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1 **Low genetic diversity associated with low prevalence of *Anaplasma marginale* in**
2 **water buffaloes in Marajó Island, Brazil**

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26 **Abstract**

27 The rickettsia *Anaplasma marginale* is the etiologic agent of bovine anaplasmosis, an
28 important tick-borne disease affecting cattle in tropical and subtropical regions of the
29 world. In endemic regions, the genetic diversity of this pathogen is usually related to the
30 high prevalence of the disease in cattle. The major surface protein 1 alpha (MSP1a) has
31 been used as a marker to characterize the genetic diversity and for geographical
32 identification of *A. marginale* strains. The present study reports the characterization of
33 *A. marginale* MSP1a diversity in water buffaloes. Blood samples were collected from
34 200 water buffaloes on Marajó Island, Brazil where the largest buffalo herd is located in
35 the Western hemisphere. Fifteen buffaloes (7.5%) were positive for *A. marginale msp1α*
36 by PCR. Four different strains of *A. marginale* with MSP1a tandem repeat structures (4-
37 63-27), (162-63-27), (78-24-24-25-31) and (τ-10-10-15) were found, being (4-63-27)
38 the most common. MSP1a tandem repeats composition in buffalos and phylogenetic
39 analysis using *msp1α* gene showed that the *A. marginale* strains identified in buffaloes
40 are closely related to *A. marginale* strains from cattle. The results demonstrated low
41 genetic diversity of *A. marginale* associated with low bacterial prevalence in buffaloes
42 and suggested that buffaloes may be reservoirs of this pathogen for cattle living in the
43 same area. The results also suggested that mechanical transmission and not biological
44 transmission by ticks might be playing the major role for pathogen circulation among
45 water buffaloes in Marajó Island, Brazil.

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47 **Keywords:** *Anaplasma marginale,* Buffalo, MSP1a, genetics, anaplasmosis

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49 **Introduction**

50 *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) is the most prevalent pathogen
51 transmitted by ticks worldwide, distributed on the six continents and responsible for
52 high morbidity and mortality in cattle in temperate, subtropical, and tropical regions
53 (Vidotto et al., 1998; Kocan et al., 2010). Bacteria of the genus *Anaplasma* are obligate
54 intracellular pathogens that can be transmitted biologically by ticks, mechanically by
55 hematophagous insects and blood-contaminated fomites and less frequently
56 transplacentally (Kocan et al., 2010).

57 The global distribution and high pathogenicity of *A. marginale* is due to the diversity
58 and genetic variability of this bacterium (de la Fuente et al., 2007). This pathogen has
59 over 20 proteins capable of inducing protective immunity (Suarez and Noh, 2011) from
60 which major surface proteins (MSPs) have been extensively characterized (Kocan et al.,
61 2010). Among the major surface proteins (MSPs), special attention has been directed to
62 MSP1a because it is involved in the interaction of the bacterium with vertebrate and
63 invertebrate host cells (de la Fuente et al., 2010). Several strains of *A. marginale* have
64 been identified worldwide and these strains differ in their morphology, MSP1a amino
65 acid sequence, antigenic characteristics, and ability to be transmitted by ticks (de la
66 Fuente et al., 2007; Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013).

67 The primary host for *A. marginale* is cattle, but other ruminants such as deer and
68 buffaloes can also be infected (Kocan et al., 2010). Approximately 300,000 buffaloes
69 are geographically isolated on Marajó Island, Brazil, representing the largest buffalo
70 herd in the Western hemisphere, and these animals have been used as a primary source
71 of meat, milk, and leather, besides being used to plow the land and to transport people
72 and crops (IBGE., 2012). Serological and molecular detection of *A. marginale* in water
73 buffaloes in Brazil have shown a prevalence of 49.0% and 5.4%, respectively (Silva et

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74 al., 2014). However, although the *A. marginale msp1α* genetic diversity has been
75 characterized in Brazilian cattle (de la Fuente et al., 2007; Estrada-Peña et al., 2009;
76 Cabezas-Cruz et al., 2013; Pohl et al., 2013), a similar study has not been conducted in
77 buffaloes.

78 In this study, we characterized the *A. marginale msp1α* genetic diversity in naturally
79 infected water buffaloes on Marajó Island, Brazil. The results demonstrated low genetic
80 diversity of *A. marginale* associated to low prevalence of the bacteria in water buffaloes
81 and suggested that buffaloes may be a reservoir of this pathogen for cattle living in the
82 same area. The results also suggested that mechanical transmission and not biological
83 transmission by ticks might be playing an essential role for pathogen circulation among
84 water buffaloes in Marajó Island, Brazil.

85 **Materials and methods**

86 *Experimental design and study site*

87 A cross-sectional molecular study was conducted sampling buffalo herds in four
88 provinces of Marajó Island, Brazil (Soure, Salvaterra, Muaná, and Chaves) between
89 January and December 2012. The Marajó Island hosts the largest water buffalo
90 population in the Western hemisphere. The vegetation on this island is predominantly
91 provided by the Amazon tropical rainforest (Furtado et al., 2009). The buffaloes are
92 vaccinated against brucellosis and foot-and-mouth disease, but endo and ectoparasite
93 control is rarely used. Large areas of bog and grassland along the floodplains of rivers
94 are found on Marajó Island (Furtado et al., 2009). These animals are reared using an
95 extensive system. The main tick species found on animals are *Amblyomma cajennense*,
96 *Rhipicephalus (Boophilus) microplus*, *Dermacentor nitens* and *A. maculatum*. These
97 tick species can be found on buffaloes with low infestation rates throughout the year
98 (Silva et al., 2014).

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99 *Sample collection and DNA extraction*

100 Two hundred female water buffaloes with approximately 3 years of age were randomly
101 selected in at least three farms from each province included in the study. Blood samples
102 were collected from the caudal or jugular veins of individual animals. DNA extraction
103 was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA)
104 following manufacturers recommendations.

105 *A. marginale msp1α PCR and DNA sequencing*

106 The primers 1733F (5' TGTGCTTATGGCAGACATTTCC 3'), 3134R (5'
107 TCACGGTCAAAACCTTTGCTTACC 3'), and 2957R (5'
108 AAACCTTGTAGCCCCAACTTATCC 3') were used to amplify *A. marginale msp1α*
109 as reported previously (Lew et al., 2002). Briefly, primers 1733F and 3134R were used
110 in the first PCR amplification, while 1733F and 2957R were used in a nested-PCR
111 reaction. For both reactions, 12.5 µl PCR Master Mix (Qiagen, Valencia, CA, USA), 20
112 pmol of each primer and 5 µl genomic DNA (first reaction) were used in a final volume
113 of 25 µl. For the second reaction 1 µl of the DNA amplified in the first reaction was
114 used as template. Control reactions were performed in a similar way but without DNA
115 added to it. After the PCR reaction, amplicons were purified with the Silica Bead DNA
116 Gel Extraction Kit (Fermentas Life Sciences, Sao Paulo, Brazil) following
117 manufacturer's instructions and sequenced. The *A. marginale msp1α* sequences obtained
118 in this study from water buffaloes are available in GenBank with accession numbers
119 KJ575588 - KJ575602.

120 *A. marginale msp1α sequence analysis*

121 A microsatellite is located at the *msp1α* 5' untranslated region (UTR) between the
122 putative Shine-Dalgarno (SD) sequence (GTAGG) and the start codon (ATG).
123 The general microsatellite structure is as previously reported GTAGG (**G/A TTT**)m

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124 (**GT**)n **T** ATG (Estrada-Peña et al., 2009) where microsatellite sequence is in bold
125 letters. The SD-ATG distance was calculated according to the equation $(4 \times m) + (2 \times$
126 $n) + 1$. Based on the structure of this microsatellite eleven genotypes (named with Latin
127 alphabet letters from A to K) of *A. marginale msp1α* have been previously identified
128 (Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013). Theoretical translation of *msp1α*
129 DNA into amino acid sequences was performed using the Expasy Translation Tool
130 (http://expasy.hcuge.ch/ tools/dna.html). Tandem repeats were identified and named
131 according to the nomenclature proposed by de la Fuente et al. (2007) and updated by
132 Cabezas-Cruz et al. (2013). Tandem repeat sequences were aligned using MUSCLE
133 (v3.7) (Edgar., 2004). Codon based alignment was performed using the codon suite
134 server (Schneider et al., 2007). Detection of selection pressure on individual codons was
135 calculated using two methods, single likelihood ancestor counting (SLAC) and fixed
136 effects likelihood (FEL) implemented in Datamonkey webserver (Delport et al., 2010).
137 Positive or negative selection was assigned to codons where ω=dN (non-synonymous
138 substitutions)/dS (synonymous substitutions) ratio was higher or lower than 1,
139 respectively. As recommended in Datamonkey webserver (Delport et al., 2010), only
140 sites with p-value < 0.25 were considered to be under selection.

141 *Phylogenetic analysis*

142 For *msp1α* phylogenetic analysis, nucleotide sequences were aligned with MUSCLE
143 (v3.7) configured for high precision (Edgar., 2004) followed by removal of the
144 ambiguous regions with Gblocks (v0.91b) (Castresana., 2000). The phylogenetic tree
145 was constructed using the neighbor joining method implemented in Neighbor from the
146 PHYLIP package (v3.66) (Felsenstein., 1989). Internal branch confidence was assessed
147 by the bootstrapping method using 1000 bootstrap replicates. Sequences of *A.*

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148 *marginale msp1α* previously reported in cattle from Brazil and the USA were obtained
149 from Genebank and used as outgroups.

150 **Results and discussion**

151 Low prevalence of *A. marginale* was recently reported in buffaloes in Marajó Island,
152 Brazil, using the major surface antigen 5 (*msp5*) gene marker (Silva et al., 2014). The
153 results obtained in the present study using *msp1α* agreed with those reported by Silva et
154 al. (2014) and showed 7.5% (15 positive samples) prevalence of *A. marginale* in water
155 buffaloes from Marajó Island, Brazil. This prevalence could be considered low when
156 compared with the prevalence of *A. marginale* in cattle from Brazil. For example, using
157 *msp1α*, a recent study showed 70% prevalence of *A. marginale* in a herd of Brazilian
158 cattle (Pohl et al., 2013). Water buffaloes with clinical anaplasmosis were not registered
159 in the present study. The pathogenic significance of *A. marginale* for water buffaloes
160 remains to be elucidated, but the fact that buffaloes can carry *A. marginale* raise
161 concerns regarding the role of this species as reservoirs of *A. marginale* for cattle living
162 in the same area (Silva et al., 2014). Phylogenetic analysis using *msp1α* show that *A.*
163 *marginale* strains found in buffaloes are closely related to strains isolated previously
164 from cattle in Brazil (Fig. 1A), suggesting that buffaloes can be infected with the same
165 strains that infect cattle and thus buffaloes could constitute reservoir hosts for *A.*
166 *marginale* in cattle. Further research is needed to elucidate the role of water buffaloes as
167 reservoir hosts for *A. marginale* in cattle in this or other regions where both species
168 share the same space.

169 The gene *msp1α* has been extensively used for the characterization of the genetic
170 diversity of *A. marginale* in cattle (Palmer et al., 2001; de la Fuente et al., 2007; Ruybal
171 et al., 2009; Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013; Pohl et al., 2013) but
172 little is known about the genetic diversity of *A. marginale* in other species of ungulates,

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173 including buffaloes (de la Fuente et al., 2004). In order to determine the genetic
174 diversity of *A. marginale* infecting buffaloes, we sequenced the 15 *msp1α* positive
175 samples that were obtained in the present study (Table 1). The results showed that the
176 genetic diversity of *A. marginale msp1α* in buffaloes from Marajó Island is low, with
177 only four different strains identified, showing the microsatellite genotype E (Table 1).
178 In contrast, the results by Pohl et al. (2013) in cattle showed, in 13 sequenced samples,
179 8 different strains of *A. marginale* with four different microsatellite genotypes (B, D, E
180 and G). Three possibilities could be considered in order to explain the low genetic
181 diversity of *A. marginale* in buffaloes in Marajó Island: (a) in bovine anaplasmosis
182 endemic regions, low genetic diversity of *A. marginale msp1α* has been related to tick
183 absence (Ruybal et al., 2009). Most of the sampled buffaloes in this study were raised
184 on submerged wetlands, where tick attachment is rare (Silva et al., 2014) and thus tick
185 infestation rates are low and transmission of *A. marginale* is an unlikely event; (b) cattle
186 movement has been proposed as a source of genetic diversity in *A. marginale*
187 worldwide (de la Fuente et al., 2007). In Marajó Island, the entry of new buffaloes is
188 prohibited, limiting the possibility of the introduction of new strains of *A. marginale*
189 and consequently bacterial genetic diversity in this area. This phenomenon is in
190 agreement with the fact that cattle movement is limited in Australia where only one
191 strain of *A. marginale* has so far been identified in cattle (Lew et al., 2002); (c) finally,
192 it could be argued that *A. marginale* was just recently introduced in this buffalo herd,
193 which will result in low genetic diversity. Low genetic diversity of *msp1α* was reported
194 in a previously uninfected cattle herd where only a single *msp1α* genotype was found
195 (Palmer et al., 2001).

196 Despite the low genetic diversity observed for *A. marginale* in buffaloes, evidence of
197 genetic diversification was found. The *A. marginale* strains obtained from buffaloes in

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198 this study had between 3 and 5 MSP1a repeat sequences (Table 1). Tandem repeat 162
199 was found for the first time in this study (Table 1 and Fig. 1B). Tandem repeats 162 and
200 4 only differ in one amino acid at position 27 with glutamine (Q) in tandem repeat 4 and
201 leucine (L) in tandem repeat 162 (Fig. 1B). In addition, the amino acid Q in tandem
202 repeat 4 is encoded by the codon CAA and a single mutation to uracile in the second
203 adenine of the CAA codon will result in the codon CUA which encodes for the amino
204 acid L in tandem repeat 162. This finding suggested that the tandem repeat 162 may
205 have originated recently from tandem repeat 4, providing evidence for genetic
206 diversification of *A. marginale* in water buffaloes. In agreement with this hypothesis,
207 the phylogenetic analysis using *msp1α* indicated that the strain Water buffalo 15 (162,
208 63, 27) possibly evolved from strain Water buffalo 13 (4, 63, 27) (Fig. 1A). In order to
209 determine which selective pressures could be triggering MSP1a diversification in *A.*
210 *marginale* from buffaloes, the ratio ω was calculated showing that codon at position 10
211 from tandem repeat 4 was evolving under negative selection (Fig. 1B). Interestingly,
212 this amino acid position is present in an immunodominant B-cell epitope described
213 before for *A. marginale* MSP1a (Garcia-Garcia et al., 2004) (Fig. 1B). These results
214 suggested that this tandem repeat which was present in the most common strain of *A.*
215 *marginale* found in buffaloes (Table 1) may be under selective pressure by the host
216 immune system (Garcia-Garcia et al., 2004).
217 Some of the tick species found infesting buffaloes such as *Rhipicephalus* and
218 *Dermacentor* spp. have been recognized as vectors of *A. marginale* (Kocan et al., 2010).
219 However, the low tick infestation rates found in buffaloes in the study area suggested
220 that mechanical and/or transplacental transmission could be playing an important role in
221 *A. marginale* transmission in this buffalo herd. The sucking lice, *Haematopinus*
222 *tuberculatus* was implicated recently in *A. marginale* transmission and outbreaks of this

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223 lice species have been reported in buffaloes (Da Silva et al., 2013). Differential tick
224 transmission fitness has been found among different *A. marginale msp1α* genotypes
225 (Palmer et al., 2004). Considering that ticks may not be playing an important role in
226 transmission among buffaloes in the study site, the most common strain found in water
227 buffaloes may be adapted to mechanical or transplacental transmission. In agreement
228 with these findings, 60% of the *A. marginale* MSP1a tandem repeats obtained here
229 presented the amino acid glycine (G) at position 20 and this amino acid was in at least
230 one of the MSP1a repeats in all the *A. marginale* strains. The negatively charged amino
231 acids aspartic acid (D) and glutamic acid (E) at position 20 were shown to be essential
232 for the binding of MSP1a to tick cells while with a G at this position no binding was
233 observed (de la Fuente et al., 2003). These amino acids affect MSP1a conformation and
234 these conformational changes were suggested to affect *A. marginale* transmission by
235 ticks (Cabezas-Cruz et al., 2013).

236 **Conclusions**

237 In this study, the genetic diversity of MSP1a in *A. marginale* was characterized in water
238 buffaloes. The *A. marginale* genetic diversity was low in buffaloes and correlated with
239 the low bacterial prevalence in this species. One major factor that could be contributing
240 to this low genetic diversity is the ecology of the studied area, which is not suitable for
241 ticks thus reducing the probability for pathogen biological transmission. Mechanical
242 transmission by hematophagous Diptera could be playing a major role in the
243 transmission of *A. marginale* in the study site. Evidence was found to support the
244 hypothesis that MSP1a is under selective pressure by the host immune system in
245 buffaloes. Finally, water buffaloes may serve as reservoir hosts of *A. marginale* for
246 cattle. These results expanded our knowledge of *A. marginale* strains and provided
247 additional support for the role of MSP1a in pathogen evolution and transmission.

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356 **Figures**

357

358 **Figure 1. Characterization of *A. marginale msp1α* sequences.** (A) Neighbor joining
359 phylogenetic tree of *A. marginale msp1α*. The tree was constructed using the neighbor
360 joining method with *A. marginale msp1a* sequences from strains identified in water
361 buffaloes and cattle. Bootstrap values are represented as percent on internal branches
362 (1000 replicates). The GenBank accession numbers of the respective sequences used for
363 the phylogenetic analysis are shown. The four different *A. marginale* strains obtained
364 from water buffaloes in this study are shown (together with tandem repeat structure in
365 parenthesis) as Water buffalo 3 (78, 24, 24, 25, 31) (black triangle); Water buffalo 13
366 (4, 63, 27) (white square); Water buffalo 15 (162, 63, 27) (black square) and Water
367 buffalo 4 (τ, 10, 10, 15) (white triangle). (B) Amino acid differences between tandem
368 repeats 4 and 162 and position evolving under negative selection. The one letter code is
369 used for the different amino acids of the tandem repeats. Conserved amino acid
370 positions are highlighted with asterisks. Substitution of glutamine (Q) in tandem repeat
371 4 by leucine (L) in tandem repeat 162 is show with an arrow. Amino acid at position 10
372 (-) evolving under negative selection ($p < 0.25$ using both FEL and SLAC methods) and
373 residues of the immunodominant B-cell epitope (Garcia-Garcia et al., 2004) (box) are
374 also shown.

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375 **Table 1.** Organization of MSP1a tandem repeats in *A. marginale* strains identified in
376 water buffaloes.

A. marginale strain identification	No. of animals infected with this strain
Brazil/Marajó Island/ E - (4, 63, 27)	9
Brazil/Marajó Island/ E - (78, 24^2, 25, 31)	3
Brazil/Marajó Island/ E - (τ, 10^2, 15)	2
Brazil/Marajó Island/ E - (162, 63, 27)	1

377

378 Strain identification is based on *msp1α* and includes Country/Locality/microsatellite
379 genotype – (tandem repeats structure). Superscripts represent the number of times that a
380 tandem repeats are repeated. The new MSP1a tandem repeat 162 was named following
381 the system proposed by de la Fuente et al. (2007) and updated by Cabezas-Cruz et al.
382 (2013).

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Figure

A

A. marginale Minas 12. JX844214
A. marginale Brazil 5. AY283198
A. marginale Brazil 9. AY283199
A. marginale Brazil 12. AY283200
A. marginale Minas 3. JX844207
A. marginale Jaboticabal. KJ398398
A. marginale Parana 2. AY998120
A. marginale Minas 2. JX844206
A. marginale Water buffalo 3. KJ575588 ▲
A. marginale Water buffalo 13. KJ575600 □
A. marginale Water buffalo 15. KJ575602 ■
A. marginale Minas 5. JX844209
A. marginale Water buffalo 4. KJ575591 △
A. marginale Brazil. AF428092
A. marginale Florida. M32871
A. marginale St. Maries. AY010245

61 96 59 96 99 49 95 100 91 100 72 50 95 100

0.02

B

```
4      TDSSSASGQQQESSVLSQSGQASTSSQLG
162    TDSSSASGQQQESSVLSQSGQASTSSLLG
       ************************** **
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