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Extensively variable surface antigens of *Sarcocystis* spp. infecting Brazilian marsupials in the genus *Didelphis* occur in myriad allelic combinations, suggesting sexual recombination has aided their diversification

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ABSTRACT

Sarcocystis neurona and *Sarcocystis falcatula* are very similar species of Apicomplexan protozoa that use marsupials of the genus *Didelphis* as definitive hosts. These mammals can serve as definitive hosts not only for these two parasites, but for other *Sarcocystis* such as *Sarcocystis speeri* and *Sarcocystis lindsayi*. *Sarcocystis* shed by opossums (with the exception of *S. neurona*) can cause disease in a great variety of birds, being commonly associated with acute pulmonary sarcocystosis in zoos. *S. neurona* is the most commonly associated parasite with the equine protozoal myeloencephalitis in horses. Herein we assessed the variability of *Sarcocystis* spp. isolated from opossums of the state of Rio Grande do Sul, Brazil, by sequencing fragments of genes coding for glycosylphosphatidylinositol-anchored surface antigens (termed surface antigen or SAG), SAG2, SAG3 and SAG4. Two genetic groups were identified, one of them related to *S. falcatula* and the other related to *S. neurona*. Various allelic combinations of SAG2, SAG3 and SAG4 occur among *S. falcatula* related isolates and strong evidences suggest that such isolates may exchange high divergent alleles in possible sexual recombination processes. Regarding the group *S. neurona*-like (isolates G37 and G38), none of the individuals in this group share alleles with individuals of the other group. Comparing G37 and G38 strains and North American strains of *S. neurona*, four polymorphisms were identified at SAG-3, five at SAG-2 and three at SAG-4. Gene sequences of locus SAG-3 from isolates G37 and G38 differed from the other sequences by an insertion 81 bp long. This insertion contains several dinucleotide repeats of AT, resembling a microsatellite locus and has already been detected in SAG3 sequences of *S. neurona* from North America. When aligned against North American strains of *S. neurona*, G37 and G38 isolates have a deletion of 8 nucleotides within this intron which indicate that *S. neurona* strains of South America are divergent from that of North America. From the results obtained so far, we have shown extensive variability in surface antigens coding sequences among *Sarcocystis* eliminated by mammals of the genus *Didelphis* spp. In addition, such divergent alleles may be exchanged in possible sexual recombination processes between different isolates of *S. falcatula* related isolate. The evolutionary relationships within *S. falcatula* related isolates will

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be best clarified after markers less subjected to selection pressures are analyzed in conjunction with surface antigen genes. These results may have a striking impact on the knowledge of the *Sarcocystis* species that infect opossums in Brazil and also in the epidemiology of the infections caused by these protozoans.

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1. Introduction

Sarcocystis neurona and *Sarcocystis falcatula* are very similar species that use the marsupial of the genus *Didelphis* as definitive hosts (Dubey and Lindsay, 1998, 1999). Some studies revealed how complex is the genetic structure of these parasites and confusion about the species identity within these species may be attributed to the many uncertainties related to the complex nature of the parasite genetic structure (Rosenthal et al., 2001; Elsheikha et al., 2005).

Sarcocystis shed by opossums (with the exception of *S. neurona*) can cause disease in a great variety of birds, being commonly associated with acute pulmonary sarcocystosis in zoos (Smith et al., 1990; Page et al., 1992; Dubey et al., 2001). *S. neurona* is the most commonly associated parasite with the equine protozoal myeloencephalitis in horses (Dubey et al., 1991).

Molecular characterization of *S. neurona* strains from opossums (*Didelphis virginiana*) and intermediate hosts from Central California revealed a striking lack of polymorphism across genes encoding glycosylphosphatidylinositol-anchored surface antigens (SAG2, SAG3, and SAG4), even though the authors demonstrated by analyzing other loci that such strains segregated into three genetically distinct groups (Rejmanek et al., 2010). Limited genetic diversity among *S. neurona* strains infecting marine mammals at the three SAGs were also found elsewhere (Wendte et al., 2010). Except for *S. neurona* strains prevalent in the US (Rejmanek et al., 2010; Wendte et al., 2010) little is known about the genetic make-up of *Sarcocystis* shed by opossums from other parts of the Americas.

Surface antigens of apicomplexan parasites are often immunodominant and have been widely studied because of their value for diagnostic purposes (Howe et al., 1998; Atkinson et al., 2000). The surface antigens genes are the most rapidly evolving of all gene families in all Apicomplexa examined (Wasmuth et al., 2009) and important species-specific differences were identified in this gene family between *Toxoplasma gondii* and *Neospora caninum*, two very closely related cyst-forming Coccidia species and members of the phylum Apicomplexa (Reid et al., 2012). The number of SAG related sequences and differences in their transcriptions were temptatively correlated to the extraordinary differences between the host ranges of both parasites. Thus, it is expected that the comparative analysis of SAGs of *Sarcocystis* shed by opossums can put some light on the knowledge of this group of parasites.

Here, we present the results of an experimental work aiming to enhance knowledge of diversity of SAG2, SAG3, and SAG4 among protozoan genus *Sarcocystis* spp. isolated from intestine of marsupials of the genus *Didelphis* spp. The sequences were compared to each other and with fragments of related sequences available in GenBank, revealing great diversity and suggesting that different isolates of *Sarcocystis* spp. may exchange highly divergent alleles.

2. Materials and methods

2.1. Isolates of *Sarcocystis* spp.

Twenty seven samples of oocysts and sporocysts of *Sarcocystis* spp. isolated from South American opossums of the genus *Didelphis* (*Didelphis aurita* and *Didelphis albiventris*) were surveyed from animals captured in the State of Rio Grande do Sul, Brazil (Southern Brazil). The oocysts and sporocyst had been purified as follows: 11 mL of sucrose solution was added to 1 g of homogenized contents of the small intestine or feces. The material was transferred to 15 mL Falcon® tubes and centrifuged at 1500 × g for 10 min. The sporocysts and oocysts present on the topmost of the suspension were recovered with the aid of a platinum loop and transferred to microtubes previously filled with 1.5 mL of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0). The oocysts/sporocysts were washed 2 times with TE and the resulting pellet was subjected to DNA extraction.

2.2. DNA extraction from oocysts and sporocysts

The parasites were resuspended in lysis buffer (10 mM Tris–HCl, 100 mM NaCl, 25 mM EDTA, 1% SDS) to obtain a final volume of 590 μL. The suspensions were freezed and thawed three times for the disruption of oocysts/sporocysts. Then, 5 μL of proteinase K (10 μg/μL) was added and the samples were incubated for 4 h at 56 °C. DNA were purified with phenol–chloroform–isoamyl alcohol and ethanol precipitated as described previously (Sambrook et al., 1989).

2.3. Polymerase chain reaction (PCR)

The primers were designed from multiple alignments between sequences of *S. falcatula* and *S. neurona*. The set of primers were as follows SAG2 (F) GGT CAG AGC TTT GTG CTG AA; SAG2 (R) ACA ACA CTG TGA GAG ATG CGA; SAG3 (F) CTC GCA GTT GCC TGC CTT G; SAG3 (R) ATC CCA CGG ACC CGT TCC C; SAG4 (F) CCG AGG TAC AGT TCA AGG CG; SAG4 (R) CGA CGA CGA TAC CCA ATG CC. The following reaction conditions were used for amplifying all three SAG genes: initial denaturation at 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s. For a 50 μL reaction it was used 2.5 mL of each primer (10 pmol/μL) 5 μL 10× PCR buffer, 1.5 mL $MgCl_2$ (50 mM), 8 μL dNTP (1.25 mM), 0.3 mL of Taq polymerase 1 μL and DNA extracted from the sample target. PCR products were prepared for sequencing using the kit GFXTM (Amersham Biosciences), according to manufacturer's instructions.

2.4. DNA sequencing and phylogenetic analysis

The PCR products were sequenced using the original primers and the Big Dye® chemistry (Applied Biosystems, Foster City, CA, USA). Sequencing products were analyzed on an ABI377 automated sequencer. Both strands of each PCR products were sequenced at least four times in both directions to increase the confidence of sequencing. The sequences were assembled and the contig formed with the phred-base calling and the phrap-assembly tool available in the suite Codoncode aligner v.1.5.2. (Codoncode Corp. Dedham, MA, USA). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was calculated as described elsewhere (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

2.5. Genetic diversity among isolates of Sarcocystis spp.

By using Simpson's diversity index (D) (Simpson, 1949), the genetic diversity among different isolates of *Sarcocystis* spp. was assessed. Simpson's diversity index is a measurement of diversity and its values ranges between 0 and 1. This index estimates the probability that two individuals randomly selected from a sample will belong to different genotypes and is calculated as follows:

$$D = 1 - \left(\frac{1}{N(N-1)}\right)\sum_{n=1}^{s} n_j(n_j - 1)$$

where N is the total number of isolates; s is the total number of genotypes; and n_j is the number of isolates belonging to the J_{th} genotype.

The analysis of nucleotide polymorphism from aligned DNA sequence data were made with the help of DnaSP v5 (Librado and Rozas, 2009). With DnaSP the following parameters of DNA sequence variation were estimated: the ratio between the estimates of *Ka* (the number of nonsynonymous substitutions per nonsynonymous site) and *Ks* (the number of synonymous substitutions per synonymous site), number of replacement changes, number of synonymous changes, number of segregating sites, number of mutations, nucleotide diversity (Pi) and standard deviation of Pi.

3. Results

Gene sequencing yielded the following results: SAG-2, 276–279 nucleotides and six alleles for 26 samples; SAG-3, 415–501 nucleotides (351–357 coding nucleotides) and five alleles for 21 samples; SAG-4, 276–279 nucleotides and 11 alleles for 25 samples. The alleles were named with characters in Roman numerals and each isolate was assigned to multilocus genotype (#1–#15) (Table 1). Sequences were deposited in GenBank under accession numbers JN185345–JN185416.

Table 1
Sarcocystis spp. genotypes identified at the SAG-2, SAG-3, and SAG-4 genes of isolates obtained from feces of the opossum genus *Didelphis* spp.

Isolate	SAG 2		SAG 3		SAG 4		MLG
Sne	I	●	I	●	I	●	#1
G37	II	●	II	●	II	●	#2
G38	II	●	II	●	II	●	
G17	III	●	III	■	XI	■	#3
G23	III	●	III	■	XI	■	
G25	III	●	III	■	XI	■	
G30	III	●	III	■	XI	■	
G27	III	●	IV	■	VII	●	#4
G10	III	●	V	▲	III	●	#5
G28	III	●	V	▲	IV	●	#6
G02	III	●	V	▲	V	●	#7
G26	III	●	*nd*		IV	●	*nd*
Sfa	IV	●	IV	■	IV	●	#8
G16	IV	●	IV	■	X	■	#9
G19	IV	●	VI	▲	X	■	#10
G11	IV	●	*nd*		X	■	*nd*
G07	IV	●	*nd*		XI	■	*nd*
G29	V	▲	V	▲	XI	■	#11
G21	VI	■	V	▲	VI	●	#12
G04	VI	■	V	▲	VII	●	#13
G32	VI	■	V	▲	VII	●	
G33	VI	■	V	▲	VII	●	
G34	VI	■	V	▲	VII	●	
G22	VI	■	V	▲	VIII	▲	#14
G03	VI	■	V	▲	*nd*		*nd*
G13	VI	■	*nd*		*nd*		*nd*
G36	VI	■	*nd*		VII	●	*nd*
G31	VII	■	V	▲	IX	■	#15
G18	*nd*		*nd*		XI	■	*nd*

nd: allele not determined.
Sne: *Sarcocystis neurona*; AY191006 (SAG-2); AY191007 (SAG-3); AY191008 (SAG-4).
Sfa: *Sarcocystis falcatula*; GQ851953 (SAG-2); GQ851956 (SAG-3); GQ851959 (SAG-4).
Each allele was marked with signals (●,■,▲), according to which clade they clustered in the genealogies represented in Fig. 1.

The ancestry relationships among different alleles at each locus were reconstructed using the Neighbor-Joining method. Each genealogy separated the alleles into three clades and each allele was marked with signals (●,■,▲), according to which clade they clustered (Fig. 1). The evolutionary reconstructions revealed a population with high genetic diversity and the value of *D* for the whole isolates surveyed (excluding G37, G38, and *S. neurona*) was 0.87.

With the exception of the alleles I and II identified for SAG2, SAG3, and SAG4, the rest of the alleles were shown to occur in various combinations. In many cases, two isolates have identical alleles at one locus and highly divergent alleles at another locus (i.e. G27/G30; G16/G19; G21/G29). Conversely, isolates G37 and G38 do not share any allele with other isolates and both are very closely related to *S. neurona* at the three loci.

Gene sequences of locus SAG-3 from isolates G37 and G38 differed from the other sequences by an insertion 81 bp long. The insertion contains several dinucleotide repeats of AT, resembling a microsatellite locus. Still considering SAG-3, insertions/deletions (indels) were observed between positions 381 and 384 (using AY191007 as reference).

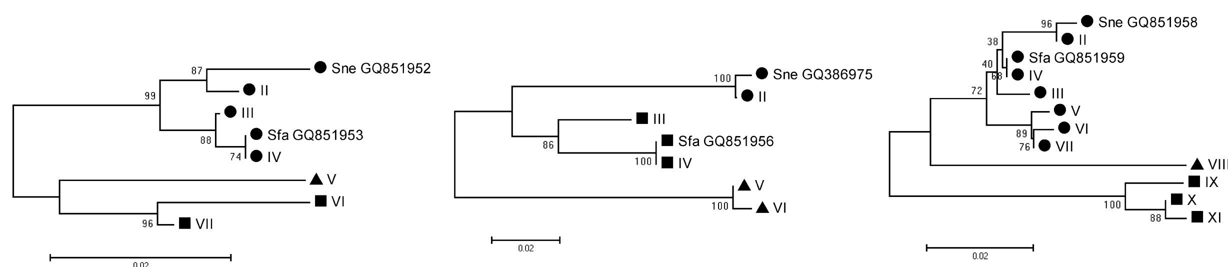

Fig. 1. Evolutionary relationships of taxa using data from SAG2 (left), SAG3 (middle), and SAG4 (right). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Kimura 2-parameter method). All positions containing gaps and missing data were eliminated. Each genealogy separated the alleles into three clades and each allele was marked with signals (●,■,▲), according to which clade they clustered. There were a total of 273 (SAG2), 415 (SAG3), and 277 (SAG4) nucleotide positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

These indels occur in multiples of three nucleotides and represent complete removal of one or two codons. Indels were also observed in alignments of SAG-2 and SAG-4, at the positions 394/395/396 (using AY191006 as reference) and at the positions 655/656/657 (using AY191008 as reference).

The alignment between the haplotypes for each locus revealed that fragments of SAG2 are more conserved than those of SAG4. The highest nucleotide diversity was found among haplotypes of SAG3 and analysis of nucleotide diversity shows that the number of non-synonymous substitutions is higher than the number of synonymous substitutions at all loci (Table 2). However, replacement changes occur more often in the alignment between haplotypes SAG2. The ratio between the number of non-synonymous substitutions per nonsynonymous site and synonymous substitution per synonymous substitution (*Ka*/*Ks*) for each pair of sequence is shown in Table 3.

4. Discussion

Herein we assessed the variability of *Sarcocystis* spp. isolated from opossums of the state of Rio Grande do Sul, Brazil, by sequencing fragments of genes coding for glycosylphosphatidylinositol-anchored surface antigens (termed surface antigen or SAG), SAG2, SAG3 and SAG4. Two genetic groups were identified, one of them related to *S. falcatula* and the other related to *S. neurona*. Strong evidences suggest that isolates related to *S. falcatula* may exchange high divergent alleles in possible sexual recombination processes.

Table 2
Extent of DNA polymorphism and the number of synonymous and non-synonymous (replacement) substitutions on each data file.

	SAG 2	SAG 3[a]	SAG 4
No. of sites excluding gaps	273	369	277
Haplotypes	7	6	11
No. of segreagating sites	25	70	35
No. of mutations	29	71	36
Synonymous changes	7	23	19
Replacement changes	22	48	17
Nucleotide diversity (Pi)	0.03977	0.09937	0.05067
Standard deviation of (Pi)	0.00663	0.01529	0.00893

[a] Only data on coding positions was computed.

The Simpson's diversity index is commonly used for bacterial typing and should be ideally between 0.90 and 1.00 in order to be satisfactorily useful in molecular epidemiology (van Belkum et al., 2007). Although barely used for protozoan typing (Soares et al., 2011), this index strongly indicates that the diversity among *S. falcatula* related isolates is above the expected for species ongoing clonal propagation.

Various allelic combinations of SAG2, SAG3 and SAG4 occur among *S. falcatula* related isolates. For example, some isolates have SAG2 sequences with high similarity to homologous genes of *S. neurona*, but, otherwise have SAG4 sequences more similar to that of *S. falcatula*. This unlinked allelic distribution is indicative of an absence of linkage disequilibrium (LD), i.e. a non-random association between alleles in different isolates of a population. The occurrence of LD in groups within a population may indicate the absence of gene exchange and hence sexual isolation of individuals from that particular group (Tibayrenc, 1999). If *S. falcatula* related isolates shed by opossums exchange genes by sexual recombination, they are exchanging highly divergent alleles and this would confer to the protozoan variability with important consequences in fitness of the parasite and the epidemiology of the infection as well.

Multilocus genotyping has been widely used to reconstruct the evolutionary history of *T. gondii* by direct sequencing or RFLP analysis of a variety of different loci including coding regions for housekeeping genes, antigens, and selectively neutral introns, showing that population structure of this parasite was found to consist of clonal lineages and recombinants among them (Ajzenberg et al., 2002; Lehmann et al., 2006; Sibley and Ajioka, 2008; Su et al., 2012; Minot et al., 2012). Novel recombined genotypes form when a felid is infected with multiple strains that exchange genetic material during the sexual development commencing in enterocytes of the small intestine of the definitive host. Sexual recombination plays an important role in defining *T. gondii*'s genetic structure, especially in South America (Sibley and Ajioka, 2008), where lineages show greater diversity and divergence between groups when compared to the eminently clonal population found in Europe and North America (Khan et al., 2011; Minot et al., 2012).

Table 3
Ratio between *Ka* (number of nonsynonymous substitutions per nonsynonymous site) and *Ks* (number of synonymous substitutions per synonymous site) for each pair of alleles (SAG2 upper, SAG3 middle, and SAG4 bottom).

	I	II	III	IV	V	VI	VII	VIII	IX	X
II	0.32									
	a									
	b									
III	0.32	0.97								
	0.65	0.59								
	0.71	0.53								
IV	0.43	1,30	*c*							
	0.97	0.90	3,04							
	d	*e*	0.00							
V	1,03	2,11	1,29	1,29						
	0.47	0.47	0.57	0.72						
	1,06	0.88	0.17	0.35						
VI	1,05	1,97	1,31	1,42	1,16					
	0.51	0.51	0.62	0.78	*f*					
	1,06	0.88	0.17	0.35	*g*					
VII	1,97	*h*	2.98	3,32	3,23	0.48				
	-	-	-	-	-	-				
	0.89	0.71	0.09	0.17	*i*	*j*				
VIII	-	-	-	-	-	-	-			
	-	-	-	-	-	-	-			
	0.37	0.33	0.23	0.29	0.29	0.37	0.33			
IX	-	-	-	-	-	-	-	-		
	-	-	-	-	-	-	-	-		
	0.44	0.40	0.47	0.37	0.48	0.43	0.40	0.37		
X	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-	-	
	0.33	0.30	0.34	0.27	0.33	0.33	0.30	0.30	0.23	
XI	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
	0.36	0.33	0.37	0.30	0.36	0.36	0.33	0.32	0.35	*k*

a–k: value of *Ks* were zero and value of *Ka* were, respectively, 0.0074, 0.0049, 0.0049, 0.0199, 0.0149, 0.0074, 0.0098, 0.0605, 0.0049, 0.0049, 0.0049.

By analogy, *S. falcatula* related isolates may have a population structure similar or even more diverse than that of *T. gondii* from South America. There are two arguments for this conjecture: first, SAG alleles of *S. falcatula* related isolates appear to be higher divergent from each other than SAG alleles of *T. gondii* and, second, whereas sexual recombination in *T. gondii* may take place during a brief period of gametogony (typically 5–10 days), this event in *S. falcatula* has much more time to occur due to the fact that opossums usually shed *Sarcocystis* during a much longer period (until 200 days post-infection) (Porter et al., 2001). As pointed by Sibley and Ajioka (2008), sexual recombination in *T. gondii* should occur much less often than expected because few cats are likely to be simultaneously infected with multiple strains (due to short period in gametocyte formation) and the parasite has the ability to bypass the cat in transmission by direct oral infection between intermediate hosts, limiting the opportunity for genetic exchange.

Difference between SAG allelic variations among *T. gondii* and *S. neurona* was associated to the fact that *T. gondii* has a much greater range of intermediate hosts when compared to *S. neurona* (Rejmanek et al., 2010). This suggests that the high allelic variation in *S. falcatula* related isolates would reflect a great range of intermediate hosts and should motivate further studies for the detection and identification of *S. falcatula* in several species as nothing is known concerning the host range of this Coccidia in Brazil.

Regarding the group *S. neurona*-like (isolates G37 and G38), none of the individuals in this group share alleles with individuals of the other group. However, this observation remains to be confirmed, because this analysis counted on few numbers of *S. neurona*-like sequences. SAG allelic variation among *S. neurona* strains of North America was low (Rejmanek et al., 2010; Wendte et al., 2010), as only three polymorphic sites were detected at SAG-3 and SAG-4. Comparing G37 and G38 strains and North American strains of

S. neurona, four polymorphisms were identified at SAG-3, five at SAG-2 and three at SAG-4. The SAG3 G37 and G38 sequence possessed a ~100 bp insertion in its intron as already revealed elsewhere (Wendte et al., 2010). When aligned against North American strains of *S. neurona*, G37 and G38 isolates have a deletion of 8 nucleotides within this intron. These results may indicate that *S. neurona* strains of South America are quite divergent from that of North America.

It is noteworthy emphasizing that mixed samples can occur in these cases, since a particular host may presumably be infected and simultaneously eliminating two or more variants of *Sarcocystis*. Among our sequences (all of them were direct sequenced from PCR products), we obtained chromatograms with peaks of double fluorescence only once (data not shown). Double peaks of fluorescence in the sequencing chromatogram may indicate the occurrence of heterozygous or mixture of organisms, leading to mixtures of alleles in a single sample. The absence of double chromatograms in the majority of the samples reinforces the hypothesis that the occurrence of mixed samples was a rare event.

The nucleotide variability at SAG loci was high in all cases. Also, these genes, as is expected, should have evolved under intense positive selection and this inference was made from the observation of the number of non-synonymous and synonymous substitutions on the data.

The nonsynonymous substitution rate *Ka* is the number of observed nonsynonymous substitutions divided by the total number of such type of changes that these sequences are capable of. Analogously, synonymous substitution rate *Ks* is the number of observed synonymous changes divided by the total number of synonymous changes that the sequences are capable of. Thus, *Ka*/*Ks* ratio measures the rate of adaptive evolution against neutral evolution between two sequences and is used to infer the direction and magnitude of natural selection acting on protein coding genes (Hu and Banzhaf, 2008). A ratio greater than one implies positive or Darwinian selection; less than one implies purifying (stabilizing) selection; and a ratio of one indicates neutral (i.e. no) selection. In general, ratios >1 between non-synonymous and synonymous substitutions in a given gene tend to be low because in most cases the mutations are disadvantageous for the organism. As a result, mutant individuals tend to be eliminated from the population or even will not be viable. On the other hand, ratio >1 between non-synonymous substitutions/synonymous can be seen when natural selection favors a change in a given protein (Nei and Kumar, 2000; Hu and Banzhaf, 2008). In our data, even though the number of non-synonymous substitutions were higher than the number of synonymous substitutions, the majority of pairwise comparison of sequences revealed *Ka*/*Ks* <1, except for data from SAG2. Nevertheless, caution is needed in interpreting the ratios between non-synonymous and synonymous substitutions recorded here, because SAG2, SAG3, and SAG4 coding genes were partially sequenced. In fact, when complete SAG sequences of *S. neurona* and *S. falcatula* were compared, the *Ka*/*Ks* ratio of SAG2, SAG3 and SAG 4 were 0.8, 1.6, and 4.76, respectively (data not shown). A combination of positive and purifying selection at different points within the gene or at different times along its evolution may cancel each other giving an average value that may be lower, equal or higher than one (Yang and Bielawski, 2000).

From the results obtained so far, we have shown extensive variability in surface antigens coding sequences among *Sarcocystis* eliminated by mammals of the genus *Didelphis* spp. In addition, such divergent alleles may be exchanged in possible sexual recombination processes between different isolates of *S. falcatula* related isolate. The evolutionary relationships within *S. falcatula* related isolates will be best clarified after markers less subjected to selection pressures are analyzed in conjunction with surface antigen genes.

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References

Ajzenberg, D., Bañuls, A.L., Tibayrenc, M., Dardé, M.L., 2002. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. Int. J. Parasitol. 32, 27–38.

Atkinson, R., Harper, P.A.W., Reichel, M.P., Ellis, J.T., 2000. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. Parasitol. Today 16, 110–114.

Dubey, J.P., Davis, S.W., Speer, C.A., Bowman, D.D., de Lahunta, A., Granstrom, D.E., Topper, M.J., Hamir, A.N., Cummings, J.F., Suter, M.M., 1991. *Sarcocystis neurona* n. sp. (Protozoa: Apicomplexa), the etiologic agent of equine protozoal myeloencephalitis. J. Parasitol. 77, 212–218.

Dubey, J.P., Garner, M.M., Stetter, M.D., Marsh, A.E., Barr, B.C., 2001. Acute *Sarcocystis falcatula*-like infection in carmine bee-eater (*Merops nubicus*) and immunohistochemical cross reactivity between Sarcocystis falcatula and Sarcocystis neurona. J. Parasitol. 87 (4), 824–832.

Dubey, J.P., Lindsay, D.S., 1998. Isolation in immunodeficient mice of *Sarcocystis neurona* from opossum (*Didelphis virginiana*) faeces, and its differentiation from *Sarcocystis falcatula*. Int. J. Parasitol. 28, 1823–1828.

Dubey, J.P., Lindsay, D.S., 1999. *Sarcocystis speeri* n. sp. (Protozoa: Sarcocystidae) from the opossum (*Didelphis virginiana*). J. Parasitol. 85, 903–909.

Elsheikha, H.M., Schott II, H.C., Mansfield, L.S., 2005. Genetic variation among isolates of *Sarcocystis neurona*, the agent of protozoal myeloencephalitis, as revealed by amplified fragment length polymorphism markers. Infect. Immun. 74, 3448–3454.

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

Howe, D.K., Crawford, A.C., Lindsay, D., Sibley, L.D., 1998. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. Infect. Immun. 66, 5322–5328.

Hu, T., Banzhaf, W., 2008. Nonsynonymous to Synonymous Substitution Ratio ka/ks: Measurement for Rate of Evolution in Evolutionary Computation. In: Rudolph, G., Jansen, Th., Lucas, S.M., Poloni, C., Beume, N. (Eds.) 2008. Parallel Problem Solving from Nature – PPSN X 10th International Conference Dortmund, Germany, pages 448–457, September 13–17, Proceedings Series: Lecture Notes in Computer Science, vol. 5199, Springer, Subseries: Theoretical Computer Science and General Issues, XX, 1164.

Khan, A., Dubey, J.P., Su, C., Ajioka, J.W., Rosenthal, B.M., Sibley, L.D., 2011. Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. Int. J. Parasitol. 41, 645–655.

Kimura, M.A., 1980. Simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Lehmann, T., Marcet, P.L., Graham, D.H., Dahl, E.R., Dubey, J.P., 2006. Globalization and the population structure of *Toxoplasma gondii*.

In: Proceedings on National Academy of Sciences USA, 103, pp. 11423–11428.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.
Minot, S., Melo, M.B., Li, F., Lu, D., Niedelman, W., Levine, S.S., Saeij, J.P., 2012. Admixture and recombination among *Toxoplasma gondii* lineages explain global genome diversity. In: Proceedings on National Academy of Sciences USA, 109, pp. 13458–13463.
Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.
Page, C.D., Schmidt, R.E., English, J.H., Gardiner, C.H., Hubbard, G.B., Smith, G.C., 1992. Antemortem diagnosis and treatment of sarcocystosis in two species psittacines. J. Zoo Wildl. Med. 23, 77–85.
Porter, R.A., Ginn, P.E., Dame, J.B., Greiner, E.C., 2001. Evaluation of the shedding of *Sarcocystis falcatula* sporocysts in experimentally infected Virginia opossums (*Didelphis virginiana*). Vet. Parasitol. 95, 313–319.
Reid, A.J., Vermont, S.J., Cotton, J.A., Harris, D., Hill-Cawthorne, G.A., Könen-Waisman, S., Latham, S.M., Mourier, T., Norton, R., Quail, M.A., Sanders, M., Shanmugam, D., Sohal, A., Wasmuth, J.D., Brunk, B., Grigg, M.E., Howard, J.C., Parkinson, J., Roos, D.S., Trees, A.J., Berriman, M., Pain, A., Wastling, J.M., 2012. Comparative genomics of the apicomplexan parasites *Toxoplasma gondii* and *Neospora caninum*: Coccidia differing in host range and transmission strategy. PLoS Pathogens 8, e1002567.
Rejmanek, D., Miller, M.A., Grigg, M.E., Crosbie, P.R., Conrad, P.A., 2010. Molecular characaterisation of *Sarcocystis neurona* strains from opossums and intermediate hosts from Central California. Vet. Parasitol. 170, 20–29.
Rosenthal, B.M., Lindsay, D.S., Dubey, J.P., 2001. Relationships among *Sarcocystis* species transmitted by new world opossums (*Didelphis* spp.). Vet. Parasitol. 95, 133–142.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
Sibley, L.D., Ajioka, J.W., 2008. Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. Annu. Rev. Microbiol. 62, 329–351.
Simpson, E.H., 1949. Measurement of diversity. Nature 163, 688.
Smith, J.H., Craig, T.M., Dillard III, E.A., Neill, P.J.G., Jones, L.P., 1990. Naturally occurring apicomplexan acute interstitial pneumonitis in thick-billed parrots (*Rhynchopsitta pachyrhyncha*). J. Parasitol. 76, 285–288.
Soares, R.M., Silveira, L.H., da Silva, A.V., Ragozo, A., Galli, S., Lopes, E.G., Gennari, S.M., de Jesus Pena, H.F., 2011. Genotyping of *Toxoplasma gondii* isolates from free range chickens in the Pantanal area of Brazil. Vet. Parasitol. 178, 29–34.
Su, C., Khan, A., Zhou, P., Majumdar, D., Ajzenberg, D., Dardé, M.L., Zhu, X.Q., Ajioka, J.W., Rosenthal, B.M., Dubey, J.P., Sibley, L.D., 2012. Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. In: Proceedings of the National Academy of Sciences, 109, pp. 5844–5849.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Tibayrenc, M., 1999. Toward an integrated genetic epidemiology of parasitic protozoa and other pathogens. Annu. Rev. Genet. 33, 449–477.
van Belkum, A., Tassios, P.T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N.K., Fussing, V., Green, J., Feil, E., Gerner-Smidt, P., Brisse, S., Struelens, M., 2007. European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group on Epidemiological Markers (ESGEM). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin. Microbiol. Infect. 13 (Suppl. 3), 1–46.
Wasmuth, J., Daub, J., Peregrin-Alvarez, J.M., Finney, C.A., Parkinson, J., 2009. The origins of apicomplexan sequence innovation. Genome Res. 19, 1202–1213.
Wendte, J.M., Miller, M.A., Nandra, A.K., Peat, S.M., Crosbie, P.R., 2010. Limited genetic diversity among *Sarcocystis neurona* strains infecting southern sea otters precludes distinction between marine and terrestrial isolates. Vet. Parasitol. 169, 37–44.
Yang, Z., Bielawski, J.P., 2000. Statistical methods for detecting molecular adaptation. Trends Ecol. Evol. 15, 496–503.