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Phylogeography of *Rhipicephalus sanguineus* sensu lato and its relationships with climatic factors

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Abstract Brown dog ticks morphologically identifiable as *Rhipicephalus sanguineus* sensu lato, are distributed world-wide and their systematics is controversial. Results of genetic and reproductive compatibility studies of geographically distinct populations of *R. sanguineus* s.l. indicate that the *R. sanguineus* complex is paraphyletic. To further elucidate systematic relationships within *R. sanguineus* s.l. and geographic boundaries of its lineages, we conducted a phylogeographical study of 136 tick specimens from 23 countries. Voucher specimens were morphologically identified. A phylogenetic tree was constructed using concatenated partial mitochondrial 12S and 16S rDNA gene sequences and analyzed by the Neighbor-Joining method. A set of 19 bioclimatic variables within the WorldClim dataset were extracted and analyzed to assess correlations between distribution of *R. sanguineus* s.l. lineages and climatic variables. The following four branches are clearly recognized on the phylogenetic tree: *R. sanguineus* s.l.—tropical and temperate clades, *R. leporis,* and *R. turanicus*. DNA sequences of *Rhipicephalus* ticks from Israel differ from those of other groups. Strong association between geographical locations of major clades of *R. sanguineus* s.l. and temperature was identified. The tropical clade of *R. sanguineus* s.l. occupies areas with the annual mean temperature >20 °C, whereas the

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temperate clade is present in areas with the annual mean temperature <20 °C. Our results indicate that ticks in two closely related phylogenetic clades are adapted to different environmental conditions and support proposals for re-classification of *R. sanguineus* complex. Differences in *R. sanguineus* s.l. ecology and human/animal pathogens transmitted by different taxa of brown dog tick need to be studied.

Keywords *Rhipicephalus sanguineus* · Genetic diversity · Phylogenetic analysis · Phylogeographic pattern · Climate variables

Background

Rhipicephalus sanguineus (Latreille), the brown dog tick, is one of the most widely distributed tick species closely associated with dogs and consequently humans. In addition to being a highly prolific ectoparasite, *R. sanguineus* s.l. has both medical and veterinary importance as a recognized vector of a number of pathogens capable of infecting humans and animals (Walker et al. 2000; Dantas-Torres et al. 2012; Eremeeva et al. 2011). At the same time, the taxonomic status of the brown dog ticks has been repeatedly questioned and debated (Gray et al. 2013; Dantas-Torres and Otranto 2015; Nava et al. 2015). Several factors contribute to existing uncertainties: the very limited original description of the species (Latreille 1809), absence of a type specimen, and a high level of morphological similarity among ticks within the *R. sanguineus* complex. As a result, multiple attempts to re-evaluate and re-describe this species as well as the whole *R. sanguineus* complex have been done in the past (Neumann 1911; Zumpt 1939, 1940; Feldman-Muhsam 1952; Hoogstraal 1956; Morel and Vassiliades 1963; Pegram et al. 1987; Filippova 1997; Walker et al. 2000).

Ambiguity in taxonomy of the brown dog tick was reiterated when molecular methods became widely used for phylogenetic analysis. In the last decade several morphological, genetic, and biological studies concluded that *R. sanguineus* s.l. is paraphyletic (Szabó et al. 2005; Liu et al. 2013; Burlini et al. 2010; Moraes-Filho et al. 2011; Levin et al. 2012; Nava et al. 2012). Current data suggests the existence of at least 2 groups of *R. sanguineus* s.l.—so-called temperate and tropical lineages. Results obtained by different researchers reveal a very similar pattern of molecular-level differentiation between groups of samples collected from different geographic locations. The tropical group is represented by *R. sanguineus* s.l. collected in such countries as Brazil, Thailand, Cuba, Colombia, Mozambique and the temperate group includes ticks from Spain, France, Italy, Germany, Argentina (Dantas-Torres et al. 2013). Differences among those geographic sites point to the possible link between lineage of *R. sanguineus* s.l. and environmental variables (climate, habitats or host specificity) associated with the particular locality. We hypothesized that adaptation to different climatic factors might be the key element determining divergence of *R. sanguineus* s.l. species. To test this hypothesis we acquired brown dog ticks from 23 countries around the world, assessed the existing genetic diversity among those ticks, and evaluated correlations between phylogenetic lineages of *R. sanguineus* s.l. and climatic variables.

Table 1 Geographic origin, type, number and morphological identification of *Rhipicephalus sanguineus* s.l. specimens (n = 136)

Country	Location(s)	Specimen type	No. of ticks per site	Morphological ID
Afghanistan	Mazar-e-Sharif	Tick	5	*R. turanicus*
Argentina	Santa Fe, Villa Ocampo	Tick	3	n/d
	Chubut	Tick	2	n/d
Aruba	Alto Vista	Tick	3	*R. sanguineus* s.l.
Brazil	Brasilia	Tick	5	*R. sanguineus* s.l.
Burkina Faso	Bobo-Dioulasso	Tick	5	*R. sulcatus*/ *R. sanguineus* s.l.[a]
Costa Rica	Limon	Tick	2	*R. sanguineus* s.l.
	Hone Creek	Tick	3	*R. sanguineus* s.l.
Djibouti	Djibouti City	Tick homogenate (in ATL buffer)	6	n/d
France	Gard	Tick	3	*R. sanguineus* s.l.
France, Reunion (Africa)	Central part	Tick	5	*R. sanguineus* s.l.
Ghana	Accra	Tick	4	*R. sanguineus* s.l.[a]
Iraq	Ramadi, Al Anbar	Tick homogenate	3	n/d
	Kirkuk	(in ATL buffer)	2	n/d
Israel	Jerusalem	Tick	2	*R. sanguineus* s.l.
Japan	Okinawa Prefecture	Tick	5	*R. sanguineus* s.l.
Kenya	Central part	DNA	3	n/d
Kuwait	Ali Al Salem Air base	Tick	5	*R. leporis*
Kyrgyzstan	Chuy province	Tick	2	n/d
Marshall Islands	Majuro	Tick	5	*R. sanguineus* s.l.
Mexico	Mexicali	DNA	3	n/d
	Hermosillo	Tick	5	*R. sanguineus* s.l.
South Africa	Cape Town	Tick	2	*Rhipicephalus* sp.
Saint Kitts and Nevis	Basseterre	Tick	5	*R. sanguineus* s.l.
Spain	Zaragoza	Tick	2	*R. sanguineus* s.l.[a]
Taiwan	Nantou county	Tick	5	*R. sanguineus* s.l.
Thailand	Central part	Tick	5	*R. sanguineus* s.l.
USA American Samoa	Aunu'u and Tutuila	Tick	4	*R. sanguineus* s.l.
USA AZ	San Carlos	Tick	3	*R. sanguineus* s.l.
USA GA	Atlanta	Tick	5	*R. sanguineus* s.l.
USA Guam	Mongmong-Toto-Maite	Tick	5	*R. sanguineus* s.l.
USA FL	Central part	Tick	5	*R. sanguineus* s.l
USA FL North	St. Johns county	Tick	4	*R. sanguineus* s.l.
USA OK	Oklahoma City	Tick	4	*R. sanguineus* s.l.
USA TX	Central part	Tick	4	*R. sanguineus* s.l.
USA NM	Chuska Mountains	Tick	2	*R. sanguineus* s.l.

[a] Samples presented additional challenges in morphological identification

Materials and methods

Tick collection and morphological identification

Our collection included ticks obtained from dogs in Aruba, Israel, Mexico (Hermosillo), St. Kitts and Nevis, South Africa, and USA (Arizona and Oklahoma states). Additional tick specimens from multiple locations around the world were generously provided by our colleagues (see the acknowledgement). Ticks were collected from dogs, preserved in 70 % ethanol, and sent to our laboratory (Table 1). All specimens suitable for morphological identification were speciated by experienced entomologists either at Georgia Southern University or at the Centers for Disease Control (CDC) using published keys (Filippova 1997; Walker et al. 2000). When acquisition of morphologically identifiable samples was impossible either homogenates in lysis buffer or extracted DNA of brown dog ticks were accepted from the total of four locations. If DNA samples from outside laboratories were used or ticks were damaged and not suitable for identification, only molecular data were analyzed. The complete list of samples and their geographical distribution are presented in Table 1 and https://www.google.com/maps/d/viewer?mid=zB332ow3a1XY.k5opvv0LTo8U&usp=sharing.

Molecular and phylogenetic analysis

DNA from 2 to 6 tick samples per location, depending on sample availability (Table 1) were individually extracted using DNeasy Blood & Tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols and eluted in 100 µl (final volume).

Polymerase chain reactions (PCRs) were performed using primers and cycling conditions for the 12S and 16S gene targets as described by Beati and Keirans (2001) and Mangold et al. (1998), respectively. Reactions were set up using Taq PCR Master Mix kit (Qiagen Inc., Valencia, CA) and run on a Veriti 96 thermal cycler (Applied BioSystems, Foster City, CA). Two negative controls (distilled water) were included into each run. Amplification products were visualized in the 1.5 % Gene Pure LE agarose (ISC Bio Express, Keysville, UT) stained with Ethidium bromide (Sigma-Aldrich, St. Louis, MO) and their size was compared to the 100 bp DNA ladder molecular weight marker (Promega, Madison, WI). Amplicons were purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI). Sequencing reactions were performed using an ABI PRISM 3.0 BigDye Terminator Cycle Sequencing kit (Applied BioSystems, Foster City, CA) as recommended by the manufacturer on an Applied BioSystems 3130*xl* genetic analyzer.

Partial sequences of the 12S mitochondrial rDNA were obtained for multiple locus sequence typing analysis. Sequences of samples from the same sampling sites were compared to each other to identify inter/intra species variations. If sequences of all samples were identical, partial sequences of the 16S mitochondrial rDNA were subsequently obtained for two samples from each site. If sequences of 12S gene demonstrated divergence within the collection site, sequences of the 16S mitochondrial rDNA were obtained and compared for all samples from that site.

For quality and consistency purposes only sequencing data generated in our laboratory were included into phylogenic and geographic analyses. Sequences were assembled using DNASTAR Lasergene 9 and Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was applied to detect homologous sequences. Alignment was performed using DNASTAR Lasergene 12 SeqMan Pro

software. Two genes were concatenated (12S + 16S) and a total of 742–746 nucleotide base pairs were analyzed for each site. Phylogenetic analysis was carried out by Neighbor-Joining statistical method (Saitou and Nei 1987) using MEGA5 software (Tamura et al. 2007). Individual and joined phylogenetic trees were tested using 5000 replicate bootstrap values (Felsenstein 1985). Sequences of *Rhipicephalus* sp. collected in South Africa, were used as an outlier and were not included into climatic analysis.

Climatic analysis

Climate data for each tick sample location were extracted from the WorldClim 10 arc-minute spatial resolution global climate dataset (http://www.worldclim.org/current). These data represent the recent long-term average climatic conditions (1950–2000). A set of 19 bioclimatic variables within the WorldClim dataset (http://www.worldclim.org/bioclim) widely used for ecological niche modeling were analyzed. Values for each of the bioclimatic variables were extracted from the grid point nearest to each latitude/longitude coordinate estimated using Google Earth and corresponding to the tick collection locations. The mean and standard deviation were calculated for all data points falling within tropical and temperate lineages of *R. sanguineus* s.l. identified via phylogenetic analysis. T-tests were used to assess whether the means for each climate variable were statistically different ($p < 0.05$) between the 2 lineages.

Annual mean temperature from the WorldClim dataset (BIO1) was used to create a map showing the distribution of the *R. sanguineus* s.l. lineages in relation to global mean temperature.

Results

Morphological identification

In total, 136 ticks from 36 collection sites representing 23 countries on 5 continents and several oceanic non-continental islands were selected for analysis. Out of those, ticks from 28 locations were suitable for morphological identification. Samples from Argentina and Kyrgyzstan were not suited for morphological analysis and species identification was determined by molecular methods as for tick DNA and tick homogenate samples acquired from Djibouti, Iraq, Kenya, and Mexicali, Mexico (Table 1). Specimens from Afghanistan were morphologically identified as *R. turanicus* and ticks from Kuwait identified as *R. leporis*. Ticks from a majority of locations satisfied identification criteria for *R. sanguineus* s.s according to the standard keys (Filippova 1997; Walker et al. 2000). However, considering on-going debates on taxonomy of *R. sanguineus* complex and in accordance with the proposal by Nava et al. (2015) we designated all specimens fitting those criteria as *R. sanguineus* s.l. Ticks from Burkina Faso, Ghana and Spain were recorded as *R. sanguineus* s.l. by convenience, since their morphological identification was not clear due to current insufficient data as recently revised (Nava et al. 2015; Table 1).

Molecular analysis

Partial sequences of 12S (GB accession numbers KT382479–KT382512) and 16S (KT382445–KT382478) mitochondrial rDNA were obtained for all tick samples as

described above and included into phylogenetic analysis. Examination of DNA sequences revealed that sequences of both gene targets of ticks collected in Afghanistan and Kyrgyzstan were 99 % identical to those of *R. turanicus* from Israel (GenBank accession numbers JQ480850.1) and Uzbekistan (FJ536579.1) for 12S gene and *R. turanicus* from China (KR809581.1) for 16S. Sequences of 12S and 16S rDNA genes of all other tick samples were 98–100 % identical to those of *R. sanguineus* s.l. in the GenBank using BLAST. Sequences of the 12s rDNA gene of tick samples from Kuwait, morphologically identified as *R. leporis*, were 99 % identical to those of *R. leporis* from Iraq (FJ536557.1) and also 99 % identical to those of *R. sanguineus* s.l. from South America (JX206977). Degree of identity of the 12s rDNA sequences of ticks from Israel was 100 % to those of tick collected in Israel and identified as *R. sanguineus* s.l. (JQ480852.1) and 99 % to those identified as *Rhipicephalus* sp. from Jordan (FJ536566.1); they were also 99 % identical to sequences of *Rhipicephalus* sp. morphotype 1 (multiple haplotypes) from Greece (KC243791.1). Only 94 % of identity of 12s rDNA sequences exists between our specimens from Israel and either *R. turanicus* from Kyrgyzstan (FJ536578.1), *R. leporis* from Iraq (FJ536557.1), colony-derived *R. sanguineus* s.l. from Reunion (JQ425164.1), or *R. sanguineus* s.l. from China (JX416325.1).

To assess genetic variation within local populations, 12S rDNA sequences of samples from the same collection sites as well from different sites within one country were compared to each other. Most samples demonstrated 100 % identity. However, the pairwise distance among sequences of *R. turanicus* from Afghanistan (n = 5, one site) was 0.6 % (2/353 base pairs) and *R. sanguineus* s.l. from Kenya and Reunion (n = 3, one site) was 0.3 % (1/354). Analysis of 12S rDNA sequences obtained from ticks collected in Hermosillo (Sonora, Mexico) (n = 5) demonstrated 0.3 % difference (1/316), whereas sequences of ticks collected in Mexicali (Baja California, Mexico) were identical to each other as well as to those of 2 (out of 5) ticks collected in Hermosillo. At the same time, all sequences of the 16S rDNA gene obtained from Hermosillo and Mexicali, Mexico were identical to each other. The greatest within population diversity in 12S rDNA sequences was observed among the 5 sequences of *Rhipicephalus* sp. from Iraq. Sequences obtained from ticks collected in Ramadi and Kirkuk demonstrated 2 % nucleotide sequence difference (7/355). Examination of 16S rDNA sequences from these sites confirmed nucleotide diversity identified among 12S rDNA sequences. The pairwise distance among 16S rDNA sequences was 0.2 % (1/433) in ticks from Kenya and Reunion, 0.5 % (2/426) for Afghanistan, but 1.6 % (7/433) among the Iraq samples.

Phylogenetic analysis

Individual phylogenetic trees constructed based on partial 12S, 16S rDNA sequences (data not shown) and concatenated 12S + 16S sequences of mitochondrial rDNA gene targets (Fig. 1) revealed similar branching pattern. Samples identified morphologically and molecularly as *R. turanicus* from Afghanistan and Kyrgyzstan clustered together. In absence of morphological identification of ticks from Kirkuk, Iraq and the fact that they grouped together with ticks from Kuwait morphologically identified as *R. leporis*, justifies their placement into *R. leporis* clade. Such placement is supported by the high bootstrap value as well as their molecular divergence from ticks collected in Ramadi, Iraq, which in turn were genetically closer to *R. sanguineus* s.l. from Brazil. Majority of the analyzed sample of *R. sanguineus* s.l. phylogenetically separated into 2 distinct clades. Ticks from 20 locations in Asia, Africa, Oceania, and American tropics formed the tropical clade. The temperate clade of *R. sanguineus* s.l. was represented by samples collected in 9 locations in

Europe, North America–USA, and South America–Argentina (Fig. 1). Within-site variations in 12S and 16S rDNA among ticks collected in Kenya and northern Mexico, did not affect the shape of either individual or concatenated gene target trees and all ticks from each site co-clustered on the phylogenetic tree within the tropical clade. With an exception of tick samples collected in Florida, *R. sanguineus* s.l. from all other sites in the continental USA were clustered together with samples from Spain, France, and Argentina in temperate clade. Although tick samples from Israel were identified morphologically as *R. sanguineus* s.l., they were closer to the *R. turanicus* group than to the either clade of *R. sanguineus* s.l. on the phylogenetic tree. Considering the low degree of molecular identity based on 12s rDNA gene sequence analysis among tick samples from Israel and all other groups from our collection those ticks were presented on the dendrogram as *Rhipicephalus* sp. I as proposed by Dantas-Torres et al. (2013).

Climatic analysis

Both *R. sanguineus* s.l. groups contained samples collected in locations geographically widely distributed through the world; however, tick samples belonging to the tropical

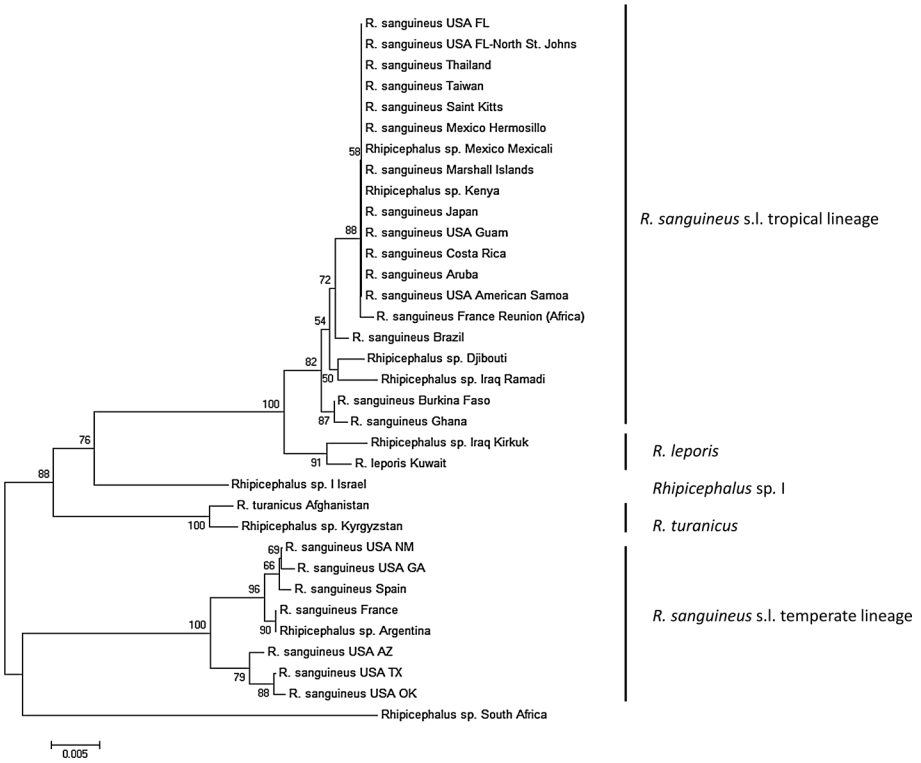

Fig. 1 Phylogenetic relationships of *Rhipicephalus sanguineus* s.l inferred from 12S + 16S rDNA. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 5000 replicates and bootstrap replicates with value less than 50 % were collapsed. Thirty four nucleotide sequences and a total of 738 bp of two genes (12S + 16S) for each sequence were analyzed. All positions containing gaps and missing data were eliminated. Bar = 0.5 % nucleotide sequence divergence. Phylogenetic analyses were conducted in MEGA5. *Rhipicephalus* sp. was used as an outlier

lineage of *R. sanguineus* s.l. were found between the latitudes 35°N and 20°S while samples identified as the temperate lineage were collected either north of the 30th parallel or south of the 43th parallel (Fig. 2).

A set of 19 bioclimatic variables within the WorldClim dataset were extracted and compared with the geographical distribution of tick samples representing the two clades. *T* test was used to evaluate differences in climatic conditions characterizing geographic locations of the two major clades of *R. sanguineus* s.l. Fourteen of the 19 analyzed bioclimatic parameters differed between the two lineages with p value of < 0.01, and the difference in the Mean Diurnal Range (BIO2) had p value of 0.03 (Table 2). Ten of the statistically different parameters including Annual Mean Temperature, Mean Diurnal Range, Isothermality, Temperature Seasonality, Min Temperature of Coldest Month, Temperature Annual Range, Mean Temperature of Wettest, Driest, Warmest and Coldest Quarters pertain to the temperature factors. Annual Precipitation, Precipitation of Wettest Month, Precipitation Seasonality (Coefficient of Variation) and Precipitation of Wettest and Warmest Quarters represent precipitation-associated factors (Table 2).

A map showing distribution of each clade of *R. sanguineus* s.l. and their correlation with annual average near surface temperature demonstrated that samples from tropical lineage are found in areas with annual average near surface temperature between 20 and 30 °C, whereas samples belonging to the temperate lineage are found in areas with annual average near surface temperature varying between 10 and 20 °C (Fig. 2).

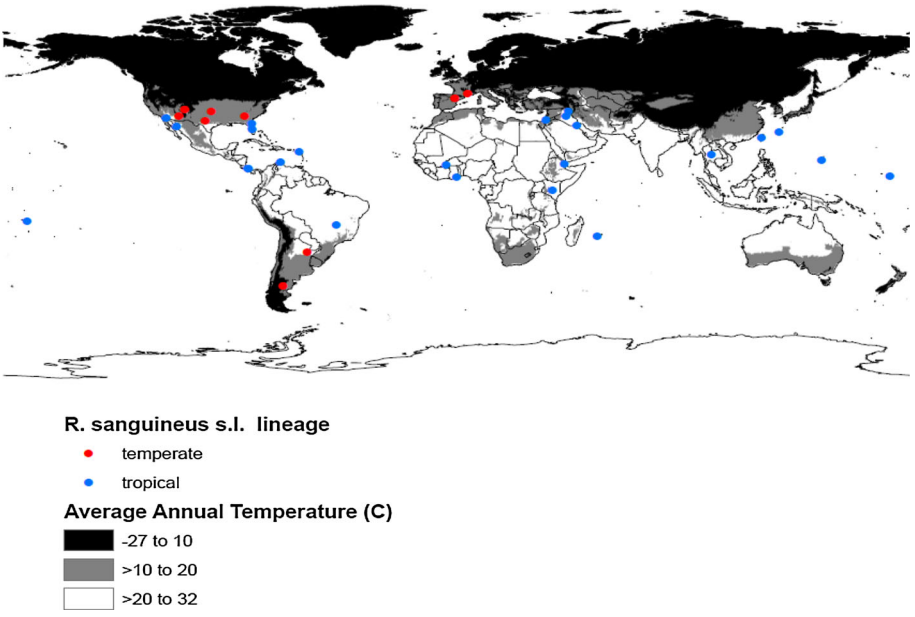

Fig. 2 Schematic map representing correlation between *Rhipicephalus sanguineus* s.l. lineages and mean annual near surface temperature

Table 2 Mean (SD) of long-term bioclimatic variables at locations of *Rhipicephalus sanguineus* s.l. specimen collection sites by tick lineage

Climate variable	*R. sanguineus* lineage		
	Temperate (n = 9)	Tropical (n = 21)	*T* test statistic (*p* value)[a]
BIO1 = Annual mean temperature	15.0 (3.7)	24 (2.5)	−8.15 (<0.01)
BIO2 = Mean diurnal range [mean of monthly (max temp − min temp)]	13.1 (2.3)	9.9 (4.1)	2.24 (0.03)
BIO3 = Isothermality (BIO2/BIO7)	0.4 (0.1)	0.6 (0.2)	−5.05 (< 0.01)
BIO4 = Temperature seasonality (standard deviation)	67.9 (15.7)	26.9 (24.6)	4.59 (<0.01)
BIO5 = Max temperature of warmest month	31.7 (3.8)	33.0 (4.3)	−0.82 (0.42)
BIO6 = Min temperature of coldest month	−0.4 (4.7)	15.4 (6.5)	−6.59 (<0.01)
BIO7 = Temperature annual range (BIO5–BIO6)	32.1 (5.8)	17.7 (9.6)	4.17 (<0.01)
BIO8 = Mean temperature of wettest quarter	17.4 (7.6)	24.9 (4.7)	−3.31 (<0.01)
BIO9 = Mean temperature of driest quarter	15.4 (5.8)	24.0 (4.8)	−4.25 (<0.01)
BIO10 = Mean temperature of warmest quarter	23.7 (3.8)	27.6 (2.7)	−3.19 (<0.01)
BIO11 = Mean temperature of coldest quarter	6.2 (4.4)	20.7 (5.1)	−7.45 (<0.01)
BIO12 = Annual precipitation	642.7 (388.7)	1485.3 (1083.6)	−3.13 (<0.01)
BIO13 = Precipitation of wettest month	88.4 (46.4)	224.4 (129.5)	−4.22 (<0.01)
BIO14 = Precipitation of driest month	25.8 (21.5)	54.3 (67.4)	−1.75 (0.09)
BIO15 = Precipitation seasonality (coefficient of variation)	36.7 (9.9)	58.0 (28.9)	−3.00 (<0.01)
BIO16 = Precipitation of wettest quarter	221.9 (123.3)	589.5 (355.9)	−4.18 (<0.01)
BIO17 = Precipitation of driest quarter	94.2 (72.2)	194.4 (227.4)	−1.82 (0.08)
BIO18 = Precipitation of warmest quarter	169.0 (114.6)	438.1 (314.3)	−3.43 (<0.01)
BIO19 = Precipitation of coldest quarter	128.0 (95.3)	253.1 (288.7)	−1.77 (0.09)

[a] Satterthwaite variance estimator was used when the *F* test for Equality of Variances was significant at $p < 0.05$

Discussion

The necessity to identify a new type specimen (neotype) of *R. sanguineus*, re-describe the species providing detailed morphological and pictorial keys, and re-evaluate the *R. sanguineus* s.l. species complex with regard to the neotype was highlighted by different authors (Dantas-Torres and Otranto 2015; Nava et al. 2015). In the present study, samples of the brown dog tick collected in multiple locations on five continents were identified and partially sequenced; sequencing data were analyzed to elucidate tick phylogeny as well as its correlation with climatic factors. Morphological identification of tick samples was problematic in several instances including ticks from Burkina Faso, Ghana and Spain. These difficulties in identification of brown dog ticks collected in different parts of the World underscore significant variations and overlaps in morphological features of the *R. sanguineus* complex already recognized in previous publications (e.g. Dantas-Torres et al. 2013; Nava et al. 2015).

Rhipicephalus turanicus was confirmed to be present in territories of Afghanistan and Kyrgyzstan, which is in agreement with results of other studies and the original description of this species by Filippova (1997). Two locations, namely Kuwait and Kirkuk, Iraq had *R. leporis*. The 12s rDNA gene target, commonly used for molecular speciation, was not sufficiently informative to resolve speciation in this case, as the 12s sequence in ticks from Kirkuk was 99 % identical to that of both *R. sanguineus* s.l. from South America as well as *R. leporis* from Iraq. Thus, molecular identification alone can be challenging and requires more comprehensive analysis. Tick specimens collected from domestic dogs in Cape Town, South Africa were morphologically identified as *Rhipicephalus* sp. Molecular analysis of 12S rDNA sequences revealed that the closest relative species was *R. zumpti* (98 % identity); several other species including *R. simus, R. pusillus,* and *R. muhsamae* demonstrated relatedness with 96–97 % of nucleotide identity.

Phylogenetic analysis revealed separation of *R. sanguineus* s.l. into 2 distinct clades with a grouping pattern similar to that reported by other researchers (Dantas-Torres et al. 2013; Nava et al. 2012). Morphological, biological, and genetic differences among populations of *R. sanguineus* s.l. from southeastern Brazil and Argentina were revealed and proposal to distinguish tropical and temperate populations of *R. sanguineus* s.l was made earlier (Oliveira et al. 2005; Szabó et al. 2005; Moraes-Filho et al. 2011). *R. leporis* group was close to *R. sanguineus* s.l. tropical lineage, but clustered separately on the phylogenetic tree with 100 % bootstrap value. *Rhipicephalus* sp. I ticks collected in Israel were unique samples in our collection; they were located on the tree between *R. leporis* and *R. turanicus* groups and demonstrated the greatest degree of molecular divergence from any samples included into analysis. Sequences of Israeli ticks were close to those of ticks collected in Greece and Italy (Dantas-Torres et al. 2013), as well as 100 % identical to those of ticks identified as *R. sanguineus* (JQ480852.1) with no information on collection site available in the GenBank. More extensive sampling and analysis of dog ticks from the Middle East is necessary to reveal if either tropical or temperate lineages are present in this geographical region.

Our work represents a first attempt to analyze correlations between phylogenetic clades of *R. sanguineus* s.l. and climatic factors. To date, this direction of the studied topic was not explored, although specific temperature range, relative humidity and day/night photoperiod are important for tick survival and those parameters vary between tick species (Troughton and Levin 2007). Climatic and microclimatic environmental conditions determine distribution and abundance of tick species on the fine scale (e.g. Barandika et al. 2011). Therefore, it is plausible that ticks populating areas with different climatic conditions would experience evolutionary pressures and be forced to adapt to those existing conditions. We analyzed and compared 19 climatic parameters describing temperature and precipitation for each of the 36 geographic locations where *R. sanguineus* s.l. were collected for this project. Fifteen out of 19 parameters were found to be statistically different between the 2 lineages of *R. sanguineus* s.l. Our data suggest that temperature parameters are the key factors as 10 of the 14 differing parameters are related to the temperature. Correlation between the annual mean temperature at the specific locations and the presence of ticks belonging to different clades indicates climatic separation in the distribution of 2 lineages of *R. sanguineus* s.l. Samples belonging to the tropical lineage of *R. sanguineus* s.l. were all acquired within the tropical belt (African and Caribbean countries, Central America, Mexico, Thailand, and Oceania) or in the areas influenced by ocean currents (Taiwan and Florida, USA) with the annual mean temperature between 20 and 30 °C. The distribution of samples of temperate lineage was confined to the areas with the annual mean temperature between 10 and 20 °C: USA and Argentina in Americas, France and

Spain in Europe. Samples from the continental USA outside of Florida belong to the temperate lineage of *R. sanguineus* s.l. Ticks collected at the two locations in northern Mexico (Hermosillo and Mexicali) and those from Florida, USA belong to the tropical lineage.

Our results suggest that the tropical clade of *R. sanguineus* s.l. occupies the circumglobal belt with the annual mean temperature > 20 °C, whereas the temperate clade is present in geographic areas with the annual mean temperature < 20 °C in both Northern and Southern hemispheres. The proposed climatic differentiation based on the annual mean temperature is supported by the fact that ticks from not distant locations in middle Georgia and northern Florida belong to the temperate and tropical clades respectively. Indeed, the annual mean temperature in northern Florida is > 20 °C (20.7 °C), whereas Georgia is located in the colder region with the annual mean temperature of 16.1 °C at the location of tick sampling. Similarly, ticks collected in the adjacent states of Arizona, USA and Sonora, Mexico (Hermosillo) fall into the temperate and tropical lineages respectively. Again, the mean annual temperatures calculated for two collection sites are 24.1 °C in Sonora and 17.1 °C in Arizona. Thus, ticks collected from neighboring sites with different annual average temperatures are phylogenetically distinct. This apparent preference directs colonization of specific geographic locations by divergent lineages of *R. sanguineus* s.l. better adapted to local climate conditions. For example, based on the > 20 °C < rule, *R. sanguineus* s.l. tropical lineage might be found in Southern Texas and Louisiana in the USA.

On the other hand, the fact that distantly located sites in Argentina included in this study with mean annual temperatures of 9.6 °C (Chubut) and 20.7 °C (Santa Fe) both have ticks of the same temperate lineage indicates that the boundaries of climatic areas are neither sharply delineated, nor static. Another study identified presence of both tropical (JX206969.1) and temperate lineages (JX206972.1) in northern Argentina (Formosa) (Nava et al. 2012). The biologic plasticity and species adaptation probably allow for overlaps in distribution of the two lineages as well as presence of non-typical populations in fringe areas. These findings merit further investigation by obtaining and analyzing samples from locations on the "border" of temperature zones. For example, collection of tick samples in southern Georgia and northern Florida with consideration for climatic factors would help to verify the proposed concept of climatic separation between the two major clades of *R. sanguineus* s.l. and provide more precise criteria.

Analysis of temperature-related climatic factors in the Mediterranean and Middle East suggests that this geographical region is more suitable for the temperate lineage of *R. sanguineus* s.l. For example, the mean annual temperature at the collection site of our Israeli samples is 18.3 °C. Interestingly, a cross-breeding study demonstrated that ticks from Israel were able to breed with ticks from Oklahoma, USA, whereas progeny of Israel-Reunion line was infertile (Levin et al. 2012).

Understanding of the climatic separation between lineages of *R. sanguineus* s.l. may also have important implications for public health. For example, a comparative analysis of the vector competence of 2 different populations of *R. sanguineus* s.l. from Brazil, one from Uruguay and one from Argentina for *E. canis* was performed by Moraes-Filho and colleagues (Moraes-Filho et al. 2015). Authors conclude that the absence or scarcity of cases of Canine Monocytic Ehrlichiosis due to *E. canis* in the South America southern cone is a result of vector incompetence of the *R. sanguineus* s.l. that belong to the temperate lineage. Similarly, some evidence has been accumulated regarding divergent strains of pathogenic *Rickettsia* spp. being transmitted by *R. sanguineus* s.l. in different geographic regions. Thus, it is possible that different lineages (or taxons) of this tick are responsible for transmission of specific pathogen genotypes. In particular, it was demonstrated that the

concurrent RMSF outbreaks in Arizona, USA and Mexicali, Baja California, Mexico, while both associate with the brown dog tick, were independent episodes involving genetically different strains of *R. rickettsii* (Eremeeva et al. 2011). Considering that our analysis separates ticks from Arizona and Baja California into different phylogenetic clades, diversion of pathogen isolates is in agreement with the above hypothesis. There is, however, no consensus on this topic. Some authors propose that a particular genotype of *R. rickettsii* is linked to a geographical distribution of the corresponding tick vector, whereas others postulate that phylogeographic pattern of *R. rickettsii* is not dependent on the range of the specific vector (Karpathy et al. 2007; Eremeeva and Dasch 2009; Paddock et al. 2014). To date, there is not enough data to draw definite conclusions and this topic is worth future in-depth study.

Altogether, results of our study emphasize the urgent need for re-classification and re-description of tick species belonging to the *R. sanguineus* complex. Phylogeographic analysis reported here should help in delineation of the geographical distribution of the new taxons. It also provides the basis for future evaluation of the vector competence of different lineages of *R. sanguineus* s.l. for different species and genotypes of human and animal pathogens.

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