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

Jenevaldo B. Silva¹, Adivaldo H. Fonseca², José D. Barbosa³, Alejandro Cabezas-Cruz^{4,5}, José de la Fuente^{4,6,*}

¹Departamento de Patologia Veterinária, Universidade Estadual Paulista, Jaboticabal, São Paulo, Brasil.

²Departamento de Clínica Veterinária, Universidade Federal do Pará, Castanhal, Pará, Brasil

³Departamento de Parasitologia, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil.

⁴SaBio. Instituto de Investigación en Recursos Cinegéticos IREC, CSIC-UCLM-JCCM, Ronda de Toledo s/n, 13005 Ciudad Real, Spain.

⁵Center for Infection and Immunity of Lille (CIIL), INSERM U1019 – CNRS UMR 8204, Université Lille Nord de France, Institut Pasteur de Lille, Lille, France.

⁶Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA.

***Corresponding author:** José de la Fuente. SaBio. Instituto de Investigación en Recursos Cinegéticos, Ronda de Toledo s/n, 13005 Ciudad Real, Spain. Phone: +34 926295450. E-mail: Jose_delafuente@yahoo.com

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 msp1a*
 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 *msp1a* 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

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

74 al., 2014). However, although the *A. marginale msp1a* 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 msp1a* 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).

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' TGTGCTTATGGCAGACATTTC 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

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 msp1a* have been previously identified
 128 (Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013). Theoretical translation of *msp1a*
 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 (Delpont et al., 2010).
 137 Positive or negative selection was assigned to codons where $\omega = dN$ (non-synonymous
 138 substitutions)/ dS (synonymous substitutions) ratio was higher or lower than 1,
 139 respectively. As recommended in Datamonkey webserver (Delpont et al., 2010), only
 140 sites with p-value < 0.25 were considered to be under selection.

141 *Phylogenetic analysis*

142 For *msp1a* 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.*

148 *marginale msp1a* previously reported in cattle from Brazil and the USA were obtained
 149 from Genbank 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 *msp1a* 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 *msp1a*, 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 *msp1a* 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 *msp1a* 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,

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

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

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 uracil 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 *msp1a* 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

lice species have been reported in buffaloes (Da Silva et al., 2013). Differential tick transmission fitness has been found among different *A. marginale msp1a* genotypes (Palmer et al., 2004). Considering that ticks may not be playing an important role in transmission among buffaloes in the study site, the most common strain found in water buffaloes may be adapted to mechanical or transplacental transmission. In agreement with these findings, 60% of the *A. marginale* MSP1a tandem repeats obtained here presented the amino acid glycine (G) at position 20 and this amino acid was in at least one of the MSP1a repeats in all the *A. marginale* strains. The negatively charged amino acids aspartic acid (D) and glutamic acid (E) at position 20 were shown to be essential for the binding of MSP1a to tick cells while with a G at this position no binding was observed (de la Fuente et al., 2003). These amino acids affect MSP1a conformation and these conformational changes were suggested to affect *A. marginale* transmission by ticks (Cabezas-Cruz et al., 2013).

Conclusions

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

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253 **References**

254

255 Cabezas-Cruz, A., Passos, L.M.F., Lis, K., Kenneil, R., Valdés, J.J., Ferrolho, J., Tonk,
 256 M., Pohl, A.E., Grubhoffer, L., Zweggarth, E., Shkap, V., Ribeiro, M.F.B.,
 257 Estrada-Peña, A., Kocan, K.M., de la Fuente, J., 2013. Functional and
 258 immunological relevance of *Anaplasma marginale* major surface protein 1a
 259 sequence and structural analysis. PLoS ONE 8, e65243.

260

261 Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their
 262 use in phylogenetic analysis. Mol. Biol. Evol. 17, 540-552.

263

264 Da Silva, A.S., Lopes, L.S., Diaz, J.D., Tonin, A.A., Stefani, L.M., Araújo, D.N., 2013.
 265 Lice outbreak in buffaloes: evidence of *Anaplasma marginale* transmission by
 266 sucking lice *Haematopinus tuberculatus*. J. Parasitol. 99, 546-547.

267

268 de la Fuente, J., Van Den Bussche, R.A., Prado, T., Kocan, K.M., 2003. *Anaplasma*
 269 *marginale* major surface protein 1a genotypes evolved under positive selection
 270 pressure but are not markers for geographic strains. J. Clin. Microbiol. 41, 1609-
 271 1616.

272

273 de la Fuente, J., Vicente, J., Höfle, U., Ruiz-Fons, F., Fernández de Mera, I.G., Van Den
 274 Bussche, R.A., Kocan, K.M., Gortazar, C., 2004. *Anaplasma* infection in free-
 275 ranging Iberian red deer in the region of Castilla - La Mancha, Spain. Vet.
 276 Microbiol. 100, 163-173.

277

- 278 de la Fuente, J., Ruybal, P., Mtshali, M.S., Naranjo, V., Shuqing, L., Mangold, A.J.,
 279 Rodríguez, S.D., Jiménez, R., Vicente, J., Moretta, R., Torina, A., Almazán, C.,
 280 Mbat, P.M., Torioni de Echaide, S., Farber, M., Rosario-Cruz, R., Gortazar, C.,
 281 Kocan, K.M., 2007. Analysis of world strains of *Anaplasma marginale* using
 282 major surface protein 1a repeat sequences. Vet. Microbiol. 119, 382–390.
 283
- 284 de la Fuente, J., Kocan, K.M., Blouin, E.F., Zivkovic, Z., Naranjo, V., Almazán, C.,
 285 Esteves, E., Jongejan, F., Daffre, S., Mangold, A.J. 2010. Functional genomics
 286 and evolution of tick-*Anaplasma* interactions and vaccine development. Vet.
 287 Parasitol. 167, 175–186.
 288
- 289 Delpont, W., Poon, A.F., Frost, S.D., Kosakovsky Pond, S.L., 2010. Datamonkey 2010:
 290 a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics 26,
 291 2455-2457.
 292
- 293 Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high
 294 throughput. Nucleic. Acids. Res. 32, 1792-1797.
 295
- 296 Estrada-Peña, A., Naranjo, V., Acevedo-Whitehouse, K., Mangold, A.J., Kocan, K.M.,
 297 de la Fuente, J., 2009. Phylogeographic analysis reveals association of tick-borne
 298 pathogen, *Anaplasma marginale*, MSP1a sequences with ecological traits
 299 affecting tick vector performance. BMC. Biol. 57, 1-13.
 300
- 301 Felsenstein, J., 1989. PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics.
 302 5, 164-166.

303

304 Furtado, A.P., Do Carmo, E.S., Giese, E.G., Vallinoto, A.C., Lanfredi, R.M., Santos,
305 J.N., 2009. Detection of dog filariasis in Marajo Island, Brazil by classical and
306 molecular methods. *Parasitol. Res.* 105, 1509-1515.

307

308 Garcia-Garcia, J.C., de la Fuente, J., Kocan, K.M., Blouin, E.F., Halbur, T., Onet, V.C.,
309 Saliki, J.T., 2004. Mapping of B-cell epitopes in the N-terminal repeated peptides
310 of *Anaplasma marginale* major surface protein 1a and characterization of the
311 humoral immune response of cattle immunized with recombinant and whole
312 organism antigens. *Vet. Immunol. Immunopathol.* 98, 137-151.

313

314 IBGE, 2012. Instituto Brasileiro de Geografia e Estatística [Brazilian Institute of
315 Geography and Statistics] (in Portuguese). Available from:
316 <http://www.ibge.gov.br/home/>

317

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

320

321 Lew, A.E., Bock, R.E., Minchin, C.M., Masaka, S., 2002. A *msp1a* polymerase chain
322 reaction assay for specific detection and differentiation of *Anaplasma marginale*
323 isolates. *Vet. Microbiol.* 86, 325-335.

324

325 Palmer, G.H., Rurangirwa, F.R., McElwain, T.F., 2001. Strain composition of the
326 ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian
327 reservoir for tick transmission. *J. Clin. Microbiol.* 39, 631-635.

328

329 Palmer, G.H., Knowles Jr., D.P., Rodriguez, J.L., Gnad, D.P., Hollis, L.C., Marston, T.,
 330 Brayton, K.A., 2004. Stochastic transmission of multiple genotypically distinct
 331 *Anaplasma marginale* strains in a herd with high prevalence of *Anaplasma*
 332 infection. J. Clin. Microbiol. 42, 5381-5384.

333

334 Pohl, A.E., Cabezas-Cruz, A., Ribeiro, M.F.B., Silveira, J.A.G., Silaghi, C., Pfister, K.,
 335 Passos, L.M.F., 2013. Detection of genetic diversity of *Anaplasma marginale*
 336 isolates in Minas Gerais, Brazil. Rev. Bras. Parasitol. Vet. 22, 129-135.

337

338 Ruybal, P., Moretta, R., Perez, A., Petrigh, R., Zimmer, P., Alcaraz, E., Echaide, I.,
 339 Torioni de Echaide, S., Kocan, K.M., de la Fuente, J., Farber, M., 2009. Genetic
 340 diversity of *Anaplasma marginale* in Argentina. Vet. Parasitol. 162, 176-180.

341

342 Silva, J.B., Vinhote, W.M.S., Oliveira, C.M.C., André, M.R., Fonseca, A.H., Barbosa,
 343 J.D., 2014. Molecular and serological prevalence of *Anaplasma marginale* in
 344 water buffaloes in the northern Brazil. Ticks. Tick. Borne. Dis. 5, 100-104.

345

346 Schneider, A., Gonnet, G., Cannarozzi, G., 2007. SynPAM-a distance measure based on
 347 synonymous codon substitutions. IEEE/ACM Trans. Comput. Biol. Bioinform. 4,
 348 553-560.

349

350 Suarez, C.E., Noh, E., 2011. Emerging perspectives in the research of bovine babesiosis
 351 and anaplasmosis. Vet. Parasitol. 180, 109-125.

352

353 Vidotto, M.C., Vidotto, O., Andrade, G.M., Palmer, G., McElwain, T., Knowles, D.P.,
354 1998. Seroprevalence of *Anaplasma marginale* in cattle in Paraná State, Brazil,
355 by MSP-5 competitive ELISA. Ann. N. Y. Acad. Sci. 849, 424-426.
356

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 msp1α* 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.

375

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

<i>A. marginale</i> 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 *msp1a* 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).

383

Figure
A

B

4
162

TDS SSASGQQQESS VLSQSGQASTSSQLG
 TDSSSASGQQQESSVLSQSGQASTSSLLG
 ***** **

- ↓