. While Farms A and S presented clinical suspicion of anaplasmosis in 8 month old calves, Farm P presented clinical signs of bovine anaplasmosis in calves of 4–6 months, but without laboratory confirmation.

2.3. Hematocrit and blood smear analyses

The hematocrit was determined by the microhematocrit technique, which considers bovine animals showing a HT between 24% and 46% as non-anemic, and those showing a HT lower than 24% as anemic (Douglas and Wardrop, 2010).

The search for *A. marginale* corpuscles was performed by reading homogenous fields in Giemsa-stained blood smears (May-Grunwald-Giemsa e Wright Giemsa) (Sigma-Aldrich®, St. Louis, Missouri, U.S.A.). At least 40 fields (approximately 200 red cells per field) were evaluated microscopically (Olympus BX40 with 100× immersion objective) and the result was expressed as a % of cells carrying *A. marginale* (Ribeiro and Reis, 1981).

2.4. Indirect Immunoenzymatic Assay (iELISA) for the detection of anti-A. marginale IgG antibodies

The indirect ELISA technique (iELISA) was used for the detection of anti-*A. marginale* IgG antibodies, according to protocol established by Machado et al. (1997) for *B. bovis* and adapted to *A. marginale* by Andrade et al. (2004). The total antigen of *A. marginale* used was produced in the Laboratory of Immunoparasitology of the FCAV/UNESP Jaboticabal, in an optimal concentration with 10 µg / mL, diluted in 0.5 M bicarbonate carbonate buffer and at a pH 9.6. After incubation for 12 h at 4 °C, blocking was carried out with PBS Tween 20 (pH 7.2), and 6% skim milk powder was added (Molico®, Nestlé, Brazil). Plates (Maxisorp®; Nunc, Thermo Scientific, Brazil) were incubated for 90 min at 37 °C in a humid chamber. After three washes with PBS-Tween 20 buffer, the positive and the negative reference sera, as well as the test sera, were added to the ELISA plate, which were diluted at 1: 400 in PBS-Tween 20 solution plus 5% normal rabbit serum. The plates were again incubated at 37 °C for 90 min. After three washes with PBS-Tween 20 buffer, bovine anti-IgG (Sigma®, St. Louis, USA) alkaline phosphatase conjugate was added to the ELISA plate at the dilution of 1: 30,000 in PBS-added Tween 20 of 5% normal rabbit serum, with subsequent incubation and washing. Finally, the alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma®, St. Louis, MO, USA), was diluted at 1 mg/mL in diethanolamine buffer pH 9.8 (Sigma®, St. Louis, USA). 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. Serum samples from calves that did not ingest colostrum, as well as those calves that did ingest colostrum, but were negative in both serology and qPCR for *A. marginale,* were used as negative controls in ELISA. The cut-off point was calculated as 2.5 times the mean absorbance of the control-negative sera (Machado et al., 1997).

2.5. DNA extraction

DNA from bovine whole blood samples was extracted following a protocol previously described by Kuramae-Izioka (1997). The DNA samples were analyzed spectrophotometrically (NanoDrop, Thermo Scientific®, San Jose, CA, USA) for concentration and 260/280 and 260/230 ratios and then stored at −20 °C until the beginning of the PCR experiments.

2.6. Amplification reaction for the endogenous glyceraldehyde-3-phosphate dehydrogenase gene (gapdh)

To verify the presence of inhibitors in the DNA samples, a PCR assay was performed for the endogenous mammal-*gapdh* gene, following the protocol established by Birkenheuer et al. (2003).

2.7. Quantitative real-time PCR (qPCR) for A. marginale (msp1β gene)

Positive samples in the conventional PCR based on the *gapdh* gene were submitted to quantitative real-time PCR (qPCR) assay as described by Carelli et al. (2007), with modifications for the *msp1β* gene. The reaction had a final total volume of 10 µL, containing a mixture of 1 µL of sample DNA, 0.9 µM of each primer oligonucleotide AM–F (5′-TTG GCAAGGCAGCAGCTT-'3) and AM – R (5′-TTCCGCGAGCATGTTGCAT-3), 0.2 µM of the hydrolysis probe AM – probe (6FAM-5′-TCGGTCTA ACATCTCCAGGCTTTCAT-3′-BHQ1) (Integrated DNA Technologies®, Cedar Rapids, EUA), 5 µL of PCR buffer (GoTaq® qPCR Master Mix, Promega®, Madison, Wisconsin, United States) and sterilized ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) q.s.p. Amplification reactions were conducted on multiplate low-profile non-skirted PCR plates (BioRad®, Hercules, California, USA). Cycles were performed under the following conditions: 95 °C for 10 min and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification reactions were conducted on a CFX96 thermal cycler (BioRad®, Hercules, CA, USA). All samples were tested in duplicate. The plasmid pSMART (Integrated DNA Technologies®, Coralville, Iowa, USA) containing the 95bp target sequence for *A. marginale* DNA (*msp1β* gene) amplification was used for the quantification of the number of copies of target DNA / µL.

The efficiency of the reaction was calculated by serial dilutions (1:10) from 10^7 to 10° performed in order to construct a standard curve with different concentrations of plasmid DNA containing the target sequence (2.0×10^7 copies / µL to $2.0 \times 10^{\circ}$ copies / µL). Plasmid copy number was determined according to the formula (Xg /µL DNA / [size of the plasmid (pb) x 660]) x 6.02×10^{23} x copies of the plasmid /µL.

In addition to UltraPure sterile water (Thermofisher Scientific®, Carlsbad, California, USA), DNA extracted from blood samples of calves maintained in tick-free barns in the Departament of Veterinary Pathology at UNESP Jaboticabal and that showed to be negative for *A. marginale* in both serology and qPCR was also used as a negative control in qPCR assays. An aliquot of *A. marginale* DNA (Jaboticabal strain) was used as a positive control in qPCR assays.

2.8. Semi-nested (sn) PCR for the msp1α gene of A. marginale

The qPCR positive samples for the *msp1β* gene of *A. marginale* were submitted to the semi-nested PCR for amplification of the *msp1α* gene fragment, using the protocols described by de la Fuente et al. (2001) and Lew et al. (2002), with modifications. The first reaction was conducted with a final volume of 25 µL of the mixture containing 2.5 µL of genomic DNA, 12.5 µL of Master Mix PCR (Qiagen®, Madison, USA), 9 µL of sterile ultrapure water (Qiagen®, Madison, USA) and 0.5 µM of each of the primers 1733 F- (5′-TGTGCTTATGGCAGACATTTCC-3′) and 3134R1- (5′-TCACGGTCAAAACCTTTGCTTACC-3′) (Integrated DNA Technologies®, Cedar Rapids, USA). Subsequently, in the second reaction, a final volume of 25 µL of the mixture was used, containing 1 µL of the amplified product in the first reaction, 12.5 µL Master Mix PCR (Qiagen®, Madison, USA), 10.5 µL water (Qiagen®, Madison, USA) and 0.5 µM of each primer 1733 F- (5′-TGTGCTTATGGCAGACATTTCC-3′) and 2957R2-(5′-AAACCTTGTAGCCCCAACTTATCC-3′) (Integrated DNA Technologies®, Cedar Rapids, USA). The amplification reactions were

conducted according to thermal sequence: initial denaturation at 94 °C for 5 min, 40 cycles at 94 °C for 30 s, 63 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 2 min. In the second amplification cycle, the thermal sequence consisted of initial denaturation at 94 °C for 5 min, 40 cycles at 94 °C for 30 s, 65 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 2 min. The amplified products were subjected to horizontal electrophoresis in 1.0% agarose gel stained with ethidium bromide (0.625 µL / mL) in TEB running buffer pH 8.0 (44.58 M Tris-base; 44 M boric acid; 12.49 mM EDTA). The process was carried out at 100 V/350 mA for 50 min A 100bp molecular weight marker (Thermo Scientific®, San Jose, CA, USA) was used to determine the bp (bp) size of the amplified products. The results were visualized and analyzed through an ultraviolet light transilluminator coupled to a computer image analysis program (ChemiDoc Imaging System, BioRad®, Hercules, California, USA).

2.9. Purification and sequencing of amplified products

The amplified products were purified with a Silica Bead DNA Gel Extraction Kit (Thermo Scientific®, San Jose, CA, USA), according to the manufacturer's recommendations. Quantification of the purified material was performed on a NanoDrop 2000c spectrophotometer apparatus (Thermo Scientific®, San Jose, CA, USA). The sequencing of the purified products was performed by means of an automated system based on the dideoxynucleotide chain termination method (Sanger et al., 1977). The process was carried out in an ABI PRISM 3700 DNA Analyzer (Applied Biosystem®, Foster City, CA) sequencer in the Department of Technology of the Faculty of Agrarian and Veterinary Sciences (FCAV / UNESP), Center for Biological Resources and Genomic Biology (CREBIO), using the same oligonucleotides used in the second reaction of *A. marginale* snPCR based on the *msp1α* gene (1733 F and 2957R2) (Integrated DNA Technologies®, Cedar Rapids, USA).

2.10. Analysis of the nucleotide sequences of A. marginale msp1α

The screening of the sequenced amplicons was performed by using the Phred/Phrap/Consed program (Ewing, 1998), which evaluates the electropherograms generated in the sequencing, observing the quality of the peaks corresponding to each sequenced base and giving a value of error probability to each of the samples. Bases with a quality above 20 were considered as reliable. Consensus sequences were also generated by Phred/Phrap/Consed. The BLASTn program (Altschul et al., 1990) was used to compare the identity of the nucleotide sequences obtained with those (GenBank) (http://www.ncbi.nlm.nih.gov/genbank) (Benson et al., 2002).

The identity (as a percentage) between the nucleotide sequences of the *msp1α* gene of *A. marginale* obtained in the present study was also investigated using the Sequence identity matrix tool in the program Bioedit v.7.0.5.3 (Thompson et al., 1994).

2.11. Classification of A. marginale genotypes and assessment of genetic diversity

The genetic diversity analysis was performed using the *msp1α* sequences of *A. marginale* obtained in the present study and those available in GenBank (Cabezas-Cruz et al., 2013). A microsatellite was located in the *msp1α* gene between the putative Shine-Dalgarno sequence (GTAGG) and the translated initiation codon (ATG) (de la Fuente et al., 2001). The structure of microsatellites was determined by GTAGG (G/A TTT) m (GT) n ATG (Estrada-Peña et al., 2009). The analysis of the genotypes was performed according to the nomenclature proposed by de la Fuente et al. (2007). The SD-ATG distance was calculated according to the formula (4 x m) x (2 x n) x 1, described by Estrada-Peña et al. (2009).

The RepeatAnalyzer software was used to identify, manage and analyze tandem repeats of *A. marginale msp1α* (Catanese et al., 2016). Genetic diversity was calculated by metric indices that measure the percentage of single repeats in a region and also the regularity with which repeats are distributed. The program was also used to calculate the frequency of each short-sequence repeats (SSRs) in the geographical region under study (by the number of genotypes) and to list the sequences that were unique to the area under study (Catanese et al., 2016; Hove et al., 2018).

The genetic diversity is calculated in several different ways that were grouped into two categories. The first category of metrics (quantity-centric) measures the percentage of unique repeats in a region, while the second (distribution-centric) measures the regularity with which the repeats are distributed. The metric GD2, a variant of the quantity-centric category, corresponds to the number of unique SSRs present in a region divided by the number of identified strains. GD2b, that can be calculated with only genotype data, is defined as the number of unique SSRs divided by the number of genotypes. GDM1, a measure of distribution-centric diversity, measures the amount of unique repeats in a region, but unlike GD2 and GD2b, is unaffected by the length of genotypes in the region. While GDM2 measures how uniformly the repeat occurrence in a region are distributed. GDM1 and GDM2 come in two variants, local and global, depending on whether the metric is calculated as an average of the values for each genotype or over the entire region, respectively (Catanese et al., 2016).

2.12. Statistical analysis

The results obtained in the iELISA and qPCR for the cow and calf, as well as the frequency of positive animals per farm, were analyzed by the chi-square test (X^2). The mean value of *A. marginale msp1β* quantification estimated by qPCR was analyzed by the Wilcoxon-Mann-Whitney test, using The R Project for Statistical Computing software (R version 3.4.4). Values of $P < 0.05$ were considered as statistically significant. The general agreement index between the iELISA and qPCR tests was estimated by the kappa coefficient (Landis and Koch, 1977). The correlation between the magnitude of *A. marginale msp1β* quantification was estimated by qPCR and the HT was evaluated by the Pearson correlation index (r).

3. Results

3.1. Frequency of IgG antibodies to A. marginale

In the iELISA test for IgG antibodies to *A. marginale*, 72.2% of the samples were positive, with a mean cutoff point of 0.190. Of the 289 seropositive animals, 53.3% were cows and 46.7% calves. In the frequency of seropositivity analysis by animal age, the cows presented more seropositive animals (77%) when compared to the calves (67.5%) ($P < 0.05$; $X^2 = 0{,}044$) (Table 2).

4. Molecular testing

4.1. Frequency of positive animals for A. marginale in qPCR (msp1β) and snPCR (msp1α) assays

All 400 DNA samples extracted from bovine blood were positive according to the cPCR for the endogenous gene (*gapdh*). Among these, 56.7% were positive according to the qPCR for *A. marginale* based on the *msp1β* gene. Of the 227 positive samples, 39.2% corresponded to

Table 2
Frequency of seropositive beef cattle for *A. marginale* from the Brazilian Pantanal by iELISA (Chi-square test), according to the animal studied (Cow/Calves) and property of origin.

Property	Cows (n)***	%	Calves (n)***	%	Total	% General
Farm A	(44/45)	97.8%	(49/53)	92.4%	(93/98)	94.9%(A)**
Farm S	(35/42)	83.3%	(30/41)	73.2%	(65/83)	78.3%(B)**
Farm P	(13/39)	33.3%	(2/41)	4.9%	(15/80)	18.7%(C)
Farm N	(30/38)	78.9%	(24/34)	70.6%	(54/72)	75%(B)**
Farm C	(32/36)	88.9%	(30/31)	96.8%	(62/67)	92.5%(A)** (B) **

*Different lowercase letters on the same line represent significant difference ($P < 0.05$),

** Different capital letters in the same column represent significant difference ($P < 0.05$).

*** (n) for number of positive animals / number of animals sampled per property.

cows and 60.8% to calves. The number of positive calves (69%) was statistically higher when compared to that of cows (44.5%) ($P < 0.05$; $X^2 = 1.27 \times 10^{-6}$) (Table 3). The mean copy-number quantification of a 95 pb fragment of the *A. marginale msp1β* gene per µL of DNA was 3.9×10^4, with a mean Cq of 32.60 for cows. As for calves, the mean quantification was significantly higher, 1.3×10^6 with a mean Cq of 30.27 ($P < 0.05$; 1.12×10^{-8}). While the HT (%) of the cows ranged from 22% to 50%, that of calves ranged from 26% to 48%. The correlation between the magnitude of quantification estimated by qPCR and the hematocrit, evaluated by the Pearson correlation index (r), was -0.0032, which is considered statistically non-existent. The kappa coefficient between the iELISA and qPCR tests presented an agreement of 0.14, which is considered low.

Table 3
Frequency of positive beef cattle in the qPCR for *A. marginale* based on the *msp1β* gene in the Brazilian Pantanal (Chi-square test), according to the animal (Cow / Calves) and property of origin.

Proprerty	Cows (n)***	%	Calves (n)***	%	Total	% Gerale
Farm A	(27/45)	60%	(53/53)	100%	(80/98)	81.6%(A)
Farm S	(15/42)	35.7%	(35/41)	85.3%	(50/83)	60.2%(B)
Farm P	(13/39)	33.3%	(1/41)	2.4%	(14/80)	17.5%(C)
Farm N	(17/38)	44.7%	(21/34)	61.7%	(38/72)	52.7%(B)
Farm C	(17/36)	47.2%	(28/31)	90.3%	(45/67)	67.1%(B)
Total	**(89/200)**	**44.5%(a)**	**(138/200)**	**69%(b)**	**(227/400)**	**56.7%**

*Different lowercase letters on the same line represent significant difference ($P < 0.05$),

**Different capital letters in the same column represent significant difference ($P < 0.05$).

*** (n) for number of positive animals / number of animals sampled per property.

Of the 227 samples that were positive for *A. marginale* in the qPCR directed to the *msp1β* gene, 22.9% were positive in the snPCR based on the *msp1α* gene, of which 9.7% corresponded to the cows and 13.2% to the calves (Table 4). Based on the intensity of the bands obtained in agarose gel electrophoresis, only 50% of the amplicons obtained were submitted to sequencing.

4.2. Analysis of nucleotide identity of the msp1α gene of A. marginale

The nucleotide identity between the *A. marginale msp1α* gene sequences obtained in the present study showed a variation between 48% and 100%. When comparing the 26 *A. marginale msp1α* sequences detected in the present study with 24 retrieved from GenBank by BLASTn, an identity range of 47%–100% was observed.

4.3. Genetic diversity of A. marginale based on the msp1α gene

Using the RepeatAnalyzer software for the alphanumeric genotype classification and identification of strains, 14 strains were found, with six strains already reported in the literature (α-β-β-β-Γ; α-β-Γ; 27-27; 42-25-31; τ-22-2-13-18; τ-22-2-13) and eight never previously described (τ-10-13-13-18; τ-27-18; EV8-EV8-17; α-β-β-β-100; EV7-11-10-15; τ-11-11-27-18; τ-11-10-15; τ-27-13-18) (Table 5). The eight new strains (τ-10-13-13-18; τ-27-18; EV8-EV8-17; α-β-β-β-100; EV7-11-10-15; τ-11-11-27-18; τ-11-10-15; τ-27-13-18) were detected in cattle from Farm A (Table 6; Fig. 2).

The metric indexes of genetic diversity, GDM1- local[a] (0.854) and GDM1-Global[a] (0.339), obtained in the Brazilian Pantanal revealed high and moderate values, indicating that the repeats (SSRs) were very diverse among themselves and also within the region studied. The GDM2-Local[a] (0.051) and GDM2-Global[a] (0.037) indices revealed low values, that is, they occurred approximately the same number of times and that the distribution in the region under study was uniform (Table 7).

Values were low for both GDM2 metrics, indicating that SSRs are sparse. Comparing with studies conducted in other Brazilian states with dairy cattle in São Paulo, Minas Gerais, Paraná, Rio de Janeiro, Goiás and buffalo in Pará, the GDM1 index also presented values close to or equal to (1) (Paraná), indicating that the majority of SSRs are not equal and thus the presence of high diversity. The distribution of the repeats in these Brazilian states was also uniform, since GDM2 presented a value closer to zero. This result was similar when compared in general for Brazil and the world. The indices showed different strains in each region and little dissemination (Table 7).

The repetition 18 and τ occurred the largest number of times with five and seven repetitions, respectively, and thus being the most widespread (Fig. 3). The ratio of the number of repeats to the number of amino acids revealed that an SSR was between 22–24 amino acids in size; two SSRs had a size of 28 amino acids and two more SSRs had between 30–32 amino acids. The highest number of repeats (12–14)

Table 4
Co-positivity and co-negativity of blood samples from Brazilian Pantanal sampled cows in the serological (iELISA) and molecular (qPCR) techniques used to diagnose *A. marginale* according to the age of animals (cows / calves).

		Cows				Calves			
		qPCR							
		Positive	Negative	Total		Positive	Negative	Total	General
iELISA	**Positive**	73 (36.5%)	81 (40.5%)	154 (77%)	**Positive**	117 (58.5%)	18 (9%)	135(67.5%)	289 (72.2%)
	Negative	16 (8%)	30 (15%)	46 (23%)	**Negative**	21 (10.5%)	44 (22%)	65 (32.5%)	111 (27.7%)
		89 (44.5%)	111 (55.5%)	200 (100%)		138 (69%)	62 (31%)	200 (100%)	400 (100%)

Table 5
Characterization of the genotypes and strains of the 26 samples positive for the *msp1α* gene of *A. marginale*, with identification of the animals, property of origin and geographical distribution according to the database of the RepeatAnalyzer.

Samples number	Identification	Property	Genotypes (Estrada-Peña et al., 2009)	Strains (Catanese et al., 2016)	Distribuition of strains (Brazil/world)
MS1	cow 3	Farm A	E	τ 22-2 13	Argentina (Chaco 2 var 1; Chaco3 var1; Chaco7; Chaco 8); Brazil, Mato Grosso do Sul
MS2	cow 8	Farm A	C	τ 10 13 13 18	Brazil, Mato Grosso do Sul
MS3	cow 10	Farm A	C	τ 10 13 13 18	Brazil, Mato Grosso do Sul
MS4	cow 13	Farm A	E	τ 27 18	Brazil, Mato Grosso do Sul
MS5	cow 22	Farm A	E	α β β β Γ	Argentina (Santa fe 37,43,50,59) / Mexico, Nayarit, Santiago Ixcuintla/ Mexico, Jalisco Tapalpa/ Brazil, São Paulo/Brazil, Goiás/ Brazil, Mato Grosso do Sul
MS6	cow 24	Farm A	E	τ 27 18	Brazil, Mato Grosso do Sul
MS7	cow 26	Farm A	E	EV8 EV8 17	Brazil, Mato Grosso do Sul
MS8	cow 32	Farm A	E	α β β β 100	Brazil, Mato Grosso do Sul
MS9	cow 33	Farm A	E	27 27	China (AM3-27a); Brazil, Minas Gerais (UFMG2); Minas Gerais (Minas-5); Minas Gerais (13,273); Brazil, Mato Grosso do Sul
MS10	cow 37	Farm A	E	42 25 31	Madagascar, Sofia; Brazil, Mato Grosso do Sul
MS11	cow 31	Farm A	E	EV7 11 10 15	Brazil, Mato Grosso do Sul
MS12	cow 20	Farm A	E	EV7 11 10 15	Brazil, Mato Grosso do Sul
MS13	cow 126	Farm P	E	27 27	China (AM3-27a); Brazil, Minas Gerais (UFMG2); Minas Gerais (Minas-5); Minas Gerais (13,273);Brazil, Mato Grosso do Sul
MS14	cow 94	Farm S	E	τ 22-2 13 18	Argentina (Chaco 2 var 1; Chaco3 var1; Chaco7; Chaco 8));Brazil, Mato Grosso do Sul
MS15	calf 1	Farm A	E	27 27	China (AM3-27a); Brazil, Minas Gerais (UFMG2); Minas Gerais (Minas-5); Minas Gerais (13,273);Brazil, Mato Grosso do Sul
MS16	calf 2	Farm A	E	τ 27 13 18	Brazil, Mato Grosso do Sul
MS17	calf 3	Farm A	B	τ 11 11 27 18	Brazil, Mato Grosso do Sul
MS18	calf 5	Farm A	E	α β β β Γ	Argentina (Santa fe 37,43,50,59)/ Mexico, Nayarit, Santiago Ixcuintla/ Mexico, Jalisco Tapalpa/ Brazil, São Paulo/Brazil, Goiás/ Brazil, Mato Grosso do Sul
MS19	calf 8	Farm A	E	α β β β Γ	Argentina (Santa fe 37,43,50,59) / Mexico, Nayarit, Santiago Ixcuintla/ Mexico, Jalisco Tapalpa/ Brazil, São Paulo/Brazil, Goiás/ Brazil, Mato Grosso do Sul
MS20	calf10	Farm A	C	τ 10 13 13 18	Brazil, Mato Grosso do Sul
MS21	calf 13	Farm A	E	α β β β Γ	Argentina (Santa fe 37,43,50,59) / Mexico, Nayarit, Santiago Ixcuintla/ Mexico, Jalisco Tapalpa/ Brasil, São Paulo/Brazil, Goiás/ Brazil, Mato Grosso do Sul
MS22	calf 18	Farm A	B	τ 11 11 27 18	Brazil, Mato Grosso do Sul
MS23	calf 21	Farm A	C	α β β β Γ	Argentina (Santa fe 37,43,50,59) / Mexico, Nayarit, Santiago Ixcuintla/ Mexico, Jalisco Tapalpa/ Brasil, São Paulo/Brazil, Goiás/ Brazil, Mato Grosso do Sul
MS24	calf 22	Farm A	E	EV8 EV8 17	Brazil, Mato Grosso do Sul
MS25	calf 23	Farm A	E	τ 11 10 15	Brazil, Mato Grosso do Sul
MS26	calf 28	Farm A	E	α β Γ	Argentina (Chaco 2 var 2; Mexico, Nayarit Santiago Ixcuintla); Brazil, Mato Grosso do Sul

Table 6
Organization of the eight new *msp1α* strains of *A. marginale*, with their genotypes, absolute quantification and positive bovine identification of Farm A, Brazilian Pantanal.

Samples	Animals	New strains (Catanese et al., 2016)	Quantity	Genotypes (Estrada-Peña et al., 2009)	Absolute quantification by qPCR (*msp1β*/ µL)
MS20	calf 10	**τ-10-13-13-18**	3	**C**	PM*
MS2	cow 8				1.97×10^5
MS3	cow 10				2.20×10^6
MS4	cow 13	**τ-27-18**	2	**E**	2.77×10^3
MS6	cow 24				1.70×10^3
MS7	cow 26	**EV8-EV8-17**	2	**E**	3.45×10^3
MS24	calf 22				1.76×10^3
MS8	cow 32	**α-β-β-β-100**	1	**E**	9.85×10^3
MS1	cow 31	**EV7-11-10-15**	2	**E**	7.11×10^3
MS12	cow 20				1.45×10^3
MS17	calf 3	**τ-11-11-27-18**	2	**B**	9.53×10^5
MS22	calf 18				1.30×10^6
MS25	calf 23	**τ-11-10-15**	1	**E**	PM*
MS16	calf 2	**τ -27-13-18**	1	**E**	1.86×10^5

PM * Positive, but due to the Monte Carlo effect, it was not possible to quantify it absolutely.

presented a size of between 28 and 30 amino acids (Fig. 4). The correlation between the number of genotypes and the number of SSRs found revealed that one genotype presented two repeats; five genotypes presented three repeats; four genotypes presented four repeats, and four other genotypes presented five repeats (Fig. 5).

5. Discussion

The results obtained in the iELISA showed a high frequency of seropositive animals (72.2%) to *Anaplasma* spp. Although *A. marginale* has been the only pathogen belonging to *Anaplasma* genus confirmed as causing diseases in cattle in Brazil so far, *Anaplasma* spp. closely related to *A. phagocytophilum*, *A. bovis*, and *A. platys* has been detected in Brazilian marsh deer (*Blastocerus dichotomus*) in the Pantanal (Sacchi et al., 2012). Therefore, we can not rule out the possibility of serological cross-reactivity in crude-antigen based ELISA technique, since these newly described *Anaplasma* genotypes may also infect domestic ruminants in Brazil.

In the analysis of the frequency of seropositive animals, the cows presented higher seropositivity when compared to the calves. In the sampled farms, cows spend a large part of their lives within the herd and breeding, with increased exposure to the pathogen and, consequently, high levels of antibodies to *A. marginale* (Palmer et al., 1999; Vidotto et al., 2004). When studying beef cattle, Grau et al. (2013) found that cows persistently infected with *A. marginale* and without a history of acute anaplasmosis could infect their offspring.

In the analysis of qPCR results by animal age, a greater number of positive calves (69%) was found when compared to cows (44.5%). A higher quantity of *A. marginale msp1β* was also found in calves (1.3×10^6) when compared to cows ($3.\ 9 \times 10^4$). This finding may be due to the developing of the calves' immature immune system. According to Palmer et al. (1999), cows would be more predisposed to persistent infections, and may act as a source of infection for mechanical (flies and horse-flies) and biological vectors (Ixodidae ticks). These animals can also spread *A. marginale* from generation to generation through the transplacental route, although confirmation of this mode of transmission in the present study has not been done.

The correlation between the magnitude of *A. marginale msp1β* quantification estimated by qPCR and the HT was considered statistically non-existent, since the animals showed no clinical signs or anemia, which may suggest that the animals have some resistance to the pathogen. Bilhassi et al. (2014) investigated the influence of race in a study comparing *B. bovis* infection in Nelore, Angus and crossbred bovines from two endemic areas for babesiosis in the state of São Paulo, Southeastern Brazil. The prevalence of infection showed no significant difference, which were 96%, 100% and 98% for the three genetic groups, respectively. However, when they verified parasitemic levels estimated by qPCR, they were found to be higher in Angus animals and crossbred cattle than in Nellore animals. Future studies aiming to investigate the influence of breed on susceptibility to bovine anaplasmosis are necessary.

Analyzing the 26 *msp1α* sequences of *A. marginale* obtained in the present study, the genotype E (76.9%) was more prevalent, followed by C (15.4%) and B (7.7%) genotypes. The results are similar to those reported in crossbred cattle (Silva et al., 2015) and water buffalo and the associated ticks (*R. microplus* and *A. sculptum*) (Silva et al., 2014b) in Rio de Janeiro. Even though there was a predominance of genotype E in all studies that have been performed in Brazil so far, the genotypes B and C, previously reported only once in Brazil, were also identified in the present study. This result corroborates Estrada-Peña et al. (2009), which observed that in South America, especially in Brazil and Argentina, genotype E was the most frequently found. The prevalence of

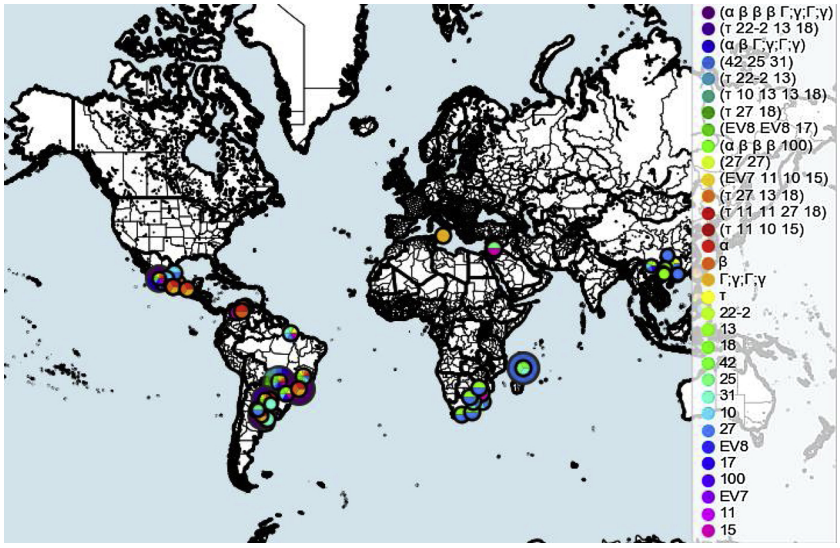

Fig. 2. Geographic visualization of repeats. The distribution of the 14 strains of *A. marginale* (*msp1α* gene) (**τ-10-13-13-18; τ-27-18; EV8-EV8-17; α-β-β-β-100; EV7-11-10-15; τ-11-11-27-18; τ-11-10-15; α-β-β-β-Γ; α-β-Γ; 27-27; 42-25-31; τ-27-13-18; τ-22-2-13-18; τ-22-2-13**) found in beef cattle in the Brazilian Pantanal. All results were generated in the RepeatAnalyzer software. The circles represent the geographic visualization of repeats. The size of a circle indicates the scope of the region it denotes. The larger the circle is, the broader the scope. While countries have the largest circles, markers for a specific county are the smallest ones.

Table 7
Metric indices of genetic diversity of *A. marginale msp1α* gene found in beef cattle in the Brazilian Pantanal compared to other Brazilian states (São Paulo, Minas Gerais, Pará), buffalo (Rio de Janeiro and Goiás), as well as Brazil (general) and world (general), according to the Software RepeatAnalyzer.

Metric	Pantanal brasileiro – MS (Souza Ramos et al., 2018)	São Paulo (Taiaçu) (Silva et al., 2016)	São Paulo (Lins) (Machado et al., 2015)	Minas Gerais (Pohl et al., 2013)	Minas Gerais (De la Fuente et al., 2004)	Pará-Marajó (Silva et al., 2014a)	Paraná (Vidotto et al., 2016)	Rio de Janeiro (Silva et al., 2014b)	RJ (Baêta et al. (2015)	Goiás (Machado et al., 2015)	Brazil (General)	World
GDM1-Local[a]	**0.854**	0.802	0.8	0.933	0.817	0.75	1	0.792	0.8	0.675	0.828	0.757
GDM1-Global[a]	**0.339**	0.3	0.75	0.909	0.375	0.75	1	0.56	0.71	0.333	0.221	0.154
GDM2-Local[a]	**0.051**	0.093	0.094	0.028	0.086	0.117	0	0.097	0.094	0.153	0.075	0.089
GDM2-Global[a]	**0.037**	0.132	0.093	0.027	0.037	0.117	0	0.05	0.11	0.157	0.034	0.008

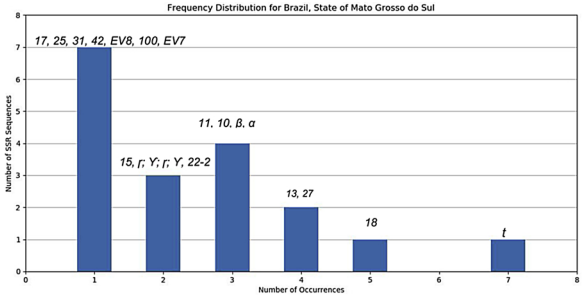

Fig. 3. Number of occurrence of each tandem repeat found in the *msp1α* gene of *A. marginale* strains in beef cattle in the Brazilian Pantanal. The **T** repeat was more diffused (seven repeats) than the others, followed by **18** (five replications).

genotype E when compared to the other genotypes may suggest a better adaptation of this genotype to cattle and ticks in Brazil. Estrada-Peña et al. (2009) also reported that the SD-ATG distance of the 23-nucleotide associated with E genotype, which has been correlated with high levels of expression of the *msp1α* protein, could be associated with a higher infectivity potential of the isolates with this tandem repeats.

In the present study, six detected strains have already been reported in the world, namely: α-β-β-β-Γ; α-β-Γ; 27-27; 42-25-31; τ-22-2-13-18; τ-22-2-13. For instance, Poohl et al. (2013) reported the strain 27-27 in cattle in the state of Minas Gerais, southeastern Brazil. On the other hand, the strain α-β-β-β-Γ has been reported in dairy cattle in the states of São Paulo (Lins and Taiaçu) (Machado et al., 2015; Silva et al., 2016), Goiás, Mambaí (Machado et al., 2015) and Rio de Janeiro (Silva et al., 2014c). The most widespread *A. marginale* strain in Brazil, namely α-β-β-β-Γ, was also the most prevalent in the present study (19.2%; 5/26), corresponding to four samples belonging to genotype E and one to genotype C. Although clinical bovine anaplasmosis was not observed in the present study, the presence of the α-β-β-β-β strain has been associated with anaplasmosis outbreaks and clinical anaplasmosis in dairy cattle in Mexico (Almazán et al., 2008) and Brazil (Silva et al., 2015). This strain has been associated with a high infestation by the *R. microplus* tick in South America (Scoles et al., 2005; de la Fuente et al., 2007).

Eight new strains, which had never been previously described, were reported in the present study (τ-10-13-13-18; τ-27-18; EV8-EV8-17; α-β-β-β-100; EV7-11-10-15; τ-11-11-27-18; τ-11-10-15; τ-27-13-18). de la Fuente et al. (2001) stated that the occurrence of independent transmission mechanisms (mechanical and biological) could reflect in high genetic diversity in endemic areas. This hypothesis has been confirmed in Brazilian endemic regions by Silva et al. (2015), which found animals infected with multiple strains of *A. marginale*.

Analyzing the GDM1 indexes of genetic diversity by the RepeatAnalyzer, a high diversity was verified among the genotypes/strains analyzed, as well as in the region in which the present study was conducted, indicating the circulation of new genotypes/strains in the country. The low values of the GDM2 indices revealed a low dispersion rate of these new strains. In a similar study conducted in South Africa, Zamantungwa et al. (2016) found that the increase in the number of new circulating genotypes/strains would indicate a greater circulation of these in nature by the action of selection pressure and consequent mutation. Comparing the results of the present study with those previously performed in other Brazilian states, a high degree of genetic diversity and low dispersion of genotypes/strains were also observed.

In endemic areas for bovine anaplasmosis in the USA, Palmer et al. (2001) showed that persistently infected cattle and ticks harbor genetically heterogeneous isolates of *A. marginale*, suggesting that different strains are maintained during their life or biological cycle, respectively. In addition, de la Fuente et al. (2001) reported that genetic heterogeneity of *A. marginale*, which results in high diversity, can

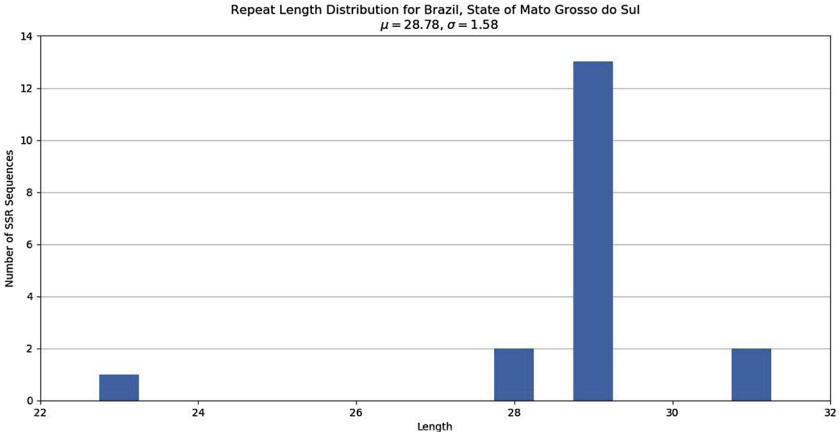

Fig. 4. Number of tandem repeats of the *msp1α* gene and their size (number of amino acids) found in strains of *A. marginale* in beef cattle in the Brazilian Pantanal. Most SSRs were between **28** and **30** amino acids in size.

Fig. 5. Correlation between the number of genotypes and the number of SSR found in strains of *A. marginale* in beef cattle in the Brazilian Pantanal. Five genotypes presented three repeats and four genotypes five repeats.

derive from different transmission mechanisms, each introducing different genotypes and strains into the bovine herd. The sale of male calves to other regions of Brazil and to other Latin American countries, such as Bolivia, may disperse the strains of *A. marginale* found in the present study. According to de la Fuente et al. (2007), cattle transfers between different geographic regions represent an important source of dispersion of different isolates of *A. marginale*, which also contributes to genetic diversity.

6. Conclusion

Beef cattle are highly exposed to *A. marginale* in the Brazilian Pantanal. Moreover, a high genetic diversity of *A. marginale,* with eight new strains, was found in the studied region.

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