Different lines of evidence used to delimit species in ticks: a study of the South American populations of *Amblyomma parvum* (Acari: Ixodidae)

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Abstract

The goal of this work was to combine different lines of evidence besides that of molecular markers to delimit species in ticks when the molecular data are not totally congruent. Two groups (Argentina, Brazil) of South American populations of *Amblyomma parvum* were compared to test whether the splitting of these two lineages suggested by genetic analyses is complete. Comparative studies of reproductive compatibility, morphological analyses of fixed characters, and comparison of population distributions in spatially defined ecological niches were performed. The morphological comparisons of both discrete and morphometric characters showed no differences among *A. parvum* ticks from Argentina and Brazil. The intercrosses and backcrosses showed evidence of pre- and post-zygotic compatibility between the two groups. No significant differences in environmental traits were found which would justify the separation of the records of *A. parvum* in distinct groups. Although the gene flow between the two groups of populations is limited, the absence of reproductive barriers, the lack of significant morphological differences, and the absence of significant differences in the niche preferences indicate that populations of *A. parvum* from Argentina and Brazil should be treated as a single species. The speciation conjectures suggested by some analyses of mitochondrial DNA sequences were not supported when different lines of evidences were compared.

Keywords: species delimitation; Ixodidae; *Amblyomma parvum*; South America

1. Introduction

Species delimitation is a major goal in systematic biology because accurate knowledge of species boundaries is a prerequisite for evolutionary, ecological, and epidemiological studies of living organisms. Species definition is a matter of ongoing theoretical debate that has led to the formulation and comparison of several species concepts. Biological (BSC), Evolutionary (ESC), Phylogenetic (PSC), Ecological (EcSC), Phenetic (PhSC), Morphological (MSC) and Cohesion (CSC), are some examples of the most recognized species concepts (Mayden, 1997, 2002). Each of these concepts has its own strengths and weaknesses, and they are often inconsistent with one another in terms of ontological demarcation of species. Moreover, the application of different concepts on the same taxon can result in contradictory results with respect to the number of species recognized (Knowlton and Weigt, 1997). The emergence of the general lineage concept (GLC) of species (de Queiroz, 1998, 2005, 2007) constitutes an attempt to reconcile alternative and incompatible species concepts. GLC defines species as separately evolving metapopulation lineages. This definition is similar to that of Wiley and Mayden (2000), where species are considered as demographically and genetically interconnected set of populations with continuity through time. In GLC, all characteristics that have been treated as emergent properties in the previous concepts are reinterpreted as contingent properties that species may or may not acquire during the course of their existence (de Queiroz, 2005, 2007). Thus, properties as phenetic distinguishability, reciprocal monophyly, ecological divergence, and pre- and postzygotic reproductive isolation are not emergent properties of species, but they provide evidence of lineage separation and serve as operational criteria to species delimitation. Strictly speaking, GLC is not a new species concept, but the explicit separation between the theoretical concept of species (as separately evolving metapopulation lineages) and the operational criteria that are used for empirical application (de Queiroz, 2007) constitute a novel approach. In this sense, Mayr (1982, 1987) has stressed the importance of considering the difference between the species as a category ("species definition") and the species as a taxon ("species delimitation"). A species taxon is a natural entity recognized and delimited by the taxonomist, and the species category is the rank given to a species taxon (Mayr, 1987).

The issue dealing with the empirical delimitation of biological entities to a specific level requires a clear definition of the operational criteria by which individuals can be tested for species membership (Sites and Crandall, 1997; Sites and Marshall, 2004; Wakes, 2006; Reeves and Richard, 2011). The use of different lines of evidence not only is necessary to formulate robust hypotheses about the specific status of a group of organisms, but also provides a source of information to prevent erroneous conclusions due to inconsistency among different characters sets. Phenotypic distinguishability, levels of genetic and ecological divergence, and reproductive isolation can be used concomitantly to infer species boundaries. The adoption of an approach that considers simultaneously independent or simply different sources of information is essential as the evolution at the species level is often reticulate (in this case monophyly of a single gene is a weak criterion for species delimitation) and because different contingent properties (e.g., fixed morphological differences, reproductive barriers, use of ecological niche space, molecular divergence thresholds) can arise at different times during lineage separation (de Queiroz, 1998; Wiens and Penkrot 2002; Markolf et al., 2013).

In the particular case of hard ticks (Acari: Ixodidae), systematic studies integrating cross-mating experiments, comparison of multiple gene sequences, and morphological analysis have been instrumental in achieving significant taxonomic reassessments in some species with public health and economic relevance. For instance, morphological, biological (cross-mating trials), ecological, and genetic evidences have shown that the taxon previously recognized as *Amblyomma cajennense* (Fabricius, 1787) encompasses at least six different species (Labruna et al., 2011a; Mastropaolo et al., 2011; Beati et al., 2013; Estrada-Peña et al., 2014a; Nava et al., 2014a), and the reinstatement of *Rhipicephalus (Boophilus) australis* Fuller, 1899, as a valid species different from *Rhipicephalus (Boophilus) microplus* (Canestrini, 1888), was carried out based on the analysis of genetic divergence, experimental crosses, and morphological dissimilarities (Labruna et al., 2009; Estrada-Peña et al., 2012).

Amblyomma parvum Aragão, 1908 is a hard tick species widely distributed from southern Mexico to northern Argentina (Nava et al., 2008). This tick is adapted to environments with low humidity and high temperatures, and its distribution is disjunct with a population found south of the

Amazonian subregion, mostly collected from the biogeographical provinces of Caatinga, Cerrado, Pantanal and Chaco, and another population located north of the Amazonian subregion, in the biogeographical provinces of Venezuelan Coast, Venezuelan Llanos, Chiapas, Eastern Central America and Western Panamanian Isthmus (Nava et al., 2008)¹. The host range of *A. parvum* adults includes several species of medium and large-sized mammals, while immature stages are principally associated with rodents of the families Caviidae and Echimyidae (Labruna et al., 2005a; Nava et al., 2006; Szabó et al., 2007; Nava et al., 2008; Horta et al., 2011; Saraiva et al., 2012). Adults of *A. parvum* are common parasites of domestic animals, cattle and goats, and are also aggressive to humans (Guglielmone et al., 2006; Nava et al., 2008). Moreover, *A. parvum* is likely to be the vector of pathogenic microorganisms, because it has been found naturally infected with *Coxiella burnetii*, *Ehrlichia* cf. *E. chaffeensis* and '*Candidatus Rickettsia andeanae*' (Pacheco et al., 2007; Tomassone et al., 2008; Labruna et al., 2011b; Pacheco et al., 2013).

A preliminary study on the population genetics of *A. parvum* in South America through analysis of mitochondrial DNA sequences showed a marked genetic divergence between Argentinean and Brazilian populations (Nava et al., 2008). This divergence varied from 3.0% to 3.7%. It was considered a noteworthy result since this value of divergence among 16S rDNA sequences is similar or higher than those reported for other Neotropical taxa of *Amblyomma* which are considered to be different species such as *Amblyomma tigrinum*-*Amblyomma triste*-*Amblyomma maculatum* (Estrada-Peña et al. 2005; Abarca et al. 2012). Lado et al. (2016) performed an extensive genetic analysis of *A. parvum* by using mitochondrial (the small and the large ribosomal subunits 12rDNA and 16SrDNA, the cytochrome oxidase I and II, and the control region or d-loop) and nuclear (inter transcribed spacer 2) markers. However, although Lado et al. (2016) also found a separation between the populations of Argentina and Brazil with mitochondrial markers, they did not find consistently a Brazilian and an Argentinian clade separated from each other when analyses of nuclear and mitochondrial markers were undertaken integrally.

Because genetic data by themselves are not enough to determine whether the splitting of two lineages is complete or not, the gathering of complementary information on independent

¹ Biogeographical provinces are named following Morrone (2006)

characters is crucial in order to better infer the underlying speciation processes (Giessler et al., 1999). It is necessary to complement genetic analyses with biological studies of reproductive compatibility, morphological analyses of fixed characters, and comparison of **population distributions** in spatially defined ecological niches. Therefore, the main goal of this work was to combine different lines of evidence besides that of molecular markers to delimit species in ticks when the molecular data are not totally congruent. The study was performed by using biological, ecological, and morphological data in order to assess whether the genetic divergence values previously recorded between South American populations of *A. parvum* was sufficiently important as to represent a complete speciation process.

2. Materials and methods

2.1 Morphological analysis

Ticks used in this study were identified as adults of *A. parvum* by following Guglielmone et al. (1990) and Barros-Battesti et al. (2006). A comparison of discrete morphological characters, known to be diagnostic within the genus *Amblyomma*, was performed among *A. parvum* adults from Argentina and Brazil. Ticks from Argentina were collected in localities of the Chaco Biogeographic Province sensu Morrone (2006), while ticks from Brazil were collected in localities belonging to the Cerrado and Pantanal Biogeographic Provinces sensu Morrone (2006) (Table 1). The following morphological features were examined: 1) Scutum punctation of males and females; 2) Shape of basis capituli of males and females; 3) Dorsal setae of the body of females; 4) Ventral plates of males; 5) Spurs of the legs (coxae and trochanter) of males and females; 6) Ventral spur of the article I of the palpi of males and females; 7) Genital aperture of females; 8) Spiracular plates of males and females; 9) Hypostome of males and females; 10) Body outline; 11) Porose areas of females.

In the analysis of morphometric characters, twenty-six morphometric variables (Table 2; Fig. 1) from 80 unfed *A. parvum* ticks (40 males and 40 females) were included. Raw measurements were log (x+1) transformed to standardize variances and improve normality.

Principal component analysis (PCA) based on a Pearson's correlation matrix was performed to identify clusters of individuals in a multivariate space without the a priori designation of groups. Canonical discriminant analysis (CDA) was also used in order to determine the extent of morphological differentiation among three groups of ticks classified according to biogeographic origin: Chaco Biogeographic Province (Argentina), Pantanal Biogeographic Province (Brazil) and Cerrado Biogeographic Province (Brazil). Males and females were separately considered in PCA and CDA. Both analyses were chosen because they are complementary, CDA optimize between-group difference relative to within-group variation, whereas PCA is used to describe differences among individuals (Zelditch et al., 2004).

2.2 Evaluation of reproductive compatibility

The cross- mating experiments were conducted with two colonies of Argentinean and Brazilian *A. parvum* ticks derived from adults collected in El Tunal (25°13'S, 64°22'W, Salta Province, Argentina) and Araguapaz (15°03'S, 50°05'W, Goiás State, Brazil), respectively. To ensure that tick groups compared in this experiment were genetically different, DNA was extracted from representative specimens (four ticks from Argentina and four ticks from Brazil) of each colony to amplify a ca. 400 bp fragment of the mitochondrial 16S rRNA gene following Mangold et al. (1998). The genetic divergences in 16S rDNA sequences between the ticks of these two colonies varied between 3 and 3.4% (Genbank accession numbers: KX618853 (haplotype from Argentina); KX618854 haplotype from Brazil)), values similar to those reported in the study of Nava et al. (2008).

Infestation with adult ticks from Argentinian (ARG) and Brazilian (BRA) laboratory colonies were conducted on rabbits, by using a feeding chamber attached on the dorsum of the hosts. All rabbits were females of the same age belonging to the "Californian" breed. Larvae and nymphs were fed on guinea pigs (*Cavia porcellus*). All rabbits and guinea pigs were maintained under the same conditions before and during the experiment. In experiment 1, 15 days old adult ticks from first laboratory generations were fed on rabbits. Four groups, each consisting of 15 couples of males (MM) and females (FF), were prepared as follows: group 1) 15 couples of adults from BRA;

group 2) 15 couples of adults from ARG; group 3) 15 couples with MM from BRA and FF from ARG; and group 4) 15 couples with MM from ARG and FF from BRA. Additionally, 4 unfed females from each strain were also fed on rabbits to control for the possible presence of parthenogenesis.

A second experiment was carried out because females of the four groups were able to lay viable eggs (see results). Hybrid larvae from each group were fed on guinea pigs, and subsequently molted into nymphs. These nymphs were also fed on guinea pigs, and the hybrid males (F1 MM) and females (F1 FF) obtained from these nymphs were used in experiment 2. The following ten groups were prepared to simulate potential mating pairs in a natural population: 1) F1 MM (MM ARG x FF BRA) x F1 FF (MM ARG x FF BRA); 2) F1 MM (MM BRA x FF ARG) x F1 FF (MM BRA x FF ARG); 3) MM BRA x F1 FF (MM ARG x FF BRA); 4) MM BRA X F1 FF (MM BRA X FF ARG); 5) MM ARG X F1 FF (MM ARG X FF BRA); 6) MM ARG X F1 FF (MM BRA X FF ARG); 7) F1 MM (MM ARG X FF BRA) X FF BRA; 8) F1 MM (MM ARG X FF BRA) X FF ARG; 9) F1 MM (MM BRA X FF ARG) X FF BRA; 10) F1 MM (MM BRA X FF ARG) X FF ARG. All groups involved 10 males and 10 females.

The engorged ticks were kept at 25° C and 83-86 % relative humidity, with a daily photoperiod of 12 h light-12 h dark. Weight of engorged females, feeding period, pre-oviposition period (number of days from female detachment to the beginning of oviposition), incubation period of eggs (number of days from the laying of the first egg until first egg hatched), reproductive efficiency index [REI = number of eggs laid / weight of the females in mg (Drummond and Whetstone, 1970)], and fecundity efficiency index [FEI = number of hatched larvae / weight of the females in mg (Aguirre et al., 2005)] were recorded. Larvae and unhatched eggs were counted as described by Guglielmone et al. (1989). The statistical significance of the differences among the means of the biological parameters was tested by using an analysis of variance (ANOVA) followed by a posterior Tukey test (Zar, 1999). The experiments with laboratory animals were approved by the Animal Experimentation Ethics Committee of the Federal University of Uberlândia (process number 097/2011).

2.3 Ecological niche

An additional goal of this work was to examine the environmental niche associated with *A. parvum* populations from Argentina, Paraguay and Brazil. The environmental traits possibly affecting the distribution of different populations of *A. parvum* were investigated. This was done in order to verify if simple regression models could detect significant differences among environmental variables and therefore help in delineating the area corresponding to the ecological niche specifically associated with the different populations. A total of 143 confirmed records of *A. parvum* in Argentina, Brazil and Paraguay were used for this study. Information was obtained from literature (see list in Nava et al., 2008) and from the Tick Collection of INTA Rafaela, Rafaela, Santa Fe, Argentina.

Selection of environmental variables is a critical step in elucidating the niche preferences of an organism (Glass et al., 1995; Guerra et al., 2002). The concerns about the automatic selection of variables by the modelling algorithms to avoid collinearly correlated variables and model overperformance (Mansfield and Helms, 1982; Legendre, 1993) were addressed by using the coefficients of the harmonic regression of temporal series of Land Surface Temperature (LST) and Normalized Difference Vegetation Index (NDVI) as explanatory variables. These two datasets were derived from the Moderate Resolution Imaging Spectroradiometer (MODIS) platform (spatial resolution of 0.05°) for the years 2000–2012. It is widely accepted that both, temperature and saturation deficit, affect tick life cycles. For remote sensing, NDVI best captures the reported distribution of some tick species (Estrada-Peña, 1999) because it is a proxy for vegetation stress, therefore reflecting water availability. We used the products MOD11C2 and MOD13C2, available at https://lpdaac.usgs.gov/products/modis_products_table (accessed December 2014). These coefficients are referred to as “environmental variables” in this study because they represent the environmental niche that may be occupied by the target species. The set of coefficients of the harmonic regressions can be retrieved from <http://antigonefp7.eu/our-results/remotely-sensed-variable-data/>, and an explanation of the environmental variables and their significance in describing the environmental traits has also been published (Estrada-Peña et al., 2014b). For modelling purposes, we used a process that randomly and recursively selects and assigns the collection points in two groups, and then applies a multi-logistic regression to check if significant

differences exist in any or several of the environmental traits. If significant differences are found, the method uses the Akaike information criterion to select the best set of environmental variables that, in combination, discriminate among the records of the allocated groups. As the process is recursive, it ensures that all records are allocated to one of the groups and compared. To account for variability in the number of records allocated to any of both assumed groups, we first ran the tests allocating the 75% of records to each group, then reducing the number of records allocated to each group to a total of 50%-25%. All the calculations were performed in the R developmental framework (R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (<http://www.R-project.org/>)).

3. Results

The comparative examination of discrete morphological characters of the adults of *A. parvum* showed no differences among ticks from Argentinean and Brazilian populations. Comparisons, made character by character, have demonstrated that adults of *A. parvum* share a rather uniform structure of discrete characters throughout their distribution in South America. The discrete morphological characters of *A. parvum* adults from Argentina and Brazil are demonstrated in figures 2 and 3 for males and females, respectively. All males examined were characterized by the presence of an inornate scutum with numerous punctations distributed uniformly (Fig. 2A), body outline oval (Fig. 2A), basis capituli sub-rectangular with posterior margin concave and with triangular cornua (Figs. 2A, 2C), presence of fine setae on ventral surface (Fig. 2C), presence of distal sclerotized ventral plates (carenas) visible in both ventral and dorsal view (Figs. 2A, 2B), coxa of leg I with 2 spurs (the external larger than internal and reaching the anterior margin of coxa II) (Fig. 2B), coxae II-IV each with triangular spur (Figs. 2B), trochanters of legs with ventral spur (most noticeable in trochanters I and II) (Fig. 2B), article I of palpi with ventral and retrograde spur (Figs. 2B, 2D), hypostome with dental formula 3/3 in files of 5 (Fig. 2D) and comma shaped spiracular plate (Fig. 2B). All females from Argentina and Brazil presented oval body outline (Fig. 3A), scutum with numerous punctations more densely distributed in the central field (Fig. 3A), basis capituli sub-triangular with posterior margin slightly concave (Figs. 3A, 3C), porose area oval (Fig. 3C), dorsal

body setae evenly distributed on dorsum (Fig. 3A), coxa of leg I with 2 spurs (the external larger than internal and reaching the anterior margin of coxa II, in unfed specimens) (Fig. 3B), coxae II-IV each with triangular spur (Fig. 3B), trochanters of legs with ventral spur (most noticeable in trochanters I and II) (Fig. 3B), hypostome with dental formula 3/3 in files of 8-9 (Fig. 3D), genital aperture "U" shaped with two distinct lateral flaps (Fig. 3B) and spiracular plate triangular with a dorsal prolongation. The length of the ventral plates in males was the only highly variable character, in some specimens they were very long and in others almost indistinct. However, this variation was also observed among individuals from a single population.

PCAs for both sexes failed to separate the Argentinean *A. parvum* from the Brazilian ones. The distributions of the groups from Chaco, Pantanal and Cerrado overlapped broadly in both males and females (Figs. 4A, 4B). In contrast, CDAs appeared to be more useful in discriminating between groups. CDA analyses of female samples distinctly separated specimens from the three biogeographic provinces (Chaco, Pantanal, and Cerrado) (Fig. 5A). However, males of *A. parvum* from Chaco were not fully separated from the males from Cerrado and Pantanal in CDA analysis, showing a partial overlap (Fig. 5B).

Results of cross- mating experiments 1 and 2 are listed in tables 3 and 4, respectively. In experiment 1, crosses between *A. parvum* from Argentina and Brazil produced viable F1 progeny. No statistically significant differences were observed in the among-group comparison for feeding period, pre-oviposition period and incubation period of eggs (Table 3). Although statistically significant differences among groups were found for weight of engorged females, REI and FEI, they were not directly related to the hybrid crosses (Table 3). In fact, values of weight of engorged females, REI and FEI of the group 4 (MM from BRA and FF from ARG) were higher than those observed in group 1 (MM and FF from Brazil) (Table 3). All backcrosses of experiment 2 also yielded viable offspring (Table 4). Statistically significant differences were only found between groups 4 and 5 for weight of engorged females and between groups 5 and 8 for REI. FEI differences were not significant in any of the comparisons (Table 4).

The recorded distribution of *A. parvum* in relation to biogeographical areas is depicted in figure 6. Most of the records of *A. parvum* were associated with the dry diagonal region of South

America. In a biogeographical context, the analysis of the distribution of *A. parvum* showed, as expected that this tick has a strong association with areas belonging to Chaco in Argentina and Paraguay, and Cerrado and Caatinga in Brazil (Fig. 6). No significant differences in environmental traits were found which would justify the separation of the records of *A. parvum* in distinct groups. The highest differences (non-significant) were found only for the slope of temperature in spring ($p=0.318$) and annual NDVI average ($p=0.411$) for the separation of samples in two groups (Argentina-Paraguay and Brazil), that reflect, approximately, a northern and a southern assemblage. Both p values are far from being significant.

4. Discussion

Species delimitations are hypotheses on evolutionary and biological differences among living organisms which are based on analyses of a given number of traits in a finite number of natural populations. Since the contingent properties related to speciation do not arise simultaneously during lineage separation (de Queiroz, 1998; Wiens and Penkrot 2002; Markolf et al., 2013), the separate analysis of different lines of evidence can lead to ambiguous conclusions. Therefore, a given taxonomic hypothesis is better tested when independent data sets are considered all together and compared.

The initial hypothesis of speciation within South American populations of *A. parvum* was formulated based on 16SrDNA sequence analysis (Nava et al., 2008). The speciation conjecture was, however, not totally supported when nuclear and mitochondrial DNA sequences were considered in a total evidence analysis (Lado et al., 2016). In this study, we used lines of evidence complementary to those provided by molecular markers to further test whether the splitting of the Argentinean and Brazilian lineages of *A. parvum* is complete or not. These lines of evidence included discrete and morphometric data sets generated by examining morphological characters, cross-mating experiments for the detection of reproductive incompatibility between populations, and comparisons of ecological niches.

Reproductive isolation among sexual taxa arises during or after speciation as a result of two distinct processes: prezygotic and postzygotic isolation (Gourbière and Mallet, 2009). Postzygotic

isolation is defined as a reduction in fertility or viability in hybrid offspring, including hatching, growth, survival and metamorphosis, whereas prezygotic isolation includes differences in behaviour, reproductive timing and gametic compatibility (Edmands, 2002). Postzygotic isolation constitutes a strong evolutionary marker of speciation, because it is likely to be permanent once established (Coyne and Orr, 2004). The intercrosses and backcrosses examined in this work represented almost all potential mating pairs that could occur in a natural population, and the results indicate pre- and post-zygotic compatibility between *A. parvum* ticks from Argentina and Brazil. All crosses performed in the two experiments yielded viable offspring, at least under experimental conditions (Tables 3, 4). Biological parameters such as engorgement weight of females and reproductive efficiency obtained in the backcrosses and intercrosses (Table 4) were similar, or in some cases better, than those obtained by Guglielmone et al., (1991), who studied the life cycle of *A. parvum* under laboratory conditions using a tick colony developed from females collected from a single locality of the Salta Province in northwestern Argentina. This comparison further reinforces the conclusions reached with the experimental cross-mating trials, confirming the lack of reproductive barriers between *A. parvum* from Argentina and Brazil.

PCA of morphometric characters demonstrated that there is no clear morphological differentiation between *A. parvum* specimens from Argentina and Brazil (Figs. 4A, 4B). A greater separation was observed when CDA was applied, but it was complete only in females from the three different areas, and between Brazilian specimens from Pantanal and Cerrado (Fig. 5B), even though that they are genetically similar. A partial separation occurred among males from Chaco, Pantanal and Cerrado with CDA, but with a certain overlap among tick individuals (Fig. 5A). This discordance between PCA and CDA is not uncommon. First, CDA optimizes between-group differences relative to within-group variation, and second, CDA describes difference between groups and the direction in which group means are most different is not necessarily the direction in which individual are most different (Zelditch et al., 2004). Therefore, results of CDA can appear to be different from the results obtained with PCA (Zelditch et al., 2004).

Discrete morphological characters did not differ between Argentinean and Brazilian populations of *A. parvum*. Comparative examination of *A. parvum* specimens from Argentina and

Brazil showed that they are morphologically almost indistinguishable, and the descriptions performed in this work perfectly coincide with earlier descriptions of Robinson (1926) and Guglielmone et al. (1990). The very variable length of the male ventral plates was observed within populations as much as between populations. A similar pattern of intraspecific variation in this character was previously reported for *A. parvum* (Guglielmone et al., 1990) and also for other *Amblyomma* species, such as *Amblyomma latepunctatum*, *Amblyomma sculpturatum*, *Amblyomma incisum* and *Amblyomma tenellum* (Labruna et al., 2005b; Nava et al., 2014b). Therefore, the most pronounced and only variation detected among discrete morphological characters is not relevant from an evolutionary perspective. Considering the morphological analyses as a whole, it may be concluded that phenotypic differences clearly distinguishing among different South American populations of *A. parvum* are missing.

Because the small inbreeding effective population size (N_e) of the mitochondrial genes may lead to coalescence of mtDNA lineages that are only temporarily isolated, producing an overresolution of mtDNA trees (Sites and Crandall, 1997; Wiens and Penkrot, 2002), sequences of a nuclear marker (ITS2:1161bp) were also included in the inter-population comparison performed by Lado et al. (2016). The phylogenetic patterns obtained by Lado et al. (2016) with nuclear and mitochondrial markers were not always concordant and did not reveal complete DNA reciprocal monophyly of the *A. parvum* clades from Argentina and Brazil. Therefore, although the gene flow between the two populations might still be limited due to geographic distance, the absence of reproductive barriers and the lack of significant morphological differences suggest that populations of *A. parvum* from Argentina and Brazil should be treated as a single species. Further evidence supporting this conclusion is provided by the absence of significant differences in the niche preferences between *A. parvum* from Argentina and Brazil, and also by the study of Gerardi et al. (2013), where *A. parvum* from Argentina and Brazil showed similar biological performances when they were fed on different mammal hosts, namely Guinea pigs, rabbits, dogs and cattle. In the light of the results presented in this work, values of genetic divergence between 3% and 4% in sequences of the mitochondrial 16S rRNA gene of *Amblyomma* ticks should not be considered as **conclusive evidence on the taxonomic status at the species level within this tick genus**. This is

relevant not only because the 16S rRNA gene is a molecular marker widely used to infer the taxonomic status of ticks at the species levels, but also because some species of the genus *Amblyomma* which are considered as different species, as for example *A. tigrinum*, *A. triste* and *A. maculatum*, have a genetic divergence among each other in their 16S rDNA sequences below 4% (see Estrada-Peña et al., 2005; Abarca et al., 2012).

This being said, *A. parvum* populations from Argentina and Brazil could at least be considered to be at the beginning of a diverging process **that could** finally result in speciation. The gradualness of the speciation process means that it is possible to find natural populations that are becoming separate species but have not yet completed the process, and where certain characters may evolve more rapidly than others (Mayr, 1996). In this sense, the strength of postzygotic isolation rises slowly with time, and reports of sterile or inviable hybrids of recently diverged taxa are unusual (Coyne and Orr, 2004). Our results show that there is no reproductive incompatibility between Argentinean and Brazilian populations of *A. parvum*, that morphological differences among them are not fixed yet, and that they share environments with similar ecological characteristics. Whether the incipient diversification of the two lineages observed in some gene sequence analyses will lead to speciation cannot be predicted. In this sense, unforeseeable future biogeographic events might even end up re-homogenizing the two mitochondrial clades and reversing the sorting process.

Acknowledgements

We acknowledge the financial support of INTA, Asociación Cooperadora EEA-INTA Rafaela, CONICET and International Cooperation Project MINCyT-CAPES (BR/11/08) and NSF grant #1026146 to LB. We wish to thank Patricia Sarmiento (Servicio de Microscopía Electrónica, Museo de La Plata, Argentina) for preparing the scanning electron micrographs.

References

- Abarca, K., López, J., Acosta-Jamett, G., Lepe, P., Soares, J.F., Labruna, M.B., 2012. A third *Amblyomma* species and the first tick-borne rickettsia in Chile. J. Med. Entomol. 49, 219-222.
- Aguirre, D.H., Gaido, A.B., Cafrune, M.M., Castelli, M.E., Mangold, A.J., Guglielmone, A.A., 2005. Eprinomectin pour-on for control of *Boophilus microplus* (Canestrini) ticks (Acari: Ixodidae) on cattle. Vet. Parasitol. 127, 157-163.
- Barros-Battesti, D.M., Arzua, M., Bechara, G.H., 2006. Carrapatos de importancia médico-veterinaria da regio Neotropical: um guia ilustrado para identificação de espécies. Vox/ICTTD-3/Butantan, São Paulo.
- Beati, L., Nava, S., Burkman, E.J., Barros-Battesti, D., Labruna, M.B., Guglielmone, A.A., Cáceres, A.G., Guzman Cornejo, C., León, R., Durden, L.A., Faccini, J.L.H., 2013. *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae), the Cayenne tick: phylogeography and evidence for allopatric speciation. BMC Evol. Biol. 13: 267.
- Boero, J.J., 1957. Las garrapatas de la República Argentina (Acarina: Ixodoidea). Departamento Editorial de la Universidad de Buenos Aires, Buenos Aires.
- Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates, Sunderland.
- de Queiroz, K., 1998. The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. In: Howard, D.J., Berlocher, S.H. (eds.), Endless forms: species and speciation. Oxford University Press, Oxford, pp. 57-75.
- de Queiroz, K., 2005. Ernst Mayr and the modern concept of species. Proc. Natl. Acad. Sci. USA. 102, 6600-6607.
- de Queiroz, K., 2007. Species concepts and species delimitation. Syst. Biol. 56, 879-886.
- Drummond, R.O., Whetstone, T.M., 1970. Oviposition of the gulf coast tick. J. Econ. Entomol. 63, 1547-1551.
- Edmands, S., 2002. Does parental divergence predict reproductive compatibility?. Trends Ecol. Evol. 17, 520-527

- 448 Estrada-Peña, A., 1999. Geostatistics as predictive tools to estimate *Ixodes ricinus* (Acari: Ixodidae)
 449 habitat suitability in the western Palearctic from AVHRR satellite imagery. Exp. Appl. Acarol.
 450 23, 337–349.
- 451 Estrada-Peña, A., Venzal, J.M., Mangold, A.J., Cafrune, M.M., Guglielmone, A.A., 2005. The
 452 *Amblyomma maculatum* Koch, 1844 (Acari: Ixodidae: Amblyomminae) tick group: diagnostic
 453 characters, description of the larva of *A. parvitarsum* Neumann, 1901, 16S rDNA sequences,
 454 distribution and hosts. Syst. Parasitol. 60, 99–112.
- 455 Estrada-Peña, A., Venzal, J.M., Nava, S., Mangold, A.J., Guglielmone, A.A., Labruna, M.B., de la
 456 Fuente, J., 2012. Reinstatement of *Rhipicephalus (Boophilus) australis* (Acari: Ixodidae) with
 457 redescription of the adult and larval stages. J. Med. Entomol. 49, 794–802
- 458 Estrada Peña, A., Tarragona, E.L., Vesco, U., De Meneghi, D., Mastropaolo, M., Mangold, A.J.,
 459 Guglielmone, A.A., Nava, S., 2014a. Divergent environmental preferences and areas of
 460 sympatry of tick species in the *Amblyomma cajennense* complex (Ixodidae). Int. J. Parasitol.
 461 44, 1081–1089.
- 462 Estrada-Peña, A., Estrada-Sánchez, A., de la Fuente, J., 2014b. A global set of Fourier- transformed
 463 remotely sensed covariates for the description of abiotic niche in epidemiological studies of
 464 tick vector species. Parasit. Vectors 7: 302.
- 465 Gerardi, M., Martins, M.M., Nava, S., Szabó, M.P.J., 2013. Comparing feeding and reproductive
 466 parameters of *Amblyomma parvum* tick populations (Acari: Ixodidae) from Brazil and
 467 Argentina on various host species. Vet. Parasitol. 197, 312–327
- 468 Glass, G.E., Schwartz, B.S., Morgan, J.M., Johnson, D.T., Noy, P.M., Israel, E., 1995. Environmental
 469 risk factors for Lyme disease identified with geographic information systems. Am. J. Publ.
 470 Health 85, 944–948.
- 471 Gourbière, S., Mallet, J., 2009. Are species real? The shape of the species boundary with
 472 exponential failure, reinforcement, and the “missing snowball”. Evolution 64, 1–24.
- 473 Guerra, M., Walker, E., Jones, C., Paskewitz, S., Cortinas, M.R., Stancil, A., Beck, L., Bobo, M.,
 474 Kitron, U., 2002. Predicting the risk of Lyme disease: habitat suitability for *Ixodes scapularis*
 475 in the north central United States. Emerg. Infect. Dis. 8, 289–297.

- 476 Guglielmone, A.A., Mangold, A.J., Aguirre, D.H., Gaido, A.B., De Olsen, A.A., 1989. The effect of
477 infection by *Babesia* sp. on some biological parameters of engorged females of *Boophilus*
478 *microplus*. *Folia Parasitol.* 36, 1-6.
- 479 Guglielmone, A.A., Mangold, A.J., Keirans, J.E., 1990. Redescription of the male and female of
480 *Amblyomma parvum* Aragao, 1908, and description of the nymph and larva, and description
481 of all stages of *Amblyomma pseudoparvum* sp.n. (Acari: Ixodida: Ixodidae). *Acarologia* 31,
482 144-159.
- 483 Guglielmone, A.A., Mangold, A.J., Garcia, M.D., 1991. The life cycle of *Amblyomma parvum* Aragao,
484 1908 (Acari: Ixodidae) under laboratory conditions. *Exp. Appl. Acarol.* 13, 129-136.
- 485 Guglielmone, A.A., Beati, L., Barros-Battesti, D.M., Labruna, M.B., Nava, S., Venzal, J.M., Mangold,
486 A.J., Szabó, M.J.P., Martins, J.R., González Acuña, D., Estrada-Peña, A., 2006. Ticks
487 (Ixodidae) on humans in South America. *Exp. Appl. Acarol.* 40, 83-100.
- 488 Horta, M.C., do Nascimento, G.F., Martins, T.F., Labruna, M.B., Machado, L.C.P., Nicola, P.A., 2011.
489 Ticks (Acari: Ixodida) parasitizing free-living wild animals in the Caatinga biome in the State
490 of Pernambuco, northeastern Brazil. *Syst. Appl. Acarol.* 16, 207-211
- 491 Knowlton, N., Weigt, L.A., 1997. Species of marine invertebrates: a comparison of the biological and
492 phylogenetic species concepts. In: Claridge, M.F., Dawah, H.A., Wilson, M.R. (eds.).
493 *Species: The units of biodiversity*. Chapman and Hall, London, pp. 199-219.
- 494 Labruna, M.B., Jorge, R.S.P., Sana, D.A., Jácomo, A.T.A., Kashivakura, C.K., Furtado, M.M., Ferro,
495 C., Perez, S.A., Silveira, L., Santos, T.S., Marques, S.R., Morato, R.G., Nava, A., Adania,
496 C.H., Teixeira, R.H.F., Gomes, A.A.B., Conforti, V.A., Azevedo, F.C.C., Prada, C.S., Silva,
497 J.C.R., Batista, A.F., Marvulo, M.F.V., Morato, R.L.G., Alho, C.J.R., Pinter, A., Ferreira, P.M.,
498 Ferreira, F., Barros-Battesti, D.M., 2005a. Ticks (Acari: Ixodida) on wild carnivores in Brazil.
499 *Exp. Appl. Acarol.* 36, 149-163.
- 500 Labruna, M.B., Keirans, J.E., Camargo, L.M.A., Ribeiro, A.F., Soares, R.M., Camargo, E.P., 2005b.
501 *Amblyomma latepunctatum*, a valid tick species (Acari: Ixodidae) long misidentified with both
502 *Amblyomma incisum* and *Amblyomma scalpturatum*. *J. Parasitol.* 91, 527-541.

- 503 Labruna, M.B., Naranjo, V., Mangold, A.J., Thompson, C., Estrada-Peña, A., Guglielmone, A.A.,
 504 Jongejan, F., de la Fuente, J., 2009. Allopatric speciation in ticks: genetic and reproductive
 505 divergence between geographic strains of *Rhipicephalus (Boophilus) microplus*. BMC Evol
 506 Biol. 9: 1-12.
- 507 Labruna, M.B., Soares, J.F., Martins, T.F., Soares, H.S., Cabrera, R.R., 2011a. Cross-mating
 508 experiments with geographically different populations of *Amblyomma cajennense* (Acari:
 509 Ixodidae). Exp. Appl. Acarol. 54, 41-49.
- 510 Labruna, M.B., Mattar, S., Nava, S., Bermudez, S., Venzal, J.M., Dolz, G., Abarca, K., Romero, L., de
 511 Sousa, R., Oteo, J., Zavala-Castro, J., 2011b. Rickettsioses in Latin America, Caribbean,
 512 Spain and Portugal. Rev. MVZ Córdoba 16, 2435-2457.
- 513 Lado, P., Nava, S., Labruna, M.B., Szabó, M.P.J., Durden, L., Bermudez, S.E., Montagna, M.,
 514 Sanchez Quirós, A.C., Beati, L., 2016. *Amblyomma parvum* Aragão, 1908 (Acari: Ixodidae):
 515 phylogeography and systematic considerations. Ticks Tick Borne Dis. 7, 817-827.
- 516 Legendre, P., 1993. Spatial autocorrelation: trouble or new paradigm? Ecology 74, 1659–1673.
- 517 Mangold, A.J., Barges, M.D., Mas-Coma, S., 1998. Mitochondrial 16S rRNA sequences and
 518 phylogenetic relationships of *Rhipicephalus* and other tick genera among Metastrata (Acari:
 519 Ixodidae). Parasitol. Res. 84, 478-484.
- 520 Mansfield, E.R., Helms, B.P., 1982. Detecting multicollinearity. Am. Stat. 36, 158-160.
- 521 Markolf, M., Rakotonirina, H., Fitchel, C., von Grumbkow, P., Brameier, M., Kappeler, P.M., 2013.
 522 True lemurs...true species- species delimitation using multiple data sources in the brown
 523 lemur complex. BMC Evol. Biol. 13: 233.
- 524 Mastropaolo, M., Nava, S., Guglielmone, A.A., Mangold, A.J., 2011. Biological differences between
 525 two allopatric populations of *Amblyomma cajennense* (Acari: Ixodidae) in Argentina. Exp.
 526 Appl. Acarol. 53, 371-375.
- 527 Mayden, R.L., 1997. A hierarchy of species concepts: the denouement in the saga of the species
 528 problems. In: Claridge, M.F., Dawah, H.A., Wilson, M.R. (eds.), Species: the units of
 529 biodiversity. Chapman and Hall, London, pp. 381-424.

- 530 Mayden, R.L., 2002. On biological species, species concepts and individuation in the natural world.
531 Fish and Fisheries 3, 171-196.
- 532 Mayr, E., 1982. The growth of biological thought: diversity, evolution, and inheritance. The Belknap
533 press of Harvard University Press, Cambridge.
- 534 Mayr, E., 1987. The ontological status of species: scientific progress and philosophical terminology.
535 Biol. Philos. 2, 145-166.
- 536 Mayr E. 1996. What is a species, and what is not?. Philos. Sci. 63, 262-277.
- 537 Morrone, J.J., 2006. Biogeographic areas and transition zones of Latin American and the Caribbean
538 islands based on panbiogeographic and cladistic analyses of the entomofauna. Ann. Rev.
539 Entomol. 51, 467-494
- 540 Nava, S., Mangold, A.J., Guglielmone, A.A., 2006. The natural hosts for larvae and nymphs of
541 *Amblyomma neumanni* and *Amblyomma parvum* (Acari: Ixodidae). Exp. Appl. Acarol. 40,
542 123-131.
- 543 Nava, S., Szabó, M.P.J., Mangold, A.J., Guglielmone, A.A., 2008. Distribution, hosts, 16S rDNA
544 sequences and phylogenetic position of the Neotropical tick *Amblyomma parvum* (Acari:
545 Ixodidae). Ann. Trop. Med. Parasitol. 102, 409-425.
- 546 Nava, S., Beati, L., Labruna, M.B., Cáceres, A.G., Mangold, A.J., Guglielmone, A.A., 2014a.
547 Reassessment of the taxonomic status of *Amblyomma cajennense* (Fabricius, 1787) with the
548 description of three new species, *Amblyomma tonelliae* n. sp., *Amblyomma interandinum* n.
549 sp. and *Amblyomma patinoi* n. sp., and reinstatement of *Amblyomma mixtum* Koch, 1844 and
550 *Amblyomma sculptum* Berlese, 1888 (Ixodida: Ixodidae). Ticks Tick Borne Dis. 5, 252-276.
- 551 Nava, S., Beati, L., Dunlop, J., Guglielmone, A.A., 2014b. Reestablishment of *Amblyomma tenellum*
552 Koch, 1844 (Acari: Ixodidae). Ticks Tick Borne Dis. 5, 620-623.
- 553 Nieri-Bastos, F.A., Lopes, M.G., Cançado, P.H., Rossa, G.A., Faccini, J.L., Gennari, S.M., Labruna,
554 M.B., 2014. Candidatus Rickettsia andeanae, a spotted fever group agent infecting
555 *Amblyomma parvum* ticks in two Brazilian biomes. Mem. Inst. Oswaldo Cruz 109, 259-261

- 556 Pacheco, R.C., Moraes-Filho, J., Nava, S., Brandao, P.E., Richtzenhain, L.J., Labruna, M.B., 2007.
 557 Detection of a novel spotted fever group rickettsia in *Amblyomma parvum* ticks (Acari:
 558 Ixodidae) from Argentina. Exp. Appl. Acarol. 43, 63-71.
- 559 Pacheco, R.C., Echaide, I.E., Alves, R.N., Beletti, M.E., Nava, S., Labruna, M.B., 2013. *Coxiella*
 560 *burnetii* in ticks, Argentina. Emerg. Infect. Dis. 19, 344-346.
- 561 Ramos, V.N., Osava, C.F., Piovezan, U., Szabó, M.P., 2014. Ticks on humans in the Pantanal
 562 wetlands, Brazil. Ticks Tick Borne Dis. 5, 497-499.
- 563 Reeves, P.A., Richards, C.M., 2011. Species delimitation under the general lineage concept: an
 564 empirical example using wild North American hops (Cannabaceae: *Humulus lupulus*). Syst.
 565 Biol. 60, 45-59.
- 566 Robinson, L.E., 1926. Ticks. A monograph of the Ixodoidea. Part IV. The genus *Amblyomma*.
 567 Cambridge University Press, London.
- 568 Saraiva, D.G., Fournier, G.F.S.R., Martins, T.F., Leal, K.P.G., Vieira, F.N., Camara, E.M.V.C., Costa,
 569 C.G., Onofrio, V.C., Barros-Battesti, D.M., Guglielmone, A.A., Labruna, M.B., 2012. Ticks
 570 (Acari: Ixodidae) associated with terrestrial mammals in the state of Minas Gerais,
 571 southeastern Brazil. Exp. Appl. Acarol. 58, 159-166.
- 572 Sites, J.W., Crandall, K.A., 1997. Testing species boundaries in biodiversity studies. Conserv. Biol.
 573 11, 1289-1297.
- 574 Sites, J.W., Marshall, J.C., 2004. Operational criteria for delimiting species. Ann. Rev. Ecol. Evol.
 575 Syst. 35, 199-227.
- 576 Szabó, M.P.J., Olegario, M.M.M., Santos, A.L.Q., 2007. Tick fauna from two locations in the Brazilian
 577 savannah. Exp. Appl. Acarol. 43, 73-84
- 578 Tomassone, L., Nuñez, P., Gurtler, R., Ceballos, L.A., Orozco, M.A., Kitron, U.D., Farber, M., 2008.
 579 Molecular detection of *Ehrlichia chaffeensis* in *Amblyomma parvum* ticks, Argentina. Emerg.
 580 Infect. Dis. 14, 1953-1955
- 581 Wakes, D.B., 2006. Problems with species: patterns and processes of species formation in
 582 salamanders. Ann. Missouri Bot. Gard. 93, 8-23.

583 Wiens, J.J., Penkrot, T.A., 2002. Delimiting species using DNA and morphological variation and
584 discordant species limits in spiny lizards (*Sceloporus*). Syst. Biol. 51, 69-91.

585 Wiley, E.O., Mayden, R.L., 2000. The evolutionary species concept. In: Wheeler, Q.D., Meier, R.
586 (eds.), Species concepts and phylogenetic theory: a debate. Columbia University Press, New
587 York, pp. 70-89.

588 Zar, J.H., 1999. Biostatistical analysis. Prentice-Hall, New Jersey.

589 Zelditch, M.L., Swiderski, D.L., Sheets, H.D., Fink, W.L., 2004. Geometrics morphometrics for
590 biologists. Elsevier, San Diego.

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Captions of figure

Figure 1. Morphometric characters of *Amblyomma parvum* ticks (adults) used in Principal Component Analysis and Canonical Discriminant Analysis. 1) Body length; 2) Breadth; 3) Scutum length; 4) Scutum width; 5) Distance between porose areas; 6) Length of basis capituli; 7) Width of basis capituli; 8) Palpal length; 9) Length palpal article I; 10) Length palpal article II; 11) Length palpal article III; 12) Width palpal article I; 13) Width palpal article II; 14) Width palpal article III; 15) Hypostome length; 16) Hypostome width; 17) Spiracular plate length; 18) Spiracular plate width; 19) Tarsus I length; 20) Tarsus I width. Figures are adapted from Boero (1957).

Figure 2. *Amblyomma parvum* male. A. Body, dorsal view; B. Body, ventral view; C. Capitulum, dorsal view; D. Hypostome. 1) Scutum punctuation; 2) Shape of basis capituli, dorsal view; 3) Ventral plates (a: dorsal view; b: ventral view); 4) Spurs of the legs (a: spurs on trochanter I; b: spurs on coxa I; c: spurs on coxa II; d: spurs on coxa III; e: spurs on coxa IV); 5) Ventral spur of the article I of the palpi; 6) Spiracular plate; 7) Hypostome. The specimen of *A. parvum* which is shown in the image belongs to an Argentinean population.

Figure 3. *Amblyomma parvum* female. A. Body, dorsal view; B. Body, ventral view; C. Capitulum, dorsal view; D. Hypostome. 1) Scutum punctuation; 2) Shape of basis capituli, dorsal view; 3) Dorsal setae of the body; 4) Spurs of the legs (a: spurs on trochanter I; b: spurs on coxa I; c: spurs on coxa II; d: spurs on coxa III; e: spurs on coxa IV); 5) Ventral spur of the article I of the palpi; 6) Genital aperture; 7) Spiracular plate; 8) Hypostome; 9) Porose areas. The specimen of *A. parvum* which is shown in the image belongs to an Argentinean population.

Figure 4. Principal components analysis of the body measurements of *Amblyomma parvum*. Fig. 4a: males. Fig. 4b: females. Characters are detailed in table 2 and fig. 1. Each point constitutes the position of each measured specimen on the reduced space. MA: males from Chaco Biogeographic Province (Argentina); MB: males from Cerrado Biogeographic Province (Brazil); MBPAN: males from Pantanal Biogeographic Province (Brazil); HA: females from Chaco Biogeographic Province (Argentina); HB: females from Cerrado Biogeographic Province (Brazil); HBPAN: females from Pantanal Biogeographic Province (Brazil).

638 **Figure 5.** Canonical discriminant analysis of *Amblyomma parvum*. Fig. 5a: males. Fig. 5b: females.

639 Characters are detailed in table 2 and fig. 1. MCH: males from Chaco Biogeographic Province

640 (Argentina); MCE: males from Cerrado Biogeographic Province (Brazil); MP: males from Pantanal

641 Biogeographic Province (Brazil); HCH: females from Chaco Biogeographic Province (Argentina);

642 HCE: females from Cerrado Biogeographic Province (Brazil); HPA: females from Pantanal

643 Biogeographic Province (Brazil).

644 **Figure 6.** Distribution of *Amblyomma parvum* in South America in relation to biogeographical areas

Table 1. Collection localities of the *Amblyomma parvum* ticks (adults) used for the comparison of morphological characters. Biogeographic provinces are listed according to Morrone (2006).

COUNTRY	PROVINCE/STATE	LOCALITY	COORDINATES	BIOGEOGRAPHIC PROVINCE
Argentina	Córdoba	Quilino	30°13'S 64°31'W	Chaco
Argentina	Córdoba	San José de las Salinas	30°04'S 64°35'W	Chaco
Argentina	Salta	El Tunal	25°14'S 64°25'W	Chaco
Argentina	Salta	Dragones	23°16'S 63°21'W	Chaco
Argentina	Santiago del Estero	Cejolao	27°29'S 62°18'W	Chaco
Brazil	Goiás	Araguapaz	15°03'S 50°05'W	Cerrado
Brazil	Mato Grosso do Sul	Fazenda Nhumirim	18°58'S 56°36'W	Pantanal
Brazil	Mato Grosso do Sul	Fazenda Alegria	19°08'S 56°46'W	Pantanal
Brazil	Mina Gerais	Formoso	15°18'S 45°49'W	Cerrado

Table 2. Morphometric characters (mean $\pm$ standard deviation) of *Amblyomma parvum* ticks (adults) used in principal component analysis and canonical discriminant analysis. See Fig. 1 to reference of each character.

	♂♂ Argentina	♂♂ Brazil	♀♀ Argentina	♀♀ Brazil
Body length	2.69 $\pm$ 0.27	2.60 $\pm$ 0.11	3.30 $\pm$ 0.10	3.41 $\pm$ 0.15
Breadth	1.94 $\pm$ 0.21	1.95 $\pm$ 0.13	2.51 $\pm$ 0.22	2.44 $\pm$ 0.18
Scutum length	2.22 $\pm$ 0.08	2.24 $\pm$ 0.10	1.44 $\pm$ 0.04	1.47 $\pm$ 0.02
Scutum width	1.60 $\pm$ 0.07	1.64 $\pm$ 0.05	1.46 $\pm$ 0.03	1.47 $\pm$ 0.04
Distance between porose areas	Not applicable	Not applicable	0.29 $\pm$ 0.09	0.33 $\pm$ 0.11
Diameter of porose areas	Not applicable	Not applicable	0.12 $\pm$ 0.01	0.15 $\pm$ 0.01
Length of basis capituli	0.26 $\pm$ 0.01	0.27 $\pm$ 0.01	0.35 $\pm$ 0.01	0.36 $\pm$ 0.01
Width of basis capituli	0.34 $\pm$ 0.02	0.36 $\pm$ 0.01	0.48 $\pm$ 0.01	0.48 $\pm$ 0.02
Palpal length	0.60 $\pm$ 0.03	0.57 $\pm$ 0.02	0.63 $\pm$ 0.03	0.60 $\pm$ 0.02
Length palpal article I	0.13 $\pm$ 0.01	0.12 $\pm$ 0.01	0.12 $\pm$ 0.01	0.12 $\pm$ 0.01
Length palpal article II	0.24 $\pm$ 0.01	0.24 $\pm$ 0.01	0.40 $\pm$ 0.01	0.39 $\pm$ 0.02
Length palpal article III	0.23 $\pm$ 0.01	0.22 $\pm$ 0.01	0.24 $\pm$ 0.01	0.24 $\pm$ 0.01
Width palpal article I	0.12 $\pm$ 0.01	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01
Width palpal article II	0.15 $\pm$ 0.01	0.15 $\pm$ 0.01	0.15 $\pm$ 0.01	0.15 $\pm$ 0.01
Width palpal article III	0.20 $\pm$ 0.01	0.19 $\pm$ 0.01	0.19 $\pm$ 0.01	0.19 $\pm$ 0.01
Hypostome length	0.36 $\pm$ 0.02	0.38 $\pm$ 0.03	0.40 $\pm$ 0.01	0.43 $\pm$ 0.01
Hypostome width	0.20 $\pm$ 0.04	0.20 $\pm$ 0.06	0.30 $\pm$ 0.08	0.26 $\pm$ 0.01
Spiracular plate length	0.45 $\pm$ 0.05	0.42 $\pm$ 0.04	0.40 $\pm$ 0.03	0.45 $\pm$ 0.02
Spiracular plate width	0.26 $\pm$ 0.01	0.27 $\pm$ 0.03	0.24 $\pm$ 0.02	0.26 $\pm$ 0.01
Tarsus I length	0.51 $\pm$ 0.02	0.47 $\pm$ 0.02	0.61 $\pm$ 0.01	0.56 $\pm$ 0.03
Tarsus I width	0.15 $\pm$ 0.02	0.15 $\pm$ 0.01	0.17 $\pm$ 0.02	0.15 $\pm$ 0.01

Table 3. Means and standard deviations of the biological parameters in engorged females obtained from the crosses of *Amblyomma parvum* from Argentina and Brazil.

	<u>♂♂ Brazil ♀♀ Brazil</u>	<u>♂♂ Argentina ♀♀ Argentina</u>	<u>♂♂ Brazil ♀♀ Argentina</u>	<u>♂♂ Argentina ♀♀ Brazil</u>
Feeding period (days) *	11.78 ± 0.66 ^a	11.48 ± 0.72 ^a	11.42 ± 0.56 ^a	11.26 ± 0.54 ^a
Engorgement weight (mg) *	305.61 ± 11.20 ^a	401.23 ± 22.13 ^b	338.91 ± 13.52 ^a	437.00 ± 18.32 ^b
Pre-oviposition period (days) *	5.26 ± 0.18 ^a	5.26 ± 0.14 ^a	5.51 ± 0.15 ^a	5.35 ± 0.19 ^a
Incubation period of eggs (days) *	35.00 ± 0.36 ^a	32.31 ± 0.26 ^a	33.57 ± 0.30 ^a	33.58 ± 0.32 ^a
Reproductive efficiency index* [†]	9.60 ± 1.19 ^a	11.05 ± 1.30 ^b	10.95 ± 1.30 ^b	11.02 ± 1.11 ^b
Fecundity efficiency index* [†]	9.37 ± 1.01 ^b	10.82 ± 1.91 ^a	7.96 ± 1.26 ^b	10.80 ± 1.62 ^a

* ANOVA. Numbers not sharing superscripts are significantly different (P < 0.01).

[†] See definition in materials and methods.

Table 4. Means and standard deviations of the biological parameters in engorged females obtained from the backcrosses of *Amblyomma parvum* from Argentina and Brazil. MM: Male; FF: Female; Ar: Argentina; Br: Brazil

	<u>Engorgement weight (mg)*</u>	<u>Reproductive efficiency index*†</u>	<u>Fecundity efficiency index*†</u>
F1 MM (MM Ar x FF Br) x FF (MM Ar x FF Br)	364,57± 15.13 ^{ab}	11,05 ± 0.27 ^{ab}	10,23 ± 0.36 ^a
F1 MM (MM Br x FF Ar) x FF (MM Br x FF Ar)	334,04 ± 17.38 ^{ab}	10,58 ± 0.37 ^{ab}	10,03 ± 0.45 ^a
MM Br x F1 FF (MM Ar x FF Br)	359,74 ± 17.19 ^{ab}	11,1 ± 0.34 ^{ab}	9,31 ± 0.85 ^a
MM Br x F1 FF (MM Br x FF Ar)	300,96 ± 18.04 ^b	10,54 ± 0.49 ^{ab}	10,27 ± 0.57 ^a
MM Ar x F1 FF (MM Ar x FF Br)	370,56 ± 14.28 ^a	11,51 ± 0.35 ^a	10,32 ± 0.36 ^a
MM Ar x F1 FF (MM Br x FF Ar)	335,61 ± 13.97 ^{ab}	10,89 ± 0.38 ^{ab}	7,17 ± 1.26 ^a
F1 MM (MM Ar x FF Br) x FF Br	365,18 ± 21.51 ^{ab}	10,63 ± 0.33 ^{ab}	9,07 ± 0.42 ^a
F1 MM (MM Ar x FF Br) x FF Ar	351,68 ± 7.70 ^{ab}	9,93 ± 0.25 ^b	9,31 ± 0.67 ^a
F1 MM (MM Br x FF Ar) x FF Br	335,70 ± 21.60 ^{ab}	10,75 ± 0.31 ^{ab}	10,12 ± 0.46 ^a
F1 MM (MM Br x FF Ar) x FF Ar	346,22 ± 9.59 ^{ab}	11,25 ± 0.33 ^{ab}	10,18 ± 0.48 ^a

* ANOVA. Numbers not sharing superscripts are significantly different (P < 0.01).

† See definition in materials and methods.

Figure 1

Figure 2

Figure 3

Figure 4a

PCA plot showing the first two principal components (F1 and F2) for 18 samples. The x-axis is F1 (40.18%) and the y-axis is F2 (22.22%).

Legend:

- HB1-HB12: HB1, HB2, HB3, HB4, HB5, HB6, HB7, HB8, HB9, HB10, HB11, HB12
- HA1-HA10: HA1, HA2, HA3, HA4, HA5, HA6, HA7, HA8, HA9, HA10
- HBAN1-HBAN4: HBAN1, HBAN2, HBAN3, HBAN4

Approximate coordinates (F1, F2) for each sample:

Sample	F1 (40.18%)	F2 (22.22%)
HB1	0.5	-0.5
HB2	-3.0	1.8
HB3	0.0	-1.0
HB4	2.0	1.0
HB5	-3.0	-2.2
HB6	-3.0	1.8
HB7	-2.0	-2.0
HB8	-0.5	-0.8
HB9	-3.0	1.8
HB10	-5.5	3.2
HB11	0.0	3.0
HB12	-3.0	1.8
HA1	0.5	0.2
HA2	0.0	0.5
HA3	0.5	-0.2
HA4	1.5	1.8
HA5	0.5	1.2
HA6	2.5	1.8
HA7	-0.5	-1.2
HA8	2.0	1.0
HA9	0.5	-0.2
HA10	3.0	1.8
HBAN1	0.0	-2.0
HBAN2	0.5	-2.2
HBAN3	0.5	-3.5
HBAN4	1.0	-3.2

Figure 5a

Figure 5b

Figure 6