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Short communication

Analysis of world strains of *Anaplasma marginale* using major surface protein 1a repeat sequences

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Abstract

Anaplasma marginale is a tick-borne pathogen of cattle that causes the disease bovine anaplasmosis worldwide. Major surface proteins (MSPs) are involved in host–pathogen and tick–pathogen interactions and have been used as markers for the genetic characterization of *A. marginale* strains and phylogenetic studies. MSP1a is involved in the adhesion and transmission of *A. marginale* by ticks and varies among geographic strains in the number and sequence of amino-terminal tandem repeats. The aim of this study was to characterize the genetic diversity of *A. marginale* strains collected from countries in North and South America, Europe, Asia, Africa and Australia, inclusive of all continents. In this study, we characterized 131 strains of *A.*

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marginale using 79 MSP1a repeat sequences. These results corroborated the genetic heterogeneity of *A. marginale* strains in endemic regions worldwide. The phylogenetic analyses of MSP1a repeat sequences did not result in clusters according to the geographic origin of *A. marginale* strains but provided phylogeographic information. Seventy-eight percent of the MSP1a repeat sequences were present in strains from a single geographic region. Strong (≥80%) support was found for clusters containing sequences from Italian, Spanish, Chinese, Argentinean and South American strains. The phylogenetic analyses of MSP1a repeat sequences suggested tick–pathogen co-evolution and provided evidence of multiple introductions of *A. marginale* strains from various geographic locations worldwide. These results contribute to the understanding of the genetic diversity and evolution of *A. marginale* and tick–pathogen interactions.

Keywords: Anaplasmosis; Major surface protein; Evolution; Tick; Genetics; Phylogenetic analysis

1. Introduction

Anaplasma marginale (Rickettsiales: Anaplasmataceae) is the causative agent of bovine anaplasmosis worldwide (Kocan et al., 2004). Ticks are biological vectors of *A. marginale* but the pathogen is often transmitted mechanically to susceptible cattle by blood-contaminated mouthparts of biting flies or fomites (Kocan et al., 2003). These obligate intracellular organisms replicate in membrane-bound parasitophorous vacuoles in bovine erythrocytes or tick cells. Both cattle and ticks become persistently infected with *A. marginale* and thus serve as reservoirs of infection (Kocan et al., 2003, 2004).

Many geographic strains of *A. marginale* have been identified, which differ in biology, genetic characteristics and transmissibility by ticks (de la Fuente et al., 2001a, 2005). The genetic diversity of *A. marginale* strains has been characterized using major surface protein (MSP) genes that are involved in interactions with vertebrate and invertebrate host cells (de la Fuente et al., 2005). These genes may have evolved more rapidly than other genes because of selective pressures exerted by the host immune system.

MSP1a, encoded by the gene *msp1α*, has thus far been identified only in *A. marginale* despite attempts to clone this gene from other *Anaplasma* spp. (de la Fuente et al., 2005). The *A. marginale* MSP1a has evolved under positive selection pressure and geographic strains of the pathogen differ in molecular weight because of a variable number of tandem 23–31 amino acid repeats (Allred et al., 1990; de la Fuente et al., 2001a, 2003a, 2005). MSP1a was shown to be an adhesin for bovine erythrocytes and tick cells (McGarey and Allred, 1994; McGarey et al., 1994; de la Fuente et al., 2001b), and the adhesion domain was identified on the variable N-terminal region containing the repeated peptides (de la Fuente et al., 2003b). MSP1a was also shown to be involved in the transmission of *A. marginale* by *Dermacentor* spp. ticks (de la Fuente et al., 2001c) and to be differentially regulated in tick cells and bovine erythrocytes (Garcia-Garcia et al., 2004).

Due to the high degree of sequence variation within endemic areas, MSP1a sequence analyses of strains from North and South America, Italy, Israel and Australia failed to provide phylogeographic information (de la Fuente et al., 2005). Nevertheless, the analyses of MSP1a repeat sequences on a global scale may provide phylogenetic and evolutionary information about *A. marginale* strains. Therefore, this study was designed for the global characterization of *A. marginale* using MSP1a repeat sequences of cattle strains from North and South America, Europe, Africa, Asia and Australia. This analysis includes new MSP1a sequences from *A. marginale* strains in Mexico, Argentina, Spain, South Africa and China, as well as MSP1a sequences reported previously from North and South America, Italy, Israel and Australia.

2. Materials and methods

2.1. A. marginale strains

A. marginale strains used in this study and Genbank accession numbers for *msp1α* sequences are listed in Fig. 1. All *A. marginale* strains in this study were originally obtained from naturally infected cattle.

(A)

Repeat form	Encoded sequence
A	DDSSSASGQQQESSVSSQSE-ASTSSQLG--
B	A*****G***********DQ**********
C	A*****G***********GQ**********
D	A****************************G*
E	A*****************************
F	T*****************GQ**********
G	******************GQ******S***
H	T*****************GQ******S***
I	******************GQ**********
J	A***L*G***********DQ**********
K	A*G***G***********DQ**********
L	AG****D***********DQ**********
M	A*****************GQ**********
m	A*****************GQ******S***
N	T*****************DQ**********
O	----**G***********DQ**********
P	T***********G****GQ**H*A*S***
Q	A*****************DQ**********
R	A*****G***H********DQ*******W**
T	AG****G***********DQ**********
U	******************DQ**********
V	A*****G***-********DQ**********
W	T*****************GQ******SR**
α	A*******------*L***GQ**********
β	T*****GD***G*G*****GQ**********
Γ	T*****************D***********
π	A*****G***********GQ******F***
Σ	A*****G************************
σ	A*****G******I*****DH**********
μ	A*******L*********GQ**********
τ	T*************L*P*GQ**********
Φ	T*****************************
1	SG************L**GGQ**********
2	T***************P**GQ**********
3	A*************L***GQ**********
4	T*************L***GQ**********
5	A*****************D***********
6	A****************H************
7	T****************H************
8	A*G***GD**********G******S***
9	A*****************D******S***
10	A*************L*P*GQ**********
11	A*************L*P*GQ********VG
12	AG************L***DQ**********
13	T*************L***DQ**********
14	T*************L***G***********
15	A***********G*L***GQ**********
16	A*****GD***G******GQ**********
17	T***********G*****GQ**********
18	T*************L***DQ******S***
19	A*****GDR****G*L***GQ**********
20	A*****GD*****G*L***GQ**********
21	A*****GD******L***GQ**********
22	A*************L*P*GQ*******S**
23	T**********K***L***SQ**********
24	A*****GN*******LP**GQ*******S**
25	A*************L***SQ**********
26	A*****GN*******LP**GQ**********
27	A*************L***DQ**********
28	AG***E*********L***GQ**********
29	T******D**********GQ**********
30	A**********K***L***SQ**********
31	A*****GN**********D***********
32	T*****G***********GQ**********
33	A*************L*K*GQ*G********
34	AN************L***DQ**********
35	T*****************GQ*G****S***
36	A*************P***************
37	T*************L***GQ******S***
38	A*************L***GQ******S***
39	***************L***DQ**********
40	AG****GD**********DQ**********
41	AS************L***DQ**********
42	T*************LP**GQ******S***
43	A*************LP**GQ******S***
44	T******A**********GQ******S***
45	T*************LP**DQ**********
46	T*************LP**GQ**********
47	A*****GD**********DQ**********

Fig. 1. Sequence of MSP1a tandem repeats in geographic strains of *A. marginale*. (A) The one letter amino acid code was used to depict the different sequences found in MSP1a repeats. Asterisks indicate identical amino acids and gaps indicate deletions/insertions. (B) The structure of the MSP1a repeats region was represented using the repeat forms described in (A) for strains of *A. marginale* from North and South America, Europe, Asia, Africa and Australia, updated after de la Fuente et al. (2005).

(B)

A. marginale strain	Country of origin	Genbank accession No.	Structure of MSP1a tandem repeats	No. of repeats
Florida	USA	M32871	A B B B B B B B	8
Idaho	USA	M32868	D D D D D E	6
Virginia	USA	AY010246	A B	2
Washington	USA	M32869	B B B C	4
Wetumka, OK	USA	AY010247	K C H	3
Cushing, OK	USA	AY127056	L C B C	4
Cushing 2, OK	USA	AY127057	K N N F H	5
Glencoe 1, OK	USA	AY127053	K F N F H	5
Glencoe 2, OK	USA	AY127054	B M F H	4
Glencoe 3, OK	USA	AY127055	T B C	3
Stillwater, OK	USA	AY127061	K F F F H	5
Stillwater 2, OK	USA	AY127062	L B C C	4
Stillwater 68, OK	USA	DQ811776	K B M F H	5
Stillwater 483, OK	USA	DQ811777	K B M H	4
Oklahoma City, OK	USA	AY127059	U	1
Okmulgee, OK	USA	AY127060	K B V C	4
Stigler, OK	USA	AY127058	T B B C	4
Pawhuska, OK	USA	AY127064	I H	2
New Castle, OK	USA	AY127063	L B C B	4
St. Maries, ID	USA	AY010245	J B B	3
California	USA	AY010242	B B C	3
Okeechobee, FL	USA	AY010244	L B C B C	5
Mississippi	USA	AY010243	D D D D E	5
Missouri	USA	AY127052	B B B B	4
Illinois	USA	AF345867	M N B M H	5
Texas	USA	AF428091	O B M P	4
Texas 198	USA	DQ811778	B B m B m	5
South Dakota	USA	AF293064	A F H	3
Oregon, Rassmusen	USA	AF293064	A F H	3
Oregon	USA	Palmer *et al.* 2001	G	1
Kansas 3261	USA	Palmer *et al.* 2004	B B	2
Kansas 4102	USA	Palmer *et al.* 2004	B B B	3
Kansas 2267	USA	Palmer *et al.* 2004	B B B B	4
Kansas 0141	USA	Palmer *et al.* 2004	B B B B B	5
Kansas 0063	USA	Palmer *et al.* 2004	B B B B B B	6
Kansas 5076	USA	Palmer *et al.* 2004	D D D D D	5
Kansas 7042	USA	Palmer *et al.* 2004	D D E	3
Kansas 4318	USA	Palmer *et al.* 2004	D D D D D E	6
Kansas 2070	USA	Palmer *et al.* 2004	D D D D D D E	7
Kansas 7030	USA	Palmer *et al.* 2004	D D D D D D D D D E	10
Kansas 0050	USA	Palmer *et al.* 2004	E M Φ	3
Canadian bison	Canada	AY253141	D Q Q R	4
U.S. bison (buffalo)	USA	AY253144	K B M F W	5
Yucatán	Mexico	AF345871	T C B B C B π	7
Mexico	Mexico	AF345868	α ß ß Γ	4
Morelos	Mexico	AF345869	α ß ß Γ	4
Veracruz	Mexico	AF345870	α ß ß Γ	4
Aguascalientes	Mexico	DQ501243	4 9 10 11 9	5
Pichucalco	Mexico	DQ501244	α ß ß Γ ß Γ	6
Puente de Ixtla	Mexico	DQ501242	12 13 14	3
Brazil 9	Brazil	AY283199	α ß τ M	4
Brazil 12	Brazil	AY283200	α ß ß N	4
Brazil 5	Brazil	AY283198	C F N	3
Brazil	Brazil	AF428092	B B Q σ μ	5
Parana	Brazil	AY602768	α ß ß ß ß Γ	6
Parana 2	Brazil	AY998120	16 F 17 13 18	5

Fig. 1. (*Continued*)

Parana 3	Brazil	AY998121	τ	10	15					3
Virasoro	Argentina	AF428094	Σ	B	Q	B	C			5
Salta	Argentina	AF428093	B	B	M					3
Entre Rios 1	Argentina	DQ833249	F	M	M					3
Entre Rios 2	Argentina	DQ833250	F	M	M					3
Entre Rios 3	Argentina	DQ833251	F	M	M					3
Entre Rios 4	Argentina	DQ833252	F	M	M					3
Santa Fe 37	Argentina	DQ833253	α	β	β	β	Γ			5
Santa Fe 43	Argentina	DQ833254	α	β	β	β	Γ			5
Santa Fe 50	Argentina	DQ833255	α	β	β	β	Γ			5
Santa Fe 59	Argentina	DQ833256	α	β	β	β	Γ			5
Santa Fe 111	Argentina	DQ833257	B	B	M					3
Santa Fe 473	Argentina	DQ833258	B	B	M					3
Santa Fe 532	Argentina	DQ833259	B	B	M					3
Santa Fe 116	Argentina	DQ833260	B	B	M					3
Chaco 2 var1	Argentina	DQ833261	τ	22	13	18				4
Chaco 2 var2	Argentina	DQ833262	α	β	Γ	Γ	β	β	Γ	7
Chaco 3 var1	Argentina	DQ833263	τ	22	13	18				4
Chaco 3 var2	Argentina	DQ833264	τ	11	10	10	11	10	15	7
Chaco 5	Argentina	DQ833265	τ	10	15					3
Chaco 7	Argentina	DQ833266	τ	22	13	18				4
Chaco 8	Argentina	DQ833267	τ	22	13	18				4
Cordoba 1	Argentina	DQ833268	23	24	25	26	27	27		6
Cordoba 2	Argentina	DQ833269	23	24	25	26	27	27		6
Quitilipi	Argentina	DQ833270	28	29	M	29	M	F		6
Mercedes	Argentina	DQ833271	23	30	31	31	31			5
Corrientes 7	Argentina	DQ833272	α	β	β	β				4
Puerto Rico	Puerto Rico	AY191826	E	Φ	Φ	Φ	Φ	Φ		6
Havana	Cuba	AY489564	A	B	B	B	B			5
Israel tailed 1FM3	Israel	AF352560	1	F	M	3				4
Israel tailed 12M3	Israel	AY295077	1	2	M	3				4
Israel non-tailed	Israel	AF352559	1	4						2
Azaria	Israel	AY846869	1	F	M	3	3			5
Lhavot-Habasan	Israel	AY846868	M	F	F	F				4
Or-Haner	Israel	AY355284	M	F	F					3
HB-A8	China	DQ811774	19	20	19	21				4
Italy 30	Italy	AY702926	M	M	M	Q				4
Italy 31	Italy	AY702927	M	M	M	Q				4
Italy 32	Italy	AY702928	5	Γ	Γ	Γ				4
Italy 16	Italy	AY702929	5	Φ	Φ	Φ				4
Italy 6	Italy	AY702930	Q	M	Q	Q	M			5
Italy 8	Italy	AY702931	Q	N	N	N				4
Italy 47	Italy	AY702932	6	7	7	7				4
Va-48	Spain	DQ811775	40	47	47	32	C	C		6
Australia Darwin	Australia	AF407542	8							1
Australia WA	Australia	AF407545	8							1
Australia F12	Australia	AF407543	8							1
Australia F72	Australia	AF407544	8							1
SA12	South Africa	DQ813536	34	13	4	37				4
SW82	South Africa	DQ813537	34	13	4	37				4
SW62	South Africa	DQ813538	34	13	4	37				4
SW162	South Africa	DQ813539	34	13	4	37				4
SW134	South Africa	DQ813540	34	13	4	37				4
SA66	South Africa	DQ813541	34	13	4	37				4
SA193	South Africa	DQ813542	34	4	37					3
SW32	South Africa	DQ813543	34	13	13	37				4
SA14	South Africa	DQ813544	34	F	4	H				4
SA10	South Africa	DQ813545	33	35	35					3
SW29	South Africa	DQ813546	3	3	38					3
SA71	South Africa	DQ813547	3	3	38					3

Fig. 1. (*Continued*)

SA302	South Africa	DQ813548	3	3	38				3
SA196	South Africa	DQ813549	3	3	38				3
SW114	South Africa	DQ813550	3	13	4	4	37		5
SW109	South Africa	DQ813551	27	4	13	13	37		5
SW44	South Africa	DQ813552	27	4	4	4	37		5
SW90	South Africa	DQ813553	27	13	4	13	4		5
SA239	South Africa	DQ813554	27	4	13	4	4		5
SA183	South Africa	DQ813555	27	13	4	44			4
SW34	South Africa	DQ813556	34	45	45	46	37		5
SA191	South Africa	DQ813557	27	37					2
SA189	South Africa	DQ813558	27	37					2
SA4	South Africa	DQ813559	27	18					2
SA63	South Africa	DQ813560	39	37	13	13	13	13	37	7
SA240	South Africa	DQ813561	40	Q	Q					3
SW113	South Africa	DQ813562	41	13	13	13	4	37		6
SW112	South Africa	DQ813563	42	43	25	31				4
SA243	South Africa	DQ813564	3	36	3	36	36	3	36	38	8

Fig. 1. (*Continued*).

2.2. *A. marginale msp1α* polymerase chain reaction (PCR) and sequencing

The *msp1α* gene was amplified from *A. marginale* DNA extracted from erythrocytes by PCR using 10 pmol of each primer MSP1aP: 5′-GCATTACAACGCAACGCTTGAG-3′ and MSP1a3: 5′-GCTTTACGCCGCCGCCTGCGCC-3′ as previously reported (de la Fuente et al., 2001d, 2003a). Amplified fragments were resin purified and cloned into pGEM-T vector (Promega) or used directly for sequencing both strands by double-stranded dye-termination cycle sequencing (Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University). Only the fragment containing the upstream and variable regions of the *msp1α* gene was sequenced. When cloned, at least three clones were sequenced from each PCR.

2.3. Sequence alignment and phylogenetic analysis

The *A. marginale* MSP1a repeat nucleotide and amino acid sequences were used for sequence alignment and phylogenetic analysis. Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) with an engine based on the Clustal W algorithm (Thompson et al., 1994). Nucleotides were coded as unordered, discrete characters with five possible character-states: A, C, G, T or N and gaps were coded as missing data. Phylogenetic analyses were implemented using MEGA version 3.0 (Kumar et al., 2004). Maximum parsimony (MP) analyses were conducted with equal weights for all characters and substitutions, heuristic searches with 10 random additions of input taxa and tree bisection-reconnection (TBR) branch swapping. Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis (Felsenstein, 1985) of 1000 replications.

3. Results and discussion

World strains of *A. marginale* ($N = 131$) were analyzed from countries in North and South America, Europe, Asia, Africa and Australia (Fig. 1A and B). Seventy-nine MSP1a repeat sequences were identified in these strains (Fig. 1A). The analysis of MSP1a repeat sequences corroborated the genetic heterogeneity of *A. marginale* strains (Fig. 1B) and extended the results of previous reports to include new geographic regions inclusive of all continents (de la Fuente et al., 2005).

Due to the high degree of sequence variation within endemic areas, MSP1a sequences fail to provide phylogeographic information (de la Fuente et al., 2005). However, although the phylogenetic analysis of MSP1a repeat sequences did not result in clusters according to the geographic origin of *A. marginale* strains, it did provide phylogeographic information

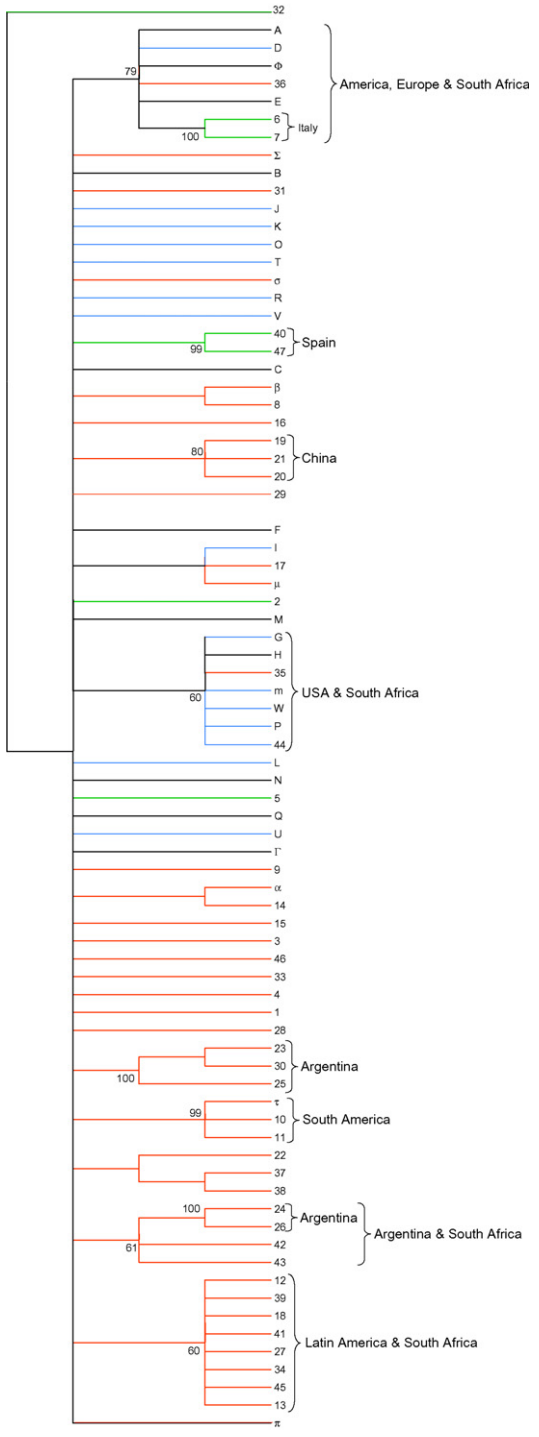

Fig. 2. Phylogenetic analyses of MSP1a repeat amino acid sequences. A condensed strict consensus of 604 MP trees was constructed using MEGA version 3.0 (Kumar et al., 2004). Numbers above/below branches indicate percent support (≥60%) for 1000 bootstrap replicates with 10 random additions of input taxa. The MSP1a repeat forms were described in Fig. 1A. Clusters by geographic origin of *A. marginale* strains are indicated. Line colors denote suggested *A. marginale*-tick vector associations (red, *Boophilus* spp.; blue, *Dermacentor* spp.; green, *Rhipicephalus* spp. and *Hyalomma* spp.; black, *A. marginale*-tick association could not be unambiguously suggested).

(Fig. 2). Seventy-eight percent of the repeat sequences (62/79) were present in strains from a single geographic region. Some MSP1a repeats clustered together and were unique to certain regions (Fig. 2). Strong (≥80%) support was found for clusters containing sequences from Italian, Spanish, Chinese, Argentinean and South American strains (Fig. 2). Clusters containing sequences from American, European and/or South African strains had a weak (60–79%) support (Fig. 2). MP analyses of MSP1a repeat amino acid and nucleotide sequences provided similar results (data not shown).

The results reported herein support the hypothesis that genetic heterogeneity observed among strains of *A. marginale* within endemic regions could be explained by cattle movement and maintenance of different genotypes by independent transmission events, due to infection exclusion of *A. marginale* in cattle and ticks, which commonly results in the establishment of only one genotype per animal (de la Fuente et al., 2005). However, when distantly related genotypes exist in the same region, infections of a single host with multiple *A. marginale* strains are possible (Palmer et al., 2004). These studies also suggest that, with the exception of Australia where a single genotype was found, multiple introductions of *A. marginale* strains from different geographic locations occurred in these regions.

Initial analysis of *A. marginale msp1α* and *Dermacentor variabilis* 16S rDNA sequences from various areas of the USA suggested tick–pathogen co-evolution (de la Fuente et al., 2001d), a result that is consistent with the biological function of MSP1a in the transmission of *A. marginale* by ticks (de la Fuente et al., 2001c). The analysis of MSP1a repeat sequences in association with tick species that may transmit *A. marginale* strains containing these repeats suggested that some sequences may have co-evolved with the tick vector and that tick–pathogen interactions could influence the presence of unique MSP1a repeats in

strains of *A. marginale* from particular geographic regions (Fig. 2). Some repeat sequences, such as #27 and #13 were present in strains from different geographic regions (Latin America and South Africa), but with a common tick vector, *Boophilus* spp. (Fig. 2). These results are consistent with the observation that *B. microplus* populations from America may have an African origin while Australian *B. microplus* could have originated in Asia (Soler et al., 2005). However, the role of other tick species and mechanical transmission of *A. marginale* strains could also play an important role in the evolution of *A. marginale* (Kocan et al., 2004).

In summary, the results reported in this study provided information about the evolution of *A. marginale* strains using MSP1a repeats sequences. Herein, we characterized the genetic diversity of *A. marginale* strains in endemic areas and corroborated the genetic heterogeneity of *A. marginale* at a global scale. The phylogenetic analyses of MSP1a repeat sequences suggested tick–pathogen co-evolution and provided worldwide evidence of multiple introductions of *A. marginale* strains from different geographic locations. Cattle movement and maintenance of different genotypes by independent transmission events most likely contributed to the genetic diversity in endemic regions. This study extends our understanding of *A. marginale* diversity, evolution and tick–pathogen interactions.

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