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Molecular dissimilarities of *Rhipicephalus sanguineus* (Acari: Ixodidae) in Brazil and its relation with samples throughout the world: is there a geographical pattern?

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Abstract In this study the genetic variability of *Rhipicephalus sanguineus* within Brazil and its relation with ticks of the same group from different continents was evaluated. Mitochondrial 12S and 16S rDNA fragments of *R. sanguineus* from seven Brazilian States were sequenced and compared to GenBank sequences of *R. sanguineus* and *R. turanicus* ticks from Africa, Asia, Europe, South America and USA. Results indicate a relatively high intra-specific variability between Brazilian samples but also a global latitude linked distribution pattern of at least two major *R. sanguineus* groups; one group distributed from latitude 25°N to 22°S including *R. sanguineus* from Brazil, Taiwan and Thailand and *R. turanicus* from Zambia and Zimbabwe, and the other group found closer to the poles, roughly above 29°N and below 30°S with ticks from Argentina, Uruguay, France, Oklahoma (USA), Israel and Egypt.

Keywords Genotypic variability · *Rhipicephalus sanguineus* · Brazil · 12S mt-rDNA · 16S mt-rDNA · Latitude

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Introduction

Rhipicephalus sanguineus (Latreille) is probably the most widely distributed tick species in the world (Walker et al. 2000). Its worldwide distribution can be attributed to its preference for dogs and for the same reason it is also referred to as the ''kennel tick'' or ''brown dog tick''. Its origin is, however, uncertain and both an African and Mediterranean origin have been proposed (Pegram et al. 1987a). Besides its global distribution it is a competent vector of several disease agents to both humans and animals. In fact *R. sanguineus* is known to transmit major diseases such as canine ehrlichiosis and babesiosis as well as boutonneuse fever and Rocky Mountain spotted fever to humans (Walker et al. 2000; Demma et al. 2005).

Rhipicephalus sanguineus is not a tick of Neotropical origin and different routes are supposed to be involved in colonization of South America with this tick. According to Aragão (1936), in 1907 this species was already known in the Northern and Northeastern States of Brazil, from Pará until Bahia, meanwhile it was rare in Rio de Janeiro and did not occur in São Paulo, Minas Gerais and Southern States from Brazil. From 1907 to 1936, it became abundant in Rio de Janeiro, Minas Gerais and São Paulo and spread to all other Southern States.

Until recently it was believed that *R. sanguineus sensu stricto* was the only representative of the genus in South America. However, morphological variations among *R. sanguineus* of several states in Brazil were shown to exist (Ribeiro et al. 1996). Later, Szabó et al. (2005) called the attention to the fact that published data on *R. sanguineus* biology throughout the world noticeably varies. Moved by this feeling, Szabó et al. (2005) and Oliveira et al. (2005) compared a population of *R. sanguineus* from Jaboticabal, São Paulo—Brazil with another from Rafaela, Santa Fe—Argentina. Oliveira et al. (2005) observed differences in the external morphology of semi-engorged females (body size, shape of the genital pore and sensory structures, for example) using electron microscopy. Szabó et al. (2005) highlighted that major biological, genotypic and morphological differences exist between both populations and that interbreeding led to non fertile females. An unexpected result of that work was the strong genetic relationship found between Brazilian *R. sanguineus* and *Rhipicephalus turanicus* Pomerantsev from Zimbabwe. These observations raised questions about the origin and distribution of these ticks in both countries and the likelihood of the existence of two dissimilar *Rhipicephalus* species in South America.

Since differences among *R. sanguineus* populations might be linked to differing vectoring capacity and susceptibility to control methods (i.e. acaricides), characterization of *R. sanguineus* populations within Brazil, a country with continental dimensions, is of utter importance. At the same time, determining the relationship of Brazilian strains with *R. sanguineus* group ticks throughout the world may explain its origin and factors involved in its distribution and colonization of the country. This knowledge might help the understanding of regional differences in the epidemiology of diseases transmitted by *R. sanguineus*, as well as to develop better control methods. Thus, the aim of this work was to evaluate the genetic diversity of *R. sanguineus* ticks in the country by comparing the mitochondrial DNA (12S and 16S) of seven different States and four geographical regions of Brazil. Further, the genetic relationship of the Brazilian strains and those from other countries and continents throughout the world was assessed by the analysis of genetic divergence of DNA sequences from the GenBank. *R. turanicus* samples were included in the analysis considering its close and many times unresolved genetic and morphological relationship with *R. sanguineus* ticks (Nava et al. 2009).

Materials and methods

Locality of research

This work was conducted in the Laboratório de Ixodologia (LABIXOD) of the Estação para Pesquisas Parasitológicas W. O. Neitz, Laboratório de Biologia Molecular (LABMOL) and Laboratório de Acarologia, from the Departamento de Parasitologia Animal (DPA), of the Instituto de Veterinária (IV), of the Universidade Federal Rural do Rio de Janeiro (UFRRJ), Seropédica, Rio de Janeiro—Brazil. The sequencing and phylogenetic analysis were conducted at EMBRAPA Seropédica—Agrobiologia, Laboratório de Genoma and Laboratório de Genética e Bioquímica, Seropédica, Rio de Janeiro—Brazil.

Tick DNA samples

DNA samples were obtained from fasting larvae. For this purpose either engorged females or larvae preserved in 70% ethanol (Rondônia sample) were generously provided by researchers from seven different States of five regions of the country (Table 1). Engorged females were collected either from naturally infested dogs (*Canis familiaris*) or from the environment that surrounded dogs. Ticks were identified using the dichotomous keys of Rageau (1953) and Walker et al. (2000). Engorged *R. sanguineus* females were washed in a solution of 2% sodium hypochlorite and then in distilled H_2O. After drying, they were stored in petri dishes and kept in a climatic chamber at the temperature of 27 ± 1°C and relative humidity of 80 ± 10% for egg laying and larval eclosion. Finally, 15 days old fasting larvae were put in glass vials in 70% ethanol and kept at room temperature until use.

Extraction and comparison of *Rhipicephalus sanguineus* tick mitochondrial 12S and 16S rDNA sequences

The DNA was extracted from a sample of 100 mg *R. sanguineus* larvae (≈500 larvae) obtained from a single female by location.

Table 1 State and region in Brazil of the *Rhipicephalus sanguineus* samples used

State/region	City	Coordinates	Provided by	Date
Pará/north	Castanhal	1°17′ 49″ S 47°55′ 19″ W	Dr. Alessandra Scofield Amaral	03/22/07
Rio Grande do Norte/northeast	Mossoró	5°11′ 15″ S 37°20′ 38″ W	Dr. Silvia Maria Mendes Ahid	03/14/07
Rondônia/north	Porto Velho	8°45′ 34″ S 63°54′ 24″ W	Dr. Fábio Barbieri	03/27/07
Goiás/central west	Goiânia	16°40′ 46″ S 49°15′ 18″ W	Dr. Lígia Borges	03/20/07
Mato Grosso do Sul/central west	Campo Grande	20°26′ 40″ S 54°38′ 51″ W	Dr. Carina Elisei and Dr. Renata Madureira	02/16/07
Espírito Santo/southeast	Alegre	20°45′ 46″ S 41°32′ 01″ W	Dr. Isabella Martins	05/11/07
Rio de Janeiro/southeast	Rio de Janeiro	22°53′ 59″ S	MSc. Leonardo Burlini and Graziela Savastano	04/29/07

The DNA extracted were PCR amplified and sequenced using primers previously described by Szabó et al. (2005) and Black and Piesman (1994) of the mitochondrial 12S rDNA and 16S rDNA sequences, respectively. The complete sequence of each primers used were: forward, 5′-AAA CTA GGA TTA GAT ACC CTA TTA TTT TAG-3′; reverse, 5′-CTA TGT AAC GAC TTA TCT TAA TAA AGA GTG-3′ (Szabó et al. 2005) and forward 5′-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′; reverse, 5′-TTA CGC TGT TAT CCC TAG AG-3′ (Black and Piesman 1994), respectively. All PCR reactions were performed in a 50 µl volume. Negative controls (no template) were always run simultaneously. PCR conditions included an initial denaturation step at 94°C for 2 min followed by 35 cycles for 45 s at 94°C, 45 s for primer annealing at 55°C and 45 s for primer extension at 72°C. A final extension step was carried out for 7 min at 72°C. A 5 µl volume of the reaction mixture was examined by 1% agarose-gel electrophoresis followed by staining with ethidium bromide. Amplified DNA was purified for sequencing. For this purpose 32 µl of the PCR product, 8 µl NaCl (5 M) and 40 µl PEG 8000 (22%) were used and the solution was gently homogenized and kept at 4°C overnight. The following day, the material was centrifuged at 13,200 rpm for 15 min at 4°C, the supernatant was removed and the tubes were washed with 500 µl of 70% ethanol, and ethanol excess was eliminated by drying at the end. Finally, the precipitate was suspended in 20 µl Nuclease-Free Water (Promega Corporation, Madison, WI, USA) and stored at −20°C. Purified PCR products were sequenced using the DYEnamic TM ET Dye terminator kit (Pharmacia Biotech) and capillary analysis in a Mega Bace 1000 sequencer.

Seventeen-mitochondrial 12S rDNA sequences and eight-mitochondrial 16S rDNA sequences of *R. sanguineus* and *R. turanicus* available in the GenBank were also used for comparative sequence analysis (Table 2). Sequences were aligned and examined using the computer program MEGA Version 4.0 (Tamura et al. 2007). Similarity matrices were constructed and neighbor-joining trees were generated from Kimura two-parameter distance measure.

Results

Comparison of mitochondrial 12S and 16S rDNA sequences of tick strains from Brazil

The absolute nucleotide differences between *R. sanguineus* group ticks sequences are shown in Tables 3, 4 and 5, and their alignment is shown in Figs. 1 and 2. Variability between Brazilian samples obtained in this work ranged from 0 to 6.6% for the 12S gene and from 0 to 2.7% in the case of the 16S. The 12S gene of the Espírito Santo sample (EU346676, 324 bp) presented the highest dissimilarity in relation to the other Brazilian representatives meanwhile the 16S gene sample from Rio de Janeiro (EU346687, 242 bp) presented the highest divergence values in relation to the others.

In the comparison of the samples obtained in this work to those of the genebank, dissimilarities between 12S rDNA and 16S rDNA gene sequences of the *R. sanguineus* and *R. turanicus* samples of various countries ranged, respectively, from 0 to 15.9% (Table 5) and 0 and 9.8% (Data not shown).

For the 12S gene, there was no variation between sequences of *R. sanguineus* from Rio de Janeiro (EU346680, 322 bp), São Paulo, Thailand and Taiwan (0%) and between the populations from Uruguay, France and Argentina (0%). Low intra-specific variation occurred among the samples form Rio Grande do Norte (EU346678, 352 bp) and Rio de Janeiro, São Paulo, Thailand and Taiwan (0.4%). In the case of the *R. turanicus* 12S gene

Table 2 *Rhipicephalus sanguineus* and *R. turanicus* 12S and 16S rDNA-mt sources and access codes in GenBank

Species	Gene	Source	Geografical origin	Genbank code of access	References
R. sanguineus	12S DNAr-mt	*Canis familiaris*	France	AF150020	Beati and Keirans (2001)
R. sanguineus	12S DNAr-mt		Jerusalem, Israel	U95915	Norris et al. (1999)
R. sanguineus	12S DNAr-mt	*Canis familiaris*/colony	Argentina	AY559841	Szabó et al. (2005)
R. sanguineus	12S DNAr-mt	*Canis familiaris*/colony	São Paulo, Brazil	AY559842	Szabó et al. (2005)
R. sanguineus	12S DNAr-mt	Laboratory colony	Cairo, Egypt	AF133056	Murrell et al. (2000)
R. sanguineus	12S DNAr-mt		Taiwan and Kinmen	DQ003004	Tsai (2006)
R. sanguineus	12S DNAr-mt		Thailand	AY987377	
R. sanguineus	12S DNAr-mt		Uruguay	AY559843	Szabó et al. (2004)
R. turanicus	12S DNAr-mt	Unknown host	Israel	AF150015	Beati and Keirans (2001)
R. turanicus	12S DNAr-mt	Unknown host	Israel	AF150014	Beati and Keirans (2001)
R. turanicus	12S DNAr-mt	Unknown host	Israel	AF150013	Beati and Keirans (2001)
R. turanicus	12S DNAr-mt	*Capra hircus*	Zimbabwe	AF150017	Beati and Keirans (2001)
R. turanicus	12S DNAr-mt	*Equus caballus*	France	AF150018	Beati and Keirans (2001)
R. turanicus	12S DNAr-mt		Jerusalem, Israel	U95916	Norris et al. (1999)
R. turanicus	12S DNAr-mt		Konya, Turkey	AF133057	Murrell et al. (2000)
R. turanicus	12S DNAr-mt		Zambia	DQ849232	
R. sanguineus	16S DNAr-mt		Jerusalem, Israel	L34302	Norris et al. (1999)
R. sanguineus	16S DNAr-mt	Laboratory colony	Spain	Z97884	Mangold et al. (1998)
R. sanguineus	16S DNAr-mt		Taiwan and Kinmen	AY883880	
R. sanguineus	16S DNAr-mt		Taiwan and Kinmen	AY883863	
R. sanguineus	16S DNAr-mt		Thailand	DQ016293	
R. turanicus	16S DNAr-mt		Jerusalem, Israel	L34303	Norris et al. (1999)
R. turanicus	16S DNAr-mt	Laboratory colony	Spain	Z97885	Mangold et al. (1998)
R. sanguineus	Complete DNAr-mt		Oklahoma, US	AF081829/NC002074	Black IV and Roehrdanz (1998)

Table 3 Matrix of absolute nucleotide differences (in bold) and matrix of sequence divergence (in italics), on pair wise comparisons and use of the Kimura-2-parameters logarithm, of the 12S rRNA gene for seven *Rhipicephalus sanguineus* isolated from different states

Samples of *R. sanguineus* 12S rRNA from different states	ES	GO	PA	RO	MS	RJ	RN
Espírito Santo (ES)		*0.065*	*0.055*	*0.065*	*0.066*	*0.055*	*0.055*
Goiás (GO)	**18**		*0.007*	*0.011*	*0.022*	*0.011*	*0.011*
Pará (PA)	**15**	**2**		*0.011*	*0.015*	*0.004*	*0.000*
Rondônia (RO)	**18**	**3**	**3**		*0.018*	*0.007*	*0.015*
Mato Grosso do Sul (MS)	**18**	**6**	**4**	**5**		*0.011*	*0.015*
Rio de Janeiro (RJ)	**15**	**3**	**1**	**2**	**3**		*0.004*
Rio Grande do Norte (RN)	**15**	**3**	**0**	**4**	**4**	**1**	

Table 4 Matrix of absolute nucleotide differences (in bold) and matrix of sequence divergence (in italics), on pair wise comparisons and use of the Kimura-2-parameters logarithm, of the 16S rRNA gene for seven *Rhipicephalus sanguineus* isolated from different states

Samples of *R. sanguineus* 16S rRNA from different states	RJ	RO	MS	PA	ES	GO	RN
Rio de Janeiro (RJ)		*0.018*	*0.022*	*0.027*	*0.022*	*0.027*	*0.027*
Rondônia (RO)	**4**		*0.004*	*0.009*	*0.009*	*0.013*	*0.009*
Mato Grosso do Sul (MS)	**5**	**1**		*0.004*	*0.004*	*0.009*	*0.004*
Pará (PA)	**6**	**2**	**1**		*0.000*	*0.004*	*0.000*
Espírito Santo (ES)	**5**	**2**	**1**	**0**		*0.000*	*0.000*
Goiás (GO)	**6**	**3**	**2**	**1**	**0**		*0.004*
Rio Grande do Norte (RN)	**6**	**2**	**1**	**0**	**0**	**1**	

samples, there was no difference between those of Zambia and Zimbabwe (0%), and 0.4% dissimilarity was observed between the isolates from Israel and Turkey. Surprisingly, the highest dissimilarity for this gene (15.9%) was intra-specific and found between *R. sanguineus* from Espírito Santo and the sequence of the same species from Israel. At the same time, on an inter-specific analysis of the 12S gene, divergence as low as 2.2% was found between Brazilian *R. sanguineus* sample from Rio Grande do Norte and the *R. turanicus* from Africa (Zambia and Zimbabwe).

Taking into account all 12S gene samples irrespective of the species, overall a stronger genetic relationship was detected between *R. sanguineus* strains from Brazil and Asia (Taiwan and Thailand) as well as *R. turanicus* from Africa (Zimbabwe and Zambia). On the other hand, *R. sanguineus* populations from Argentina and Uruguay appeared to be more related to the French, Egyptian and North American (USA, Oklahoma) *R. sanguineus*. A *R. turanicus* sample from France and two from Israel were also more related to this group. A third group was presented by two additional *R. turanicus* samples from Israel and one from Turkey. This group, however, was more related to the French and Argentinean samples then those from Brazil and Asia. The neighbor-joining analysis to the 12S gene yielded the tree shown in Fig. 3.

In relation to the 16S gene (Data not shown), there were no differences between the sequences from Goiás (EU346688, 331 bp) and Espírito Santo (EU346683, 300 bp); between Mato Grosso do Sul (EU346684, 227 bp), Taiwan and Thailand; between

Table 5 Matrix of absolute nucleotide differences (in bold) and matrix of sequence divergence (in italics), on pair wise comparisons and use of the Kimura-2-parameters logarithm, of the 12S rRNA gene for 24 *Rhipicephalus sanguineus* (R.s) and *R. turanicus* (R.t) isolated from different origins

Origin of the isolates	1	2	3	4	5	6	7	8	9	10	11	12
1. R. s. (ES)		*0.065*	*0.072*	*0.065*	*0.065*	*0.058*	*0.058*	*0.058*	*0.054*	*0.054*	*0.076*	*0.076*
2. R. s. (GO)	**18**		*0.014*	*0.011*	*0.022*	*0.011*	*0.011*	*0.011*	*0.011*	*0.011*	*0.029*	*0.029*
3. R. s. (PA)	**20**	**4**		*0.018*	*0.029*	*0.018*	*0.018*	*0.018*	*0.018*	*0.018*	*0.036*	*0.036*
4. R. s. (RO)	**18**	**3**	**5**		*0.018*	*0.007*	*0.007*	*0.007*	*0.007*	*0.014*	*0.032*	*0.032*
5. R. s. (MS)	**18**	**6**	**8**	**5**		*0.011*	*0.011*	*0.011*	*0.011*	*0.014*	*0.036*	*0.036*
6. R. s. Taiwan	**16**	**3**	**5**	**2**	**3**		*0.000*	*0.000*	*0.000*	*0.004*	*0.025*	*0.025*
7. R. s. Thailand	**16**	**3**	**5**	**2**	**3**	**0**		*0.000*	*0.000*	*0.004*	*0.025*	*0.025*
8. R. s. (SP)	**16**	**3**	**5**	**2**	**3**	**0**	**0**		*0.000*	*0.004*	*0.025*	*0.025*
9. R. s. (RJ)	**15**	**3**	**5**	**2**	**3**	**0**	**0**	**0**		*0.004*	*0.025*	*0.025*
10. R. s. (RN)	**15**	**3**	**5**	**4**	**4**	**1**	**1**	**1**	**1**		*0.022*	*0.022*
11. R. t. Zâmbia	**21**	**8**	**10**	**9**	**10**	**7**	**7**	**7**	**7**	**6**		*0.000*
12. R. t. Zimbabwe	**21**	**8**	**10**	**9**	**10**	**7**	**7**	**7**	**7**	**6**	**0**	
13. R. t. Israel 35	**32**	**19**	**21**	**20**	**21**	**18**	**18**	**18**	**18**	**17**	**13**	**13**
14. R. t. Turkey	**30**	**17**	**19**	**18**	**19**	**16**	**16**	**16**	**16**	**15**	**13**	**13**
15. R. t. Israel 1	**31**	**18**	**20**	**19**	**20**	**17**	**17**	**17**	**17**	**16**	**14**	**14**
16. R. t. Israel	**39**	**26**	**28**	**27**	**28**	**25**	**25**	**25**	**25**	**24**	**22**	**22**
17. R. t. Israel 63	**34**	**21**	**23**	**22**	**23**	**20**	**20**	**20**	**20**	**19**	**17**	**17**
18. R. s. France	**38**	**25**	**27**	**26**	**27**	**24**	**24**	**24**	**24**	**23**	**23**	**23**
19. R. s. Argentina	**38**	**25**	**27**	**26**	**27**	**24**	**24**	**24**	**24**	**23**	**23**	**23**
20. R. s. Uruguay	**38**	**25**	**27**	**26**	**27**	**24**	**24**	**24**	**24**	**23**	**23**	**23**
21. R. s. Egypt	**39**	**26**	**28**	**27**	**28**	**25**	**25**	**25**	**25**	**24**	**24**	**24**
22. R. s. US	**28**	**27**	**29**	**28**	**29**	**26**	**26**	**26**	**26**	**25**	**25**	**25**
23. R. t. France	**38**	**25**	**27**	**26**	**27**	**24**	**24**	**24**	**24**	**23**	**21**	**21**
24. R. s. Israel	**44**	**31**	**33**	**32**	**33**	**30**	**30**	**30**	**30**	**29**	**27**	**27**

Origin of the isolates	13	14	15	16	17	18	19	20	21	22	23	24
1. R. s. (ES)	*0.116*	*0.108*	*0.112*	*0.141*	*0.123*	*0.138*	*0.138*	*0.138*	*0.141*	*0.138*	*0.138*	*0.159*
2. R. s. (GO)	*0.069*	*0.061*	*0.065*	*0.094*	*0.076*	*0.091*	*0.091*	*0.091*	*0.094*	*0.098*	*0.091*	*0.112*
3. R. s. (PA)	*0.076*	*0.069*	*0.072*	*0.101*	*0.083*	*0.098*	*0.098*	*0.098*	*0.101*	*0.105*	*0.098*	*0.119*
4. R. s. (RO)	*0.072*	*0.065*	*0.069*	*0.098*	*0.080*	*0.094*	*0.094*	*0.094*	*0.098*	*0.102*	*0.094*	*0.116*
5. R. s. (MS)	*0.076*	*0.069*	*0.073*	*0.102*	*0.084*	*0.099*	*0.099*	*0.099*	*0.102*	*0.106*	*0.099*	*0.120*
6. R. s. Taiwan	*0.065*	*0.058*	*0.061*	*0.091*	*0.072*	*0.087*	*0.087*	*0.087*	*0.091*	*0.095*	*0.087*	*0.108*
7. R. s. Thailand	*0.065*	*0.058*	*0.061*	*0.091*	*0.072*	*0.087*	*0.087*	*0.087*	*0.091*	*0.095*	*0.087*	*0.108*
8. R. s. (SP)	*0.065*	*0.058*	*0.061*	*0.091*	*0.072*	*0.087*	*0.087*	*0.087*	*0.091*	*0.095*	*0.087*	*0.108*
9. R. s. (RJ)	*0.065*	*0.058*	*0.062*	*0.091*	*0.073*	*0.087*	*0.087*	*0.087*	*0.091*	*0.095*	*0.087*	*0.109*
10. R. s. (RN)	*0.062*	*0.054*	*0.058*	*0.087*	*0.069*	*0.084*	*0.084*	*0.084*	*0.087*	*0.091*	*0.084*	*0.105*
11. R. t. Zâmbia	*0.047*	*0.047*	*0.051*	*0.080*	*0.062*	*0.083*	*0.083*	*0.083*	*0.087*	*0.091*	*0.076*	*0.097*
12. R. t. Zimbabwe	*0.047*	*0.047*	*0.051*	*0.080*	*0.062*	*0.083*	*0.083*	*0.083*	*0.087*	*0.091*	*0.076*	*0.097*
13. R. t. Israel 35		*0.014*	*0.018*	*0.051*	*0.033*	*0.054*	*0.054*	*0.054*	*0.061*	*0.065*	*0.047*	*0.072*
14. R. t. Turkey	**4**		*0.004*	*0.047*	*0.025*	*0.043*	*0.043*	*0.043*	*0.051*	*0.054*	*0.036*	*0.061*
15. R. t. Israel 1	**5**	**1**		*0.047*	*0.025*	*0.047*	*0.047*	*0.047*	*0.054*	*0.058*	*0.040*	*0.065*
16. R. t. Israel	**14**	**13**	**13**		*0.014*	*0.073*	*0.073*	*0.073*	*0.080*	*0.084*	*0.069*	*0.083*

Table 5 continued

Origin of the isolates	13	14	15	16	17	18	19	20	21	22	23	24
17. R. t. Israel 63	**9**	**7**	**7**	**4**		*0.058*	*0.058*	*0.058*	*0.065*	*0.069*	*0.055*	*0.076*
18. R. s. France	**15**	**12**	**13**	**20**	**16**		*0.000*	*0.000*	*0.007*	*0.011*	*0.022*	*0.054*
19. R. s. Argentina	**15**	**12**	**13**	**20**	**16**	**0**		*0.000*	*0.007*	*0.011*	*0.022*	*0.054*
20. R. s. Uruguay	**15**	**12**	**13**	**20**	**16**	**0**	**0**		*0.007*	*0.011*	*0.022*	*0.054*
21. R. s. Egypt	**17**	**14**	**15**	**22**	**18**	**2**	**2**	**2**		*0.011*	*0.029*	*0.054*
22. R. s. US	**18**	**15**	**16**	**23**	**19**	**3**	**3**	**3**	**3**		*0.033*	*0.065*
23. R. t. France	**13**	**10**	**11**	**19**	**15**	**6**	**6**	**6**	**8**	**9**		*0.061*
24. R. s. Israel	**20**	**17**	**18**	**23**	**21**	**15**	**15**	**15**	**15**	**18**	**17**	

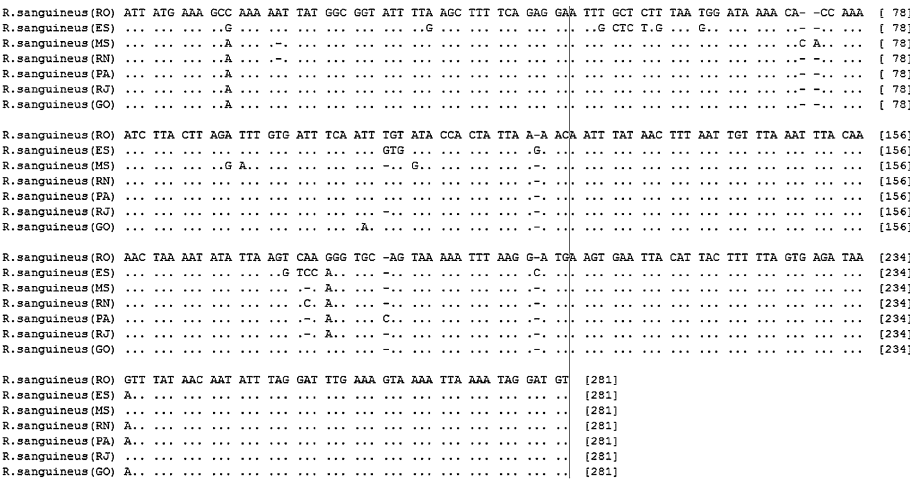

Fig. 1 Alignment of nucleotide sequences (5′-3′) of the 12S rRNA gene of *Rhipicephalus sanguineus* from different regions of Brazil. A *point* indicates that the sequence at that point is identical to the sequence of the top. A *hyphen* indicates a "gap" in alignment

Thailand and Taiwan; and between USA and Spain. Low intra-specific variations were observed between *R. sanguineus* sequences from Rio Grande do Norte (EU346685, 269 bp) and Espírito Santo, Pará (EU346686, 328 bp) and Goiás (0.5%); Pará and Espírito Santo (0.4%); Rio Grande do Norte and Mato Grosso do Sul, Thailand and Taiwan (0.4%). The greatest intra-specific divergence was found between *R. sanguineus* from Rio de Janeiro and the sequences from United States and Spain (9%).

Overall 16S gene related observations broadly agreed with those of the 12S gene with one major cluster comprising *R. sanguineus* from Brazil and Asia and a more distantly related *R. sanguineus* sequence from Israel (L34302). The other cluster comprised *R. sanguineus* from Spain and USA (Oklahoma) and two *R. turanicus* strains, one from Israel and another from Spain. The analysis by "neighbor-joining" for the 16S gene produced the tree shown in Fig. 4.

The analysis by the parameters used for the construction of the tree shown in Fig. 3 provided a strong support (97%) for a cluster containing Argentine, Uruguayan, French,

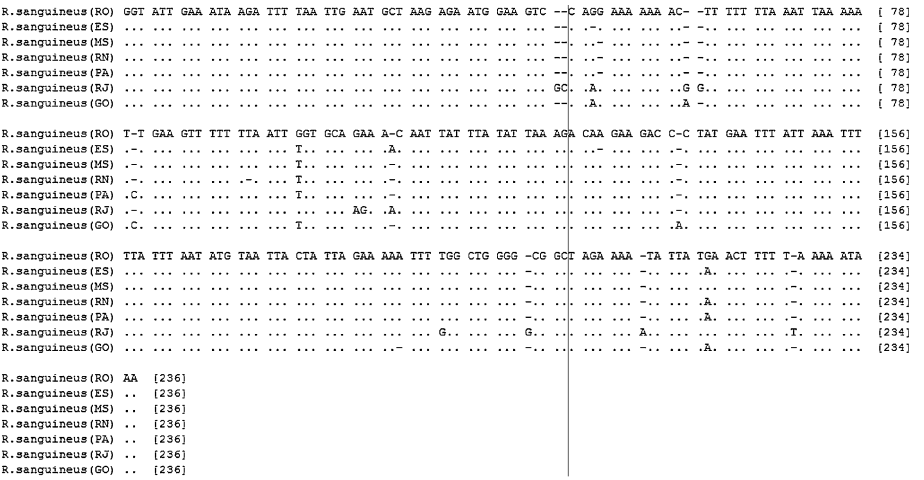

Fig. 2 Alignment of nucleotide sequences (5′-3′) of the 16S rRNA gene of *Rhipicephalus sanguineus* from different regions of Brazil. A *point* indicates that the sequence at that point is identical to the sequence of the top. A *hyphen* indicates a "gap" in alignment

North American and other Mediterranean *R. sanguineus*. A high value of "bootstrap" (100%) supported the close relationship between Brazilian *R. sanguineus* and *R. sanguineus* from Thailand and Taiwan and confirmed the proximity of the Brazilian samples with African *R. turanicus*. In addition, sequences obtained from four different populations of *R. turanicus* from Israel and one from Turkey formed two groups. The formation of four distinct groups within the Brazilian isolates was also observed; sequences from Goiás (EU346681, 390 bp), Pará (EU346679, 383 bp) and Rondônia (EU346675, 338 bp) belonging to a group; Mato Grosso do Sul (EU346677, 332 bp), São Paulo and Rio de Janeiro more Asian samples of *R. sanguineus*, another group; while sequences from Espírito Santo and Rio Grande do Norte have remained each one in a class alone.

In the 16S gene analysis, the results provided strong support (100%) for the cluster containing *R. sanguineus* from Brazil along with those of Asian origin, and showed the close proximity between *R. sanguineus* and *R. turanicus* from Spain.

Last but not least, an interesting observation was a correlation between latitude and tick genetic relationship (Figs. 3, 5). Thus, disregarding annotated tick species for each 12S gene sample, two major groups were found; one built up with ticks found between the latitudes 22°S and 25°N and another by ticks found closer to the poles, below 30°S and above 29°N. Albeit with lesser samples, the 16S gene sequences clustered in similar pattern.

Discussion

As already stated by Pegram et al. (1987b), ticks from the *R. sanguineus* group historically belong to one of the most controversial groups of the genus. The identification and distinction of two species of the genus, *R. sanguineus* and *R. turanicus*, is particularly a challenge. Several factors contribute to the controversy. The type-specimen of *R. sanguineus* has been lost and little is known of its origin. There is a lack of

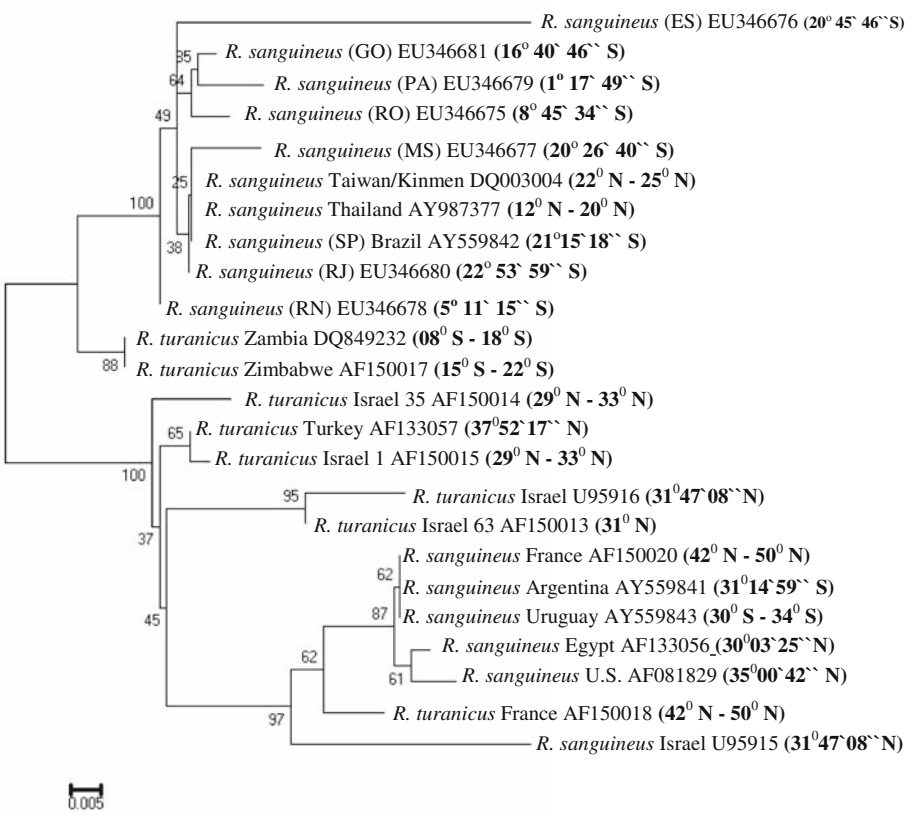

Fig. 3 Neighbor-joining tree (unrooted) of the 12S rRNA gene using Kimura two-parameter distance. *Numbers* represent the percentages of bootstrap support. Latitude for each location (city or country) is presented between *brackets*

morphological features to clearly distinguish both species, however, they are both strongly associated with dogs and are, apparently, cosmopolitan. Moreover in some locations they are found in sympatry, and ticks of a given location might present varying morphological features under the same genetic background (Pegram et al. 1987a, b; Ribeiro et al. 1996; Walker et al. 2000; Beati and Keirans 2001; Bernasconi et al. 2002; Santos-Silva et al. 2008). Furthermore, it can also be supposed that the wide geographical distribution of these tick species led to the appearance of subpopulations with distinct features.

Until not long ago, it was believed that only one species of the group, *R. sanguineus sensu stricto*, inhabited South America. However, it was recently shown that at least two very dissimilar populations of *R. sanguineus* exist in the continent, one in Rafaela, Santa Fé, Argentina and the other in Jaboticabal, São Paulo, Brazil (Szabó et al. 2005). In this work we further investigated the matter by analyzing several *R. sanguineus* populations within Brazil, sometimes as distant 2,946 km from each other. Results showed that, although genetic divergence exists in the country, it is not up to characterize different species. Brazilian samples diverged up to 6.6% for the 12S gene. In this regard, Beati and Keirans (2001) suggested that, in the case of tick 12S gene at least, divergence up to 7.8% indicate an intra-specific variation, and higher values rather than indicate inter-specific

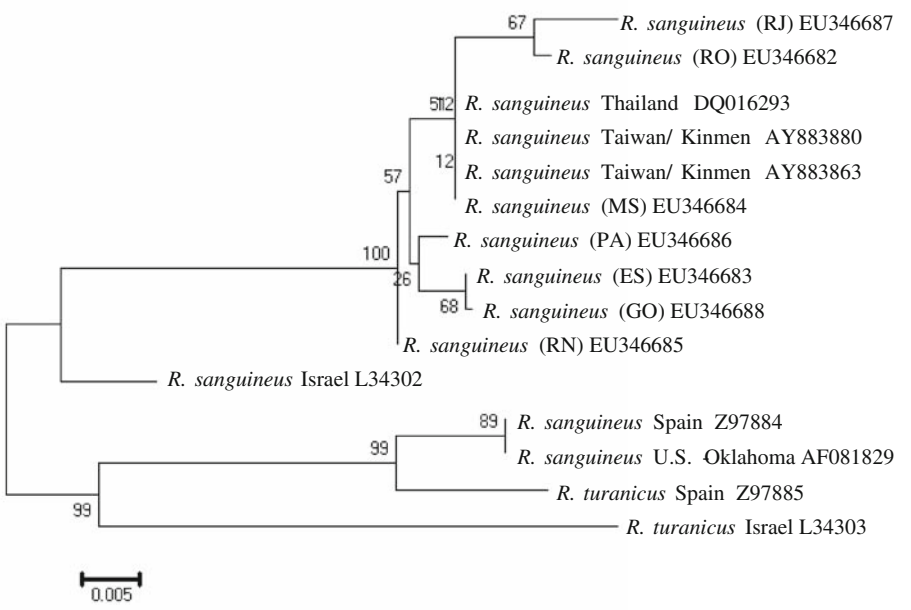

Fig. 4 Neighbor-joining tree (unrooted) of the 16S rRNA gene using Kimura two-parameter distance. *Numbers* represent the percentages of bootstrap support

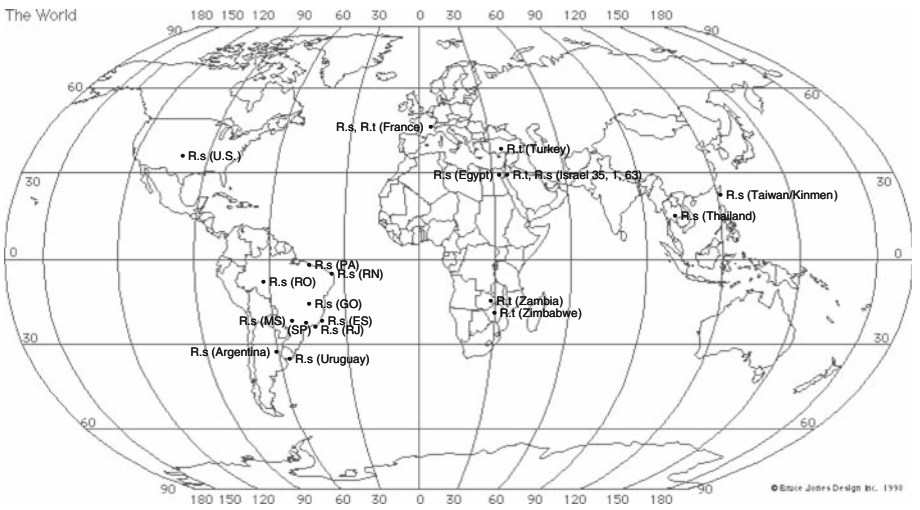

Fig. 5 12 S gene samples and their geographical location. Note that a closer genetic relationship occurs among ticks originated from latitudes from 25°N to 22°S and those below 30°S and above 29°N. *Rhipicephalus sanguineus* (R.s) and *R. turanicus* (R.t)

character. This genetic variability within the country is not unexpected if one considers the distance between tick populations which could lead, as already mentioned, to locally characteristic populations. Thus the Brazilian tick populations analyzed in this work can be

considered a single species and related to the Brazilian strain described by Szabó et al. (2005). Under this perspective, however, *R. turanicus* from both Zimbabwe and Zambia, as well as *R. sanguineus* from Thailand, Taiwan and Brazil also constitute a single tick species. Nevertheless, strong genetic relationship between ticks identified as *R. sanguineus* and *R. turanicus* has been found elsewhere. For instance, Santos-Silva et al. (2008) compared three mitochondrial (12S rDNA, cytochrome oxidase II and control region) and one nuclear (28S rDNA) genes of *R. sanguineus* and *R. turanicus* from Portugal and concluded that they are genetically indistinguishable.

In the analysis of the 16S rRNA gene, the Brazilian *R. sanguineus* samples did not have such a marked distance between them as was the case for the 12S rRNA gene. This might have occurred due to the smaller size of the compared fragments of the 16S rRNA gene. These fragments amounted to only approximately 28% of the total size of the gene, while the 12S rRNA gene fragments amounted to approximately 57% of the total size of the gene. Moreover 16S rRNA sequences of tick from *R. sanguineus* group are scarce in the genebank, thus comparisons were more limited. Results of the analysis of both genes however, overall matched.

In the work by Szabó et al. (2005) the authors raised two possible relations between *R. sanguineus* group tick populations from Rafaela, Argentina and the other from Jaboticabal, Brazil. In the first hypothesis *Rhipicephalus* populations with intermediate features between the populations from Santa Fe and Jaboticabal were to be found and in the second one, two distinct populations would be allopatrically separated or in sympatry. Preliminary data by Moraes-Filho et al. (2008) based on 16S rDNA genes showed that *R. sanguineus* group ticks in South America can be allocated into two major and one smaller clade; one with ticks from eight Brazilian States from the Central-Western, Northern and Southeastern regions (the majority of the country and overlapping geographically with ticks from the present work), Venezuela, Peru, Mexico and *R. sanguineus* from South Africa; a second major clade with ticks from two Southern States of Brazil, Uruguay, Chile, Argentina and Spain, and the smaller clade, located between the clades described above, with ticks from Central-western and Northern Brazil, Colombia and *R. turanicus* from South Africa. Thus the work by Moraes-Filho and colleagues favors the hypothesis that there are in South America at least two distinct *R. sanguineus* group populations which are allopatrically separated with one population above and the other below Southern Brazil. In fact, on a gross overview of the range of 12S gene samples analyzed in our work, latitude related pattern as the one found by Moraes-Filho et al. (2008) can also be seen on a global scale. Hence, disregarding annotated tick identifications, two major groups are found; one with ticks found between the latitudes 22°S and 25°N and another with ticks living closer to the poles, below 30°S and above 29°N. Thus the close relationship of *R. sanguineus* of Brazil with *R. turanicus* from Zambia and Zimbabwe as well as *R. sanguineus* from Taiwan and Thailand fits well in this pattern as does the intimate relationship among *R. sanguineus* group ticks from Europe, Oklahoma and South of South America.

From the above mentioned observations it is tempting to hypothesize that, considering the extensive distribution of the host and the global trade for centuries, both dog-linked *Rhipicephalus* tick populations must have circulated throughout the world. In Brazil, for instance, ticks must have been introduced from both Portugal as well as Africa due to political dependence from the European country and slave trade from the latter. Nevertheless, the establishment of these populations may have been determined by latitude related conditions (i.e. temperature/light:dark regimen). Since one or both of these tick species may survive indoors under artificial conditions, focal populations may have

established outside its latitude range. This hypothesis, however, must be confirmed by several additional observations as well as, if confirmed, there would be many additional issues to clarify. Thus, as a starting point, an analysis including several tick samples from countries all over the world and with more genes would help to unfold the issue (an approach that would help even if the latitude hypothesis is not confirmed). A particularly important issue would be to determine at borderline latitudes the sympathry or allopathry of tick populations as well as the possible presence of hybrids. For instance, South of Brazil as well as South Africa are expected to have both tick populations. Further, altitude related influences on the establishment of these *Rhipicephalus* ticks should be also assessed.

The possible presence of another subpopulation or even a third closely related species must also be kept in mind as pointed out by Nava et al. (2009). This might be the case of the smaller intermediate clade from the work of Moraes-Filho et al. (2008) or of the group formed by two *R. turanicus* samples from Israel and one from Turkey from our work. Last but not least it would be desirable to have data on biological and ecological behavior, vector capacity as well as morphological features of all samples genetically analyzed to make the proper correlations.

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References

Aragão HB (1936) Ixodidas brasileiros e de alguns países limítrofes. Mem Inst Oswaldo Cruz 31(4): 759–843

Beati L, Keirans JE (2001) Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. J Parasitol 87(1):32–48

Bernasconi MV, Casati S, Peter O, Piffaretti JC (2002) *Rhipicephalus* ticks infected with *Rickettsia* and *Coxiella* in Southern Switzerland (Canton Ticino). Infect Genet Evol 2:111–120

Black IV WC, Roehrdanz RL (1998) Mitochondrial gene order is not conserved in arthropods: prostriate and metastriate tick mitochondrial genomes. Mol Biol Evol 15(12):1772–1785

Black WC, Piesman J (1994) Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc Natl Acad Sci USA 91:10034–10038

Demma LJ, Traeger MS, Nicholson WL et al (2005) Rocky mountain spotted fever from an unexpected tick vector in Arizona. New Engl J Med 353(6):587–594

Mangold AJ, Bargues MD, Mas-Coma S (1998) Mitocondrial 16S rDNA sequences and phylogenetic relationship of species of *Rhipicephalus* and other tick genera among Metastriata (Acari:Ixodidae). Parasitol Res 84:478–484

Moraes-Filho J, Pacheco R, Ogrzewalska M, Brandão P, Richtzenhain L, Labruna M (2008) Genetic analysis of different populations of *Rhipicephalus sanguineus* from South America, Mexico, Spain and South Africa: VI international conference on ticks and tick-borne pathogens (TTP-6). Buenos Aires, Argentina 21–26, 2008. Book of Proceedings, poster 272, p. 370

Murrell A, Campbell NJH, Barker SC (2000) Phylogenetic analyses of the rhipicephaline ticks indicate that the genus *Rhipicephalus* is paraphyletic. Mol Phylogenet Evol 16(1):1–7

Nava S, Guglielmone AA, Mangold AJ (2009) An overview of systematics and evolution of ticks. Front Biosci 14:2857–2877

Norris DE, Klompen JSH, Black WC (1999) Comparison of the mitochondrial 12S and 16S ribosomal DNA genes in resolving phylogenetic relationships among hard ticks (Acari : Ixodidae). Ann Entomol Soc Am 92(1):117–129

Oliveira PR, Bechara GH, Denardi SE et al (2005) Comparison of external morphology of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidae) ticks from Brazil and Argentina. Vet Parasitol 129: 139–147

Pegram RG, Clifford CM, Walker JB et al (1987a) Clarification of the *Rhipicephalus sanguineus* group (Acari: Ixodoidea: Ixodidae). I. *R. sulcatus* Neumann, 1908 and *R. turanicus* Pomerantsev, 1936. Syst Parasitol 10:3–26

Pegram RG, Keirans JE, Clifford CM et al (1987b) Clarification of the *Rhipicephalus sanguineus* group (Acari: Ixodoidea: Ixodidae). II. R. sanguineus (Latreille, 1806) and related species. Syst Parasitol 10:27–44

Rageau J (1953) Clés pour l′identification dês tiques du Cameroun. Ann Parasitol Hum Comp 28:5–6

Ribeiro AL, Faccini JLH, Daemon E (1996) Estudo das variações morfológicas de *Rhipicephalus sanguineus* (Latreille, 1806) (Acari:Ixodidae) no Brasi. Rev Univ Rural, Sér da Vida 18(1-2):25–33

Santos-Silva M, Beati L, Vilela C, Bacellar F (2008) Re-evaluation of the systematic status of *Rhipicephalus sanguineus* group in Portugal: VI international conference on ticks and tick-borne pathogens (TTP-6). Buenos Aires, Argentina 21-26, 2008. Book of Proceedings, poster 267, p. 365

Szabó MPJ, Mangold AJ, João CF et al (2005) Biological and DNA evidence of two dissimilar populations of the *Rhipicephalus sanguineus* tick group (Acari:Ixodidae) in South America. Vet Parasitol 130:131–140

Tamura K, Dudley J, Nei M et al (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. doi:10.1093/molbev/msm092

Walker JB, Keirans JE, Horak IG (2000) The genus *Rhipicephalus* (Acari:Ixodidae): *a guide to the brown ticks of the world*. Cambridge University Press, Cambridge