Universidad Zaragoza

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Zaragoza, April 25th, 2022.

Dear Editors,

I am uploading to the web site the manuscript to be considered for publication in "Molecular Ecology" with the title "Spatial genetic homogenization of the only clade of the tick *Rhipicephalus microplus* in the Neotropics." The study deals with the unexpected low genetic variability of the *COI, 16S rDNA* and ITS2 gene in populations of the invasive tick *Rhipicephalus microplus* in the Neotropics; we further compared with Asian populations included in clusters of what is known as "*R. microplus* complex". Other species, like *R. annulatus*, and other rhipicephalids have been included for a better support of the maximum likelihood phylogeny.

Our first conclusion is that only clade A (*R. microplus* s.s.) is present in the complete Nearctic-Neotropical region, meaning for only one or a few invasive events of the tick in the region. In this part of the study study we aimed to address the phylogenetic signature of the these three genes along a gradient of environmental conditions, or regarding the spatial distance among samples (purposely collected for this study from Mexico to Argentina).

As before, we obtained unexpected results. The genetic pool of the tick is changing randomly, suggesting probable intermixing of populations from neighbor ranches in a *continuum* of changes. Further on, there is not phylogenetic conservatism in our samples, since the range of environmental traits in the regions have no effect on the phylogenetic signature of the tested genes. I hope you will find this manuscript suitable for the journal and filling its quality standards.

All the best,

Agustín Estrada-Peña

1 Spatial genetic homogenization of the only clade of the tick *Rhipicephalus microplus* in the

2 Neotropics.

3 Running title: *Rhipicephalus microplus* ticks in the Neotropics.

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Abstract

34 This study addresses the variability of the mitochondrial cytochrome oxidase subunit I (*COI*) and
35 *16S rDNA* (*16S*), and nuclear internal transcriber spacer *ITS2* (*ITS2*) genes in a set of purposely
36 collected samples of the cattle tick, *Rhipicephalus microplus* (Canestrini, 1888) introduced in the
37 Nearctic-Neotropical range (Mexico to Argentina), and in geo-referenced sequences from
38 GenBank. The main aims of the study are (i) to provide evidence of the phylogeny of the tick in
39 the region, as consequence of a single or several introductions, (b) to explore a possible impact
40 of environmental traits, and (c) to check for the effect of geographical distance on genetic
41 variability. We included published sequences of *Rhipicephalus annulatus* (Nearctic, Afrotropical
42 and Mediterranean), *R. microplus* (Afrotropical, Asia), to fully characterize the Neotropical
43 populations (total: 74 *16S*, 44 *COI*, and 49 *ITS-2* sequences included in the analysis). Only clade
44 A of *R. microplus* spread in the Nearctic-Neotropics. The K statistic, a measure of phylogenetic
45 signal, supports low divergence rates of every tested gene in populations of *R. microplus* in the
46 target region even under diverging environmental conditions. This test demonstrates that spatial
47 distance and genetic variability are negatively correlated. The low variability of these genes may
48 be due to (i) the recent introduction of the tick in the Neotropics, (ii) a high degree of panmixia
49 because exchange of populations, and (iii) low environmental pressures, promoting a lack of
50 genetic drift. These results have implications for the ecology and control of cattle tick infestations.

51 Keywords: *Rhipicephalus microplus*, *COI*, *16S rDNA*, *ITS-2*, Neotropics, genetic variability,
52 environmental traits, panmixia.

53

Introduction.

Six *Rhipicephalus (Boophilus)* tick species are currently recognized, according to Guglielmone et al. (2014): *Rhipicephalus annulatus* (Say, 1821), *Rhipicephalus decoloratus* Koch, 1844, *Rhipicephalus microplus* (Canestrini, 1888), *Rhipicephalus australis* Fuller, 1899, *Rhipicephalus kohlsi* Hoogstraal and Kaiser, 1960, and *Rhipicephalus geigyi* Aeschlimann and Morel, 1965. It has been postulated that the ancestral range of these species is the Oriental region (Roy et al., 2018). However, *R. microplus* has revealed as an invasive tick, being currently present in most of the Neotropical region and large portions of Africa, after a recent spread event coming from Neotropics (Madder et al., 2011), which suggests that the species can adapt to a wide range of environmental conditions.

Rhipicephalus microplus is regarded as one of the most serious pests affecting cattle health and production (Sutherst et al., 1983). It is considered that the species was introduced in the Neotropics from and into yet unknown sites, most probably with the cattle trade, probably around the XVII century. It is unknown if the tick was introduced only once, like *Amblyomma variegatum* in the Caribbean islands (Barré et al., 1995) or had several introductions (with different genetic signatures) at different points alongside the region. Currently, the Nearctic-Neotropical range of the cattle tick includes a territory ranging from Southern USA to Argentina, around the latitude 32°S. The southern fringe of *R. microplus* in Neotropics fluctuates because of relaxed control measures in the area and because the changing patterns of the winter since cold temperature naturally limits its spread further south (Labruna et al., 2009).

Recent phylogenetic analyses of mitochondrial genome sequences of *R. microplus* indicated the existence of a species complex (Roy et al., 2018). This complex is structured in three clades named clade A or *R. microplus* s.s. with ticks originating from southeast Asia (Burger et al., 2014a), clade B with ticks from China and northern India that are close to *R. annulatus* (Burger et al., 2014a) and clade C from Bangladesh, India, Malaysia, and Pakistan (Low et al., 2015; Roy et al., 2018). These phylogenies have been obtained using sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene, demonstrating the power that this single gene can provide to separate cryptic species. Actually, the *COI* gene has been the preferred marker together with nuclear internal transcriber 2 gene (*ITS 2*) marker, used to track the divergence of *R. microplus* populations in South Africa (Baron et al., 2018) or western Africa (Silatsa et al., 2019) Nuclear genes or single nucleotide polymorphisms (SNPs) have been proposed as suitable for resolving specific identities of ticks (i.e., Paulauskas et al., 2018; Lado et al., 2019). Individual gene markers,

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microsatellites, or SNPs have been used for some species of ticks to look at hybridization, mitochondrial introgression, phylogenetic relationships, interspecific variation, and comparison between mitochondrial and nuclear markers for species/lineages delimitation (i.e., Beati et al., 2013; Gulia-Nuss et al., 2016; Kovalev et al., 2016; Leo et al., 2014; Martins et al., 2016). However, it has been demonstrated that *ITS2*, a nuclear marker, resulted in poor resolution for the phylogeny of the cryptic species of *R. microplus* in comparison with mitochondrial genes (Burger et al., 2014).

To date, the situation has been never examined for *R. microplus* in the Neotropics (Labruna et al., 2009). In Mexico (Nearctic region), *R. microplus* coexists with *R. annulatus* in a not yet completely captured pattern of spatial variability probably due to changes in biotic and abiotic traits. There is no demonstration that *R. microplus* in the Neotropics may have a clear population divergence; indeed, it is unknown if climate patterns in this large range may impact the genetic diversification of the populations of ticks. Hutchinson (1957) defined a species fundamental niche as "all the possible combinations of environmental traits where a species can persist and maintain a viable population in the absence of predators or competitors". The fundamental niche is not itself a heritable trait. However, the characteristics of the niche are defined and constrained by species physiology, which is heritable, and as such can be analyzed in a phylogenetic framework (Wiens and Graham, 2005; Kozak and Wiens, 2010). The Neotropics have areas of rapid transition from humid to arid climates likely producing sharp changes in the diversity of tick assemblages and/or the adaptation of *R. microplus* to different environmental conditions.

Phylogenetic niche conservatism (PNC) is the tendency of lineages to retain their ancestral ecological niche through speciation events (Nee et al., 1992). This is of special interest in the context of an invasive tick species because it means that the ancestral niche could be reflected in the genetic signature of the tick and modified by local environmental traits. Some of the phylogenetic studies conducted in recent years on different organisms indicated that major aspects of the niche are more preserved during evolution and speciation than expected (Donoghue, 2008; Olalla-Tárraga et al., 2011). However, it is considered that PNC may constrain the adaptation to new climatic conditions (Wiens & Graham, 2005).

It has been suggested that phylogeny could be related to the ability to spread by alien species (i.e., Losos, 2008; Kellermann et al., 2012), which immediately suggests the restriction of their spread by biotic (the existence vertebrate hosts for ticks) or abiotic pressures. Studies have

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117 reported a spatial genetic variability of tick populations observing a large distribution range;
118 however, the correlation between environmental traits and genetic variability in ticks has been
119 described without strict comparisons between these features. In other studies, in which this issue
120 has been addressed, the PNC paradigm has supported the existence of partially overlapping
121 ecological ranges of different tick species (i.e., Nava et al., 2014). According to Cuervo et al.
122 (2021), this could be interpreted as speciation phenomena resulting in separating niches as a
123 speciation response to prevailing environmental conditions.

124 In this study, we aimed to evaluate several features of the populations of *R. microplus* in the
125 Nearctic-Neotropics using two mitochondrial genes, *COI* and *16S rDNA (16S)*, and a nuclear gene
126 *(ITS 2)*, in purposely collected populations of the tick, in addition to a core group of samples with
127 reliable sequences and available coordinates downloaded from GenBank. The main aim of the
128 study is to test several questions namely, (a) how many clades of the *R. microplus* complex exist
129 in the Neotropics? (b) is there a spatial gradient of genetic variability among samples in the target
130 region? (c) are climate traits affecting the evolution of the target genes?

131 **Materials and Methods**

132 **1. Collection of ticks and preparation of samples.**

133 All ticks were collected as engorged females at selected points of Central and South America,
134 from Mexico to Argentina (Figure 1). The number of field collections available for sequencing of
135 each gene was variable, but included specimens from Mexico, Brazil, Argentina, and Uruguay,
136 following the large distribution of biomes in the target territory. The collection points were
137 selected based on the prevailing environmental features, providing a wide representation of the
138 sites colonized by *R. microplus* for a phylogeographical analysis. Two of the samples are reference
139 strains (Mexico "La Joya" and Uruguay "Mozo") that animal health authorities keep under
140 laboratory conditions for studies on resistance of *R. microplus* to acaricides and vaccine efficacy.
141 We also included in the analysis DNA from purposely field collected strains from India and
142 Pakistan, all of them belonging to the "clade C" of *R. microplus* as previously defined by Burger
143 et al. (2014a), Low et al. (2015) and Roy et al. (2018), and DNA from field collected strains of *R.*
144 *annulatus* from the Nearctic region (Mexico) to perform a finer phylogenetic comparison. The total
145 number of "populations" of *R. microplus* purposely collected for this study in the target region
146 was 45; data were complemented by 21 sequences of *R. microplus, R. annulatus, R. geigyi* and
147 *R. decoloratus*, obtained from GenBank and originally collected in the Neotropics or the

148   Afrotropical region, as well as three other species of *Rhipicephalus spp.* (not *Boophilus*), together

149   with *Ixodes ricinus* used as an outgroup. All the samples, geographical origin, source, samples

150   IDs, and BOLD/GenBank accession numbers are provided in Supplementary File 1.

151   For field collected specimens engorged females were allowed to oviposit under controlled

152   conditions in incubators (27 °C, 85% relative humidity). Only egg masses coming from a minimum

153   of 10 females were used in this study. The eggs masses from each female were mixed and

154   processed together. Figure 1 includes the sites of surveys and the total samples collected in the

155   Nearctic-Neotropics, as well as their environmental information (for calculations of environmental

156   information, see point 4 of Methods).

157   **2.   DNA extraction, PCR and sequencing.**

158   Total DNA (mitochondrial DNA and genomic DNA) was extracted using DNeasy Blood and Tissue

159   kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. Fragments of two

160   mitochondrial genes: *16S, COI* and the nuclear *ITS2* were amplified by PCR using the primers

161   listed in Table 1. A mounting number of studies hold up the benefits of using more than one DNA

162   genetic marker to compare the congruence of *Rhipicephalus* phylogenies and increment the

163   phylogenetic resolution at species level (Murrel et al., 2000, Nava et al., 2018; Roy et al., 2018;

164   Silatsa et al., 2019).

165   Amplification of the mitochondrial *COI* was performed at total volume of 50 µl containing 2 µl of

166   DNA template, 25 µl H_2O, 5 µl NH_4, 5 µl of dNTPs (2 mM/µl), 2.5 µl of $MgCl_2$ (25 mM/ul), 0.1 µl

167   Bioline Taq Polymerase (Bioline Reagents Ltd, London, UK), 5 µl of each primer (each at 10

168   pmol/µl), and 0.38 µl of Bovine Serum Albumin (20 mg/ml). The thermocycler conditions

169   consisted of an initial denaturation step at 94°C for 1 minute, 5 cycles of pre-amplification of 94°C

170   for 1 minute, 45°C for 1.5 minutes, 72°C for 1.5 minutes, followed by 35 cycles of amplification of

171   94°C for 1 minute, 57°C for 1.5 minutes, and 72°C for one minute, followed by a final elongation

172   step of 72°C for 5 minutes. All PCR products were visualized on a 1.5% agarose gel, and samples

173   showing bands of the correct size were bidirectionally sequenced at the Sequencing Unit, Animal

174   and Plant Health Agency (APHA). Additionally, the amplification of the *16S* and *ITS2* genes was

175   performed at a total volume of 25 µl containing 12.5 µl Platinum™ II Hot-Start PCR Master Mix (2x)

176   (Invitroge), 1 µl of each primer (each at 10 pmol/µl), 10.5 µl of nuclease-free water, and 1 µl

177   genomic DNA. PCR amplification was conducted at 94 °C for 4 min, followed by 30 amplification

178   cycles of 94°C for 60 s, primer-specific annealing temperature of 54°C (*16S*) or 55 °C (*ITS2*) for

179 30 s, 72°C for 30 s, and then a final step at 72 °C for 10min. All PCR products were visualized on

180 a 1.5% agarose gel and samples showing bands of the correct size were bidirectionally

181 sequenced by SecuGen (Madrid, Spain). Due to the high frequency of indels and/or unambiguous

182 peaks some *16S* and *ITS 2* sequences were discarded for subsequent analyses.

183

184 **Table 1.** Genes, primers used for amplification and their respective sequences.
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Gene	Primer	Sequence (5′ → 3′)	Reference
COI	LCO1490	GGTCAACAAATCATAAAGATATTG G	Folmer et al., 1994
	HC02198	TAAACTTCAGGGTGACCAAAA AATCA	
16S	16+1	CTGCTCAATGATTTTTTAAATTGCTGTGG	Black and Piesman, 1994
	16-1	CCGGTCTGAACTCAGATCAAGT	
ITS2	3SA	CTA AGC GGT GGA TCA CTC GG	Barker, 1998
	JB9A	GCACTATCAAGCAACACGACTC	

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187
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3. Molecular analysis and phylogenetic reconstruction

190 Paired bi-directional sequences were combined to produce a single consensus sequence, that

191 was visually inspected, edited and trimmed to the same length to remove ambiguous ends using

192 the Geneious Prime v2.2 software (https://www.geneious.com) and aligned using MAFFT v.7.304

193 (Katoh and Standley, 2013). Full details for each specimen and sequence information can be

194 found at the Barcode of Life Database (BOLD) (https://www.boldsystems.org) within the "Human

195 Pathogens and Zoonoses Initiative", Working Group 1.4, BOLD project RHMCP DNA Barcoding

196 world *Rhipicephalus microplus* (eggs). As mentioned above we completed the alignment and

197 performed more robust phylogenies of the mitochondrial genome sequences (*COI, 16S*) and

198 nuclear gene sequences (*ITS 2*) using sequences retrieved from GenBank based on their

199 taxonomy, the quality of the sequence and their geographic origin.

200 The three phylogenetic trees (*COI, 16S, ITS 2*) were inferred according to the Maximum

201 Likelihood (ML) method with 100 bootstrap replicates using the MEGAX software (Kumar et al.,

202 2018). Phylogenetic tree annotation and visualization were performed using FigTree v1.4.4

203 (http://tree.bio.ed.ac.uk/) (Rambaut, 2014). The software jModelTest2 v.2.1.10 was used to select

204 the best-fitting model of nucleotide evolution for each gene fragment testing framework based

205 on the Akaike Information Criterion or AICc (Sugiura 1978, Darriba et al., 2012). The HKY+G

206 model was selected as the best fit available for the Nearctic-Neotropical *Rhipicephalus* phylogeny,

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207 and the GTR+G+I was the model obtained for the Asian-Neotropical *Rhipicephalus* phylogeny.

208 And the GTR+G model was selected as the best fit available for the Nearctic-Neotropical

209 *Rhipicephalus* phylogeny of the 1*6S* whereas the HKY was selected as the best fit available for the

210 Nearctic-Neotropical *Rhipicephalus* phylogeny of the *ITS2*. The GTR+G was the model obtained

211 for the Asian-Neotropical *Rhipicephalus* phylogeny of both *16S* and *ITS2*. Genetic divergence

212 values (% Identity) were obtained using the software Geneious Prime v2.2 software.

213 **4. Environmental data and environmental distance among populations of Neotropical *R.***

214 ***microplus* as driver of genetic variability.**

215 Once the maximum likelihood trees were built for each gene (*COI, 16S, ITS2*), we aimed to

216 correlate the phylogeny of the Nearctic-Neotropical samples of *R. microplus* with selected

217 environmental traits, including the temperature (which regulates the development of the molting

218 stages of the tick), and the vegetation stress (a proxy for relative humidity) (which regulates the

219 mortality). The focus is to investigate if environmental features could impact the molecular

220 divergence of marker genes of the geo-referenced strains of *R. microplus* in the target region.

221 Thus, if the environmental disparity (i.e., the relative differences among the bioclimate regions

222 occupied by the surveyed ticks) is correlated with the observed variability of the targeted genes,

223 then these effects could be measured by comparing the environmental variability with the genetic

224 distances among populations. We used the monthly values of LST, and NDVI obtained for the

225 period 1980 to 2018. These datasets were obtained from the MODIS satellite repository at

226 monthly chunks and converted to the monthly averaged values for the complete period 1980-

227 2018. This resulted in two sets of variables covering the 12 months of a natural year.

228 Separated stacks with the 12 monthly layers of the two variables before, averaged for the period

229 1980-2018, were used for an unsupervised classification of the territory (total: 24 variables). The

230 purpose is to classify and group portions of the territory on the grounds of climate. We applied

231 an unsupervised classification, but we do not provide any response data (that is, we do not identify

232 any pixel as belonging to a particular class). This is useful when there is not prior knowledge of

233 the status of an area. It is the method of choice to classify a large region into clusters. We adhered

234 to the k-means algorithm incorporated in the software Erdas Imagine (Hexagon Software:

235 https://www.hexagongeospatial.com/products/power-portfolio/erdas-imagine) to process the

236 set of raster maps resulting in a bioregionalization based on the three climate variables. To

237 implement a *k*-means classification algorithm, a target number of regions *k* was determined by

maximizing cluster validity index. The Calinski-Harabasz Variance Ratio Criterion (VRC) (Calinski and Harabasz,1974) was used to measure within-group and between-group dispersion. The classification produced a set of areas representing a unique combination of environmental values, as summarized in the Figure 1. Clusters of the same category are statistically inseparable, and clusters belonging to different categories are statistically different from others within the margins of the k-means algorithm.

We calculated the environmental distance among categories by the Schoener's D distance using the package ENMTools (Warren and Dinnage, 2020) for R (R Core Team, 2020). This calculates a Euclidean distance among categories based on the differences among the monthly variables of LST and NDVI. We obtained the phylogenetic signal in the matrix of environmental distances using the "multiPhylosignal" function of the package "picante" for R (R Core Team, 2020). The K statistic is a measure of phylogenetic signal that compares the observed signal in a trait to the signal under a Brownian motion model of trait evolution on a phylogeny (Blomberg et al. 2003). K values of 1 correspond to a Brownian motion process. K values closer to zero correspond to a random pattern of evolution, while K values greater than 1 indicate strong phylogenetic signal and conservatism of traits.

We only used geo-referenced records for this part of the study, because each sample must be ascribed to a bioclimatic region. A K value higher than 1 would indicate conservatism of the traits under different environmental traits and thus a phylogenetic signal.

5. Spatial distance among populations as driver of genetic variability.

Other than the possible molecular divergence of the marker genes according to environmental features, phylogenetic dissimilarity among Neotropical populations could occur because the spatial separation of the samples that do not interbreed. Phylogenetic signal was used to verify the correlation between the genetic variability of the target genes of the Neotropical *R. microplus* and the geographic distance of the samples. Geographic distance matrices were generated using the latitude and longitude points of each sample with the package geodist (Padgham and Sumner, 2020) in R (R Core Team, 2020). As before, "multiPhylosignal" function of the package "picante" for R was used to measure the genetic similarity and the logarithm (base 10) of the distance in km was calculated. To limit inconsistences in the results, we used logarithmic transformation of the data to adjust large variability in the distance among strains i.e., some strains

268   may be separated by a few hundred km (i.e., Uruguay and Argentina) while other may be
269   separated by thousands of km (i.e., Mexico and Argentina).

270   **Results.**

271   **1. Only *R. microplus* s.s. (clade A) exists in the Nearctic-Neotropics.**

272   The phylogeny of *R. microplus* constructed with two mitochondrial (*COI*, *16S*) and one nuclear
273   (*ITS2*) gene showed a similar structure (Figures 2-4). To note, not every tree was constructed using
274   the same number of samples, because other than populations obtained explicitly for this study,
275   the number of sequences from GenBank varied. The mitochondrial gene *COI* (Figure 2) showed
276   strong support (100%) for the clade that includes all the Nearctic-Neotropical *R. microplus*, and
277   moderate support for the clade that included the *R. microplus* strains from China, Philippines, and
278   Kenya (67%). The *R. microplus* clade is sister to the *R. annulatus* strains (96%) which are also sisters
279   to *R. microplus* India, Pakistan and China (85%). Thus, the *COI* maximum likelihood tree
280   successfully resolved the relationships within the *R. microplus* complex and supported monophyly
281   for *R. annulatus* in Neotropics (Figure 2). Meanwhile, the *16S* maximum likelihood tree (Figure 3)
282   showed weak support for *R. microplus* cluster and for its division in the clades that included *R.*
283   *microplus* from Neartic-Neotropics and African strains, plus *R. microplus* from Asian strains (less
284   than 50% bootstrap, not showed in the phylogenetic tree). However, there is good support
285   among these clades for the monophyly of *R. annulatus* from Mexico (87%) and Israel and *R.*
286   *microplus* from India (86%). The *ITS2* maximum likelihood tree (Figure 4) showed weak support
287   for the *R. microplus* clade (57%), and thus reflects a poor phylogenetic structure within the *R.*
288   *microplus* complex. A nuclear marker was unable to differentiate between the Nearctic-
289   Neotropics and the Asian clades of R. *microplus,* whereas it strongly identified the *R. annulatus*
290   clade (99%), *R. bursa* (100%), *R. sanguineus* (100%), *and R. geigyi* clades (99%) (Figure 4).

291   We plotted the mean identity among the different groups of ticks. The purpose is to group taxa
292   and localities, to know if the variability of the three genes observed in the groups of samples is
293   coherent and compatible with the current knowledge of the phylogenetic position of each
294   species. We used the main sets of *R. microplus* (Neotropical, African, Asian) and *R. annulatus*
295   (Nearctic, African, Mediterranean), testing separately against *R. australis*, *R. decoloratus*, *R. geigyi*,
296   other *Rhipicephalus* spp., and the outgroup *I. ricinus* (Figure 5). The results demonstrated the
297   high similarity of the *R. microplus* collected in Africa and the Neotropics with the samples of the
298   clade A *sensu* (Burger et al., 2014) included in the analysis. Asian representatives of clade C *sensu*

299 (Burger et al., 2014) are well separated from the samples above. Results strongly support that only
300 *R. microplus* clade A *sensu* (Burger et al., 2014) is present in the Nearctic and Neotropics. We
301 interpreted this finding as the consequence of only one or few invasive events of the species in
302 the Americas. In the same way, *R. annulatus* collected in places of the Nearctic, Africa and the
303 Mediterranean region are almost identical. This high similarity of Neotropical and African
304 populations of *R. microplus* and *R. annulatus* supports the utility of *COI* and *16S rDNA* for tracking
305 the status of populations that became separated probably hundreds of years ago, even if both
306 populations (African and Neotropical, of either *R. annulatus* or *R. microplus*) came from different
307 invasive events at different moments of the timeline. Supporting previous results, *ITS2* was unable
308 to separate the main groups of *Boophilus*; only species belonging to the main outgroup and to
309 "other rhipicephalids" were adequately separated with this nuclear marker.

310 **2. Environmental traits are not driving the mutation rates of three genes of *R.***
311 ***microplus.***

312 We argued if the gradient of climate in the Neotropics could be the driver of the small genetic
313 differences found in the samples of Neotropical *R. microplus*. The hypothesis is that most similar
314 populations should colonize similar environmental regions. A K test comparing differences of
315 climate and genetic similarities showed that climate plays no role for the variability of the
316 sequences of *COI*, *16S rDNA* and *ITS2* of the geo-referenced populations of *R. microplus* (test
317 value= 0.04, p<0.01; test value= 0.03, p<0.03; test value= 0.01, p<0.01, respectively for each
318 gen). The test value near zero and the high p-value indicated that the use of the environmental
319 niche by populations with slightly divergent sequences is almost random and that environmental
320 traits do not affect the mutation rates of neither *COI*, *16S*, nor *ITS2*.

321 **3.   There is a random pattern of genetic evolution of *R. microplus* according to**
322 **distance in Neotropics.**

323 We tested if spatial distance influences the genetic variability of the three genes included in this
324 study. The hypothesis is that more distant samples should be less related. K test of similarity
325 comparing genetic variability and geographic distance produced test value of -0.44 (p<0.01) for
326 *COI*, -0.21 (p<0.05) for *16SrDNA* and -0.05 (p=0.633) for *ITS2*. These results are indicative of a
327 random genetic evolution; in other words, closer populations are not more (dis)similar than
328 separated ones. Therefore, we hypothesize that geographical distance may not impact the small
329 genetic differentiation between the populations of *R. microplus* in the Neotropics. Closer

330 populations of *R. microplus* in the Neotropics should be more similar than those geographically

331 separated, and hypothesis not validated by our results. The values of the K statistic could be

332 explained by a mixed effect of short- and long-distance mixing, either because the contiguity of

333 ranches, or because long-distance trade of hosts. Supplementary Material 2 shows the values of

334 K statistic among each pair of samples, also including the distance in kilometers among geo-

335 referenced samples of *R. microplus* from the Neotropics.

336 **Discussion**

337 This study addressed the current phylogenetic structure of *R. microplus* complex in the

338 Neotropics. For that, the phylogeny *R. microplus* was constructed using the molecular marker

339 genes *COI*, *16S*, and *ITS2* genes, as previously described in similar studies (Hebert et al., 2003a,

340 2003b; Murrel et al., 2000, Coimbra-Dores et al., 2018; Nava et al., 2018; Roy et al., 2018; Silatsa

341 et al., 2019; Mohamed et al., 2022). We further compared the obtained sequences with data from

342 the Oriental region, the proposed center of the tick spread.

343 Overall results suggested that the *COI* gene should be the preferred molecular marker to infer

344 the relationships among the *R. microplus* complex, showing a strong support for the split of the

345 Neartic-Neotropical and Asian clades (Burger et al., 2014a; Roy et al., 2018). This study

346 demonstrated that every individual of *R. microplus* collected (or with available sequence(s)) in the

347 Neotropical region belongs to the clade A of the complex of species *sensu* (Burger et al., 2014;

348 Low et al., 2015; Roy et al., 2018). This is an important finding because, after the reclassification

349 of these cryptic species into clades, it was important to test the probable existence of different

350 clades in the Nearctic-Neotropics. This finding could support the hypothesis of one or a few

351 invasive events of *R. microplus* clade A in the Nearctic-Neotropical areas, although it is yet

352 unknown how and when the species was introduced. Meanwhile the 16S gene showed certain

353 limitations supporting clustering of Neartic-Neotropic and Asian R. *microplus* clades. The

354 phylogeny obtained using a nuclear marker (*ITS2* gene), was unable to differentiate the clades

355 within the *R. microplus* context (Burger et al., 2014a).

356 Additionally, and for comparative purposes, the phylogenetic relationships among the few

357 samples of the Asian *R. microplus*-like that were included in the study were not different with

358 samples from India and Pakistan strains, shaping altogether in a tight cluster. Only one sample

359 from China splits from that cluster. This event agrees with previous findings describing the group

360 of cryptic species existing in the Asian region (Roy et al., 2018). These clades are not yet formally

361 defined at specific level and referred to as "*R. microplus*" because of the lack of tests on crosses

362 and back-crosses under controlled conditions among representatives allowing species

363 delimitations (a fertile progeny *versus* a hybrid one). Morphological studies exist for specimens

364 of the so-called Clade C (Roy et al., 2018) as well as for *R. australis* (Estrada-Peña et al., 2012).

365 Both *COI* and *16S rDNA* sequences of *R. microplus* in the Nearctic-Neotropical regions have a

366 very low variability, some of the populations displaying 100% identity gene sequences. However,

367 both genes are enough to separate the different clades of the species, and to produce adequate

368 separation with the species used as outgroups. Actually, *COI* was chosen as the only marker for

369 providing evidence of *R. microplus* in Kenya and Sub-Saharan Africa (Kanduma et al., 2020), the

370 only mitochondrial marker in studying the taxonomic status of the complex of species (Berry,

371 2017) for checking the population variability of the tick in South Africa (Baron et al., 2018). The

372 finding of a significant negative correlation between the genetic distances of the *COI* and *16S*

373 *rDNA* genes among the geo-referenced samples of *R. microplus* and the geographic distance

374 confirms that populations tend to be similar at short distances. However, given the small

375 divergence found in sequences, these results should be taken with caution. Crosses over long

376 distances may happen, perhaps derived from cattle trade over long distances which highlights

377 inadequate tick control in some cases (Thomson et al., 2004).

378 *Rhipicephalus microplus* is distributed along a *continuum* over the continent, excluding patches

379 where the climate is locally unsuitable, adequate hosts are absent or where active control

380 campaigns persist. Under these conditions, we hypothesize that the contact among cattle herds

381 kept in contiguous ranches might be common, and thus the inter-breeding of ticks (short distance

382 similarity). The hypothesis of the use of wild ungulates as hosts for long distance travels allowing

383 mixing of distant populations should not be rejected, since the tick feeds for 24 days. This has

384 been also discussed by **Araya-Anchetta** et al. (2015) who elaborated about the fracture of the

385 genetic signature of ticks because long-distance movements of cattle. The mixing of tick

386 populations leads to a homogenization of their genetic variability that is commonly known as

387 panmixia (Beerli and Palczewski, 2010). These results are compatible with the data by Busch et al.

388 (2014) using microsatellite markers of *R. microplus* in Texas, who reported a genetic structure in

389 a pattern of isolation-by-distance.

390 Another factor that could shape the results obtained for *R. microplus* is the short time the tick has

391 been spreading into the region. The approximate date and place of introduction are unknown,

392 but the Neotropical populations are not yet significantly separated from others belonging to the

393 clade A in Asia, the presumed ancestral area of the species. Arguments for *R. annulatus* support

394 this hypothesis. *Rhipicephalus annulatus* has a disjoint distribution in Mediterranean and Sub-

395 Saharan Africa (Estrada-Peña et al., 2004). The formation of the Sahara Desert, that most probably

396 split both northern and southern populations of *R. annulatus*, is still a matter of debate (Kroepelin,

397 2006) but it is measured in a minimum of thousands of years. Considering that both *COI* and *16S*

398 of the Tropical and Mediterranean populations of *R. annulatus* are very similar, and that this

399 similarity persists in Nearctic samples (spread only about 300-400 years ago according to

400 Becklund et al., 1968), a conclusion could be that the mutation rates of both genes is low enough

401 as to measure the divergence at these timescales. Cattle trade is uncommon between sub-

402 Saharian and Mediterranean regions (Thomson et al., 2004), and even in the absence of

403 population mix, the genetic homogeneity of the tick is still observed. We think this reasoning can

404 also be applied to the high similarity found in *R. microplus* across the Nearctic-Neotropical

405 regions: the time of spread of the tick is not yet enough to measure any genetic divergence.

406 Results point to a few introductions (or only one event) of *R. microplus* supported by a suitable

407 environment in most of the surveyed region.

408 Our results revealed a strong spatial homogenization in the evolution of *R. microplus COI* and *16S*

409 *rDNA* genes in the Neotropics. Since published data suggest a high ability of *R. microplus* to adapt

410 to a large range of climate traits in sites where it has been introduced, we expected a measurable

411 genetic variability correlation with the climate. However, results did not support this hypothesis

412 as the small changes in examined sequences are not correlated with the climate features. Support

413 for this hypothesis came from an unsuspected source. Strains of *R. microplus* kept under

414 laboratory conditions at constant conditions of temperature, humidity, and light regimes, and

415 commonly used for sensitivity analyses to acaricides and vaccination trials, have a similar

416 sequence to wild strains obtained from field surveys. It appears that field populations subjected

417 to changes in the environmental traits did not show major differences in the sequences of the

418 three genes, compared with those of laboratory colonies that are kept under constant

419 environmental conditions. This is an additional clue supporting that climate is not the driver of the

420 small variability of the tested genes.

421 It remains to be investigated whether the adequate markers to track these changes were used, or

422 if other genes could be better for measuring the impact on the tick populations by these traits. It

423 could be possible that *R. microplus* could be mutating following environmental features, but we

424 did not manage to select adequate markers for these changes. As supported by previous data,
425 the nuclear *ITS2* produced poorer results when used on *Boophilus* (i.e., Burger et al., 2014), and
426 mitochondrial genes seem to work better. We encourage the finding and testing of other markers
427 that could help in establishing a relationship between the climate and genetic mutations. Perhaps
428 the sequencing of the complete mitogenome would provide a better overview of these
429 hypothetical associations between genetic structure and environmental traits (Csordas et al.,
430 2016). Future studies using for analysis of multiple genome cluster sequences may provide a
431 more comprehensive analysis of genetic variability in ticks. Additionally, gene expression and
432 protein representation analyses may provide additional information on the possible functional
433 implications of genetic variability in species of ticks subjected to a range of features of climate.

434 **Conclusions**.

435 The Nearctic-Neotropical samples of *R. microplus* s.s. belong only to clade A, and they show small
436 differences in the sequences of three target genes even if collected in sites subjected to different
437 weather annual patterns. This small variability seems to not be correlated with changes in climate.
438 Additionally, the *COI* gene is a very reliable marker of tick species and clade, and therefore its
439 use is reinforced as one of the methods of the panoply of tools available for genetic studies of
440 ticks. The *COI* and *16S rDNA* patterns of *R. microplus* in the Neotropics are consistent with the
441 spread of a monophyletic lineage and a panmixia of populations because contiguity of cattle
442 ranches, and probably livestock trade and movements of wild ungulates. The structure of the
443 Neotropical populations of *R. microplus* is thus very stable. This genetic structure supports that
444 the interventions based on tick antigens for the control of *R. microplus* cattle infestations may be
445 effective in broad areas of the Neotropics (de la Fuente and Kocan, 2003; de la Fuente et al.,
446 2007).

447 **Acknowledgments**.

450

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Figure Legends.

Figure 1: The collection sites of *Rhipicephalus microplus* used in this study in the Nearctic-Neotropical region, overlying the classification of environmental traits in 20 bioclimatic regions (colors). The colors are random and intended only to show the differences and combinations of both temperature and soil humidity. Scales of colors range from the warmer and drier site (arbitrarily named "bioclimatic region 1" or BC1) to the colder and more humid (named "bioclimatic region 20" or BC20). See Material and Methods for the calculation of the bioclimatic regions. Some populations of ticks were collected in near places, separated by no more than 100 km but subjected to different climate traits. The scale of the map does not allow for the separate plotting of each population and are included as only one point but labelled accordingly.

Figure 2: Asian-Neotropical Maximum likelihood tree inferred from partial COI mtDNA (*COI*) sequences. Sequence data generated in the present study are highlighted in bold. Retrieved sequences from GenBank with the accession numbers and geographical origin are available in the Supplementary File 1 and were included to generate a robust phylogenetic tree. Support values were indicated at each node (bootstrap <50%, are not shown). The bar represents 0.06 substitutions per site. The tree was rooted using *Ixodes ricinus* as outgroup.

Figure 3: Asian-Neotropical Maximum likelihood tree inferred from partial 16S rDNA (*16S*) sequences. Sequence data generated in the present study are highlighted in bold. Retrieved sequences from GenBank with the accession numbers and geographical origin are available in the Supplementary File 1 and were included to generate a robust phylogenetic tree. Support values were indicated at each node (bootstrap <50%, are not shown). The bar represents 0.07 substitutions per site. The tree was rooted using *Ixodes ricinus* as outgroup.

Figure 4: Asian-Neotropical Maximum likelihood tree inferred from Internal transcribed spacer (*ITS2*) sequences. Sequence data generated in the present study are highlighted in bold. Retrieved sequences from GenBank with the accession numbers and geographical origin are available in the Supplementary File 1 and were included to generate a robust phylogenetic tree. Support values were indicated at each node (bootstrap <50%, are not shown). The bar represents 0.2 substitutions per site. The tree was rooted using *Ixodes ricinus* as outgroup.

480 **Figure 5**: Heat maps displaying the percent similarity in the *COI* (A), *16S rDNA* (B) and *ITS2* (C)

481 sequences for groups of species and collection areas included in this study. A dendrogram (left)

482 is provided to show the averaged genetic similarities of each gene among samples.

483

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Data accessibility.

Sequences obtained for this study are all available in GenBank. Adequate accession numbers are provided in the manuscript. Satellite data are available in the MODIS repository website, currently (April, 2022) located at https://modis.gsfc.nasa.gov/data/.

Benefits Generated.

A research collaboration was developed with scientists from the countries providing genetic samples, all collaborators are included as co-authors, and the results of the research have been shared with them. The research addresses a priority concern, in this case the capacity building for the control of the most important arthropod pest in the Neotropical region.

Authors contributions.

AEP and JdF designed the research and decided the collection points. SGS and LHT sequenced the samples and/or recorded available samples in GenBank. All the phylogenetic treatment of the data was performed by SGS. The rest of co-authors collected samples from different countries. AEP analyzed the climate data and wrote the drfat of the paper. AEP, JdF and SGS wrote the final version of the manuscript.

Supplementary Material.

Supplementary File 1: An excel-type spreadsheet including all the samples including for the maximum likelihood reconstruction of the phylogenies based on *COI*, *16S*, or *ITS2* (in separate sheets). Each sheet includes the name as in the figures (phylogenetic trees) as well as the precedence (country), number of GenBank or BOLD accession number and reference (in case it was not from our own collections).

Supplementary File 2: An excel-type spreadsheet including the similarities among samples based on *COI*, *16S*, or *ITS2* (in separate sheets). A fourth sheet includes the distance in km among geo-referenced samples (in straight line). The fifth sheet includes the percent of similarity among the ecological regions outlined for the phylogeographic study. All the data about similarities are in the range 0-100. All the sheets are named accordingly.

R. microplus|Brazil 1
R. microplus|Uruguay 2
R. microplus|Brazil 2
R. microplus|Brazil 3
R. microplus|Brazil 4
R. microplus|Brazil 5
R. microplus|Brazil 6
R. microplus|Brazil 7
R. microplus|Brazil 8
R. microplus|Argentina 1
R. microplus|Argentina 2
R. microplus|Argentina 3
R. microplus|Argentina 4
R. microplus|Cameroon
R. microplus|Benin
R. microplus|South Africa 1
R. microplus|Brazil 11
R. microplus|Brazil 10
R. microplus|Brazil 14
R. microplus|Brazil 12
R. microplus|Uruguay 1
R. microplus|Brazil 9
R. microplus|Colombia 1
R. microplus|Panama 3
R. microplus|Panama 1
R. microplus|USA
R. microplus|Colombia 2
R. microplus|Mexico 9
R. microplus|Mexico 8
R. microplus|Mexico 7
R. microplus|Mexico 6
R. microplus|Mexico 5
R. microplus|Mexico 4
R. microplus|Mexico 3
R. microplus|Mexico 2
R. microplus|Mexico 1
R. microplus|Argentina 5
R. microplus|Panama 2
R. microplus|Philippines 1
R. microplus|Brazil 13
R. microplus|China 1
R. microplus|Philippines 2
R. microplus|Kenia
R. australis|New Caledonia
R. annulatus|Mexico 12
R. annulatus|Mexico 11
R. annulatus|Mexico 10
R. annulatus|Mexico 13
R. annulatus|Burkina Faso 1
R. annulatus|Mexico 14
R. annulatus|Mexico 17
R. annulatus|Mexico 16
R. annulatus|Mexico 15
R. annulatus|Israel
R. microplus|China 2
R. microplus|India 1
R. microplus|India 2
R. microplus|India 3
R. microplus|India 4
R. microplus|Pakistan 1
R. microplus|India 5
R. microplus|Pakistan 2
R. microplus|Pakistan 3
R. microplus|Pakistan 4
R. microplus|Pakistan 5
R. geigyi|Burkina Faso 2
R. geigyi|Burkina Faso 3
R. decoloratus|South Africa 2
R. decoloratus|Cameroon 2
R. decoloratus|Cameroon 3
R. bursa|Italy 1
R. bursa|Italy 2
R. simus|South Africa 3
R. sanguineus|France 1
R. sanguineus|France 2
I. ricunus|Switzerland

100%
100%
67%
76%
75%
60%
100%
85%
96%
93%
76%
100%
63%
100%
88%
100%
98%
65%
100%

2

0.06

R. microplus|Argentina 6
R. microplus|Bolivia 1
R. microplus|Uruguay 2
R. microplus|Uruguay 1
R. microplus|Mexico 9
R. microplus|Mexico 8
R. microplus|Mexico 7
R. microplus|Mexico 6
R. microplus|Mexico 4
R. microplus|Mexico 3
R. microplus|Mexico 2
R. microplus|Mexico 1
R. microplus|Brazil 13
R. microplus|Brazil 9
R. microplus|Brazil 7
R. microplus|Brazil 6
R. microplus|Brazil 5
R. microplus|Brazil 4
R. microplus|Brazil 3
R. microplus|Brazil 1
R. microplus|Argentina 5
R. microplus|Argentina 4
R. microplus|Argentina 3
R. microplus|Argentina 2
R. microplus|Argentina 1
R. microplus|Brazil 14
R. microplus|Costa Rica 11
R. microplus|Paraguay 1
R. microplus|Peru 1
R. microplus|Tanzania 1
R. microplus|Uruguay 3
R. microplus|Colombia 8
R. microplus|Colombia 11
R. microplus|Colombia 9
R. microplus|Colombia 10
R. microplus|Colombia 2
R. microplus|Colombia 6
R. microplus|Colombia 3
R. microplus|Colombia 4
R. microplus|Cameroon 1
R. microplus|South Africa 1
R. microplus|Colombia 1
R. microplus|Colombia 7
R. microplus|Colombia 5
R. australis|Australia 1
R. australis|New Caledonia 1
R. australis|New Caledonia 2
R. australis|Australia 2
R. annulatus|Mexico 12
R. annulatus|Mexico 13
R. annulatus|Mexico 11
R. annulatus|Mexico 10
R. annulatus|Israel 1
R. annulatus|Mexico 14
R. annulatus|Mexico 17
R. microplus|China 1
R. microplus|India 4
R. microplus|India 3
R. microplus|Pakistan 1
R. microplus|India 1
R. microplus|India 2
R. microplus|India 5
R. microplus|Pakistan 4
R. microplus|Pakistan 5
R. decoloratus|South Africa 2
R. geiyi|Burkina Faso 1
R. geiyi|Mali1
R. bursa|Italy 1
R. bursa|Spain 1
R. simus|South Africa 3
R. sanguineus|France 1
R. sanguineus|France 2
I.ricinus|

61
76
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91
86
85
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86
83
61
100
100
100

0.0

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56% **R. microplus|Uruguay 2**
R. microplus|Colombia 1
R. microplus|Mexico 12
R. microplus|Argentina 4
R. microplus|Brazil 13
R. microplus|Argentina 2
R. microplus|Mexico 1
R. microplus|Mexico 3
R. microplus|Brazil 1
R. microplus|Mexico 7
R. microplus|Brazil 14
R. microplus|Mexico 8
R. microplus|Mexico 5
R. microplus|Brazil 6
R. microplus|Mexico 9
B. microplus|South Africa 1
R. microplus|Brazil 9
R. microplus|Brazil 4
R. microplus|Brazil 5
R. microplus|Mexico 6
R. microplus|Mexico 4
R. microplus|Argentina 5
R. microplus|Mexico 2
R. microplus|Argentina 3
R. microplus|Brazil 2
R. microplus|Cambodia 1
R. microplus|China 1
57% **R. microplus|India 1**
R. microplus|India 3
R. microplus|India 2
R. microplus|Pakistan 4
R. microplus|Pakistan 2
R. microplus|Pakistan 1
B. microplus|Brazil 15
R. microplus|Pakistan 5
R. australis|Australia 1
64% *R. annulatus*|Romania 1
99% *R. annulatus*|Texas 1
84% **R. annulatus|Mexico 11**
100% *R. bursa*|Iran 1
R. bursa|Italy 1
89% *R. simus*|South Africa 2
100% *R. sanguineus*|Brazil 16
100% *R. sanguineus*|France 1
99% *R. geiyi*|Burkina Faso 1
R. geiyi|Guinea 1
I. ricinus|Russia

0.2

4