

## Exploring the ecological and evolutionary relationships between *Rickettsia* and hard ticks in the Neotropical region.

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### ABSTRACT

This study addresses a meta-analysis of the distribution of *Rickettsia* spp. in the Neotropical region, as well as their associations with ticks and vertebrates. A total of 219 published reports on *Rickettsia* in ticks in the target region were compiled, providing 599 records of 31 species of *Rickettsia* recorded in 50 species of Ixodidae. The aim is to capture the phylogenetic relationships between rickettsiae and the ticks carrying them in the target region, with a focus on the co-speciation ticks-rickettsiae. We compared the phylogeny of ticks, the records of rickettsiae, the environmental gradients colonized by ticks and the effect of the phylogenetic composition of vertebrates feeding ticks on the detection of *Rickettsia* in ticks. Results show that differences in rickettsial composition in ticks do not depend on the vertebrate's blood-source. This is the first time this result is demonstrated. This study pinpoints that some Neotropical rickettsial organisms are associated with well-defined phylogenetical clusters of ticks. Secondly, and probably only in a few cases, rickettsiae share species of phylogenetically distant ticks distributed along a gradient of environmental traits in which the ticks overlap (i.e., the different strains of *Rickettsia parkeri* sensu lato). We outline the importance of some ticks that share hosts and habitat: these ticks may act as “bridges” for the circulation of rickettsial species. There are also many species of *Rickettsia* that have been detected so far in only one tick species, pointing to a tight relationship or to the lack of data preventing conclusions about the detection of these bacteria in other ticks. Two species, namely *Rickettsia amblyommatis* and *Rickettsia bellii* have been recorded in the majority of ticks in the region (mainly *Amblyomma* spp.) and seem to be not associated with definite tick species because they may be an essential symbiont of the ticks. We conclude that an adequate analysis of rickettsiae-ticks-habitat is necessary to address the human health issues derived from the infections by rickettsiae.

### 1. Introduction

Organisms of the genus *Rickettsia* (Rickettsiales: Rickettsiaceae) are obligate intracellular alpha-proteobacteria that have been primarily associated with diverse eukaryotic hosts, including protists, leeches, and several arthropod groups (Diop et al., 2019). Among the latter, hard ticks (Acari: Ixodidae) are indeed the most important group to harbor rickettsial organisms. At least 27 of the 32 valid published *Rickettsia* species are known to be primarily associated with hard ticks (Parola et al., 2013).

*Rickettsia* species have been phylogenetically classified into four major groups: (i) the spotted fever group (SFG), represented by more than 20 valid species, all associated with ticks, including several agents impacting human health (e.g., *Rickettsia rickettsii*, *Rickettsia conorii*, *Rickettsia japonica*, *Rickettsia sibirica*, *Rickettsia africae*, *Rickettsia parkeri*); (ii) the typhus group (TG), represented by only two species, *Rickettsia prowazekii* and *Rickettsia typhi*, which are human pathogens transmitted by lice and fleas, respectively; (iii) the transitional group (TRG), which contain human pathogens that are primarily associated with ticks (*Rickettsia australis*), mites (*Rickettsia akari*) or fleas (*Rickettsia felis*); and

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(iv) the ‘Ancestral’ group (AG), represented by several tick-associated organisms (e.g., *Rickettsia bellii*, *Rickettsia canadensis*) that have never been confirmed as human or animal pathogens (Gillespie et al., 2007; Parola et al., 2013; Diop et al., 2019).

Tick-associated rickettsiae are usually vertically transmitted from engorged female ticks to their offspring (transovarial transmission), making this the most important mechanism driving permanent foci of rickettsiae among tick populations (Burgdorfer and Brinton, 1975). On the other hand, some *Rickettsia* species can negatively affect tick population size, by decreasing female reproductive performance and/or molting success rates (Labruna et al., 2011a; Nieri-Bastos et al., 2013; Krawczak et al., 2016; Gerardi et al., 2019). In such cases, it is generally accepted that the long-term perpetuation of *Rickettsia* species in nature would depend on availability of vertebrate amplifying hosts, which would serve as infection source (horizontal transmission) for creating new cohorts of infected ticks (Burgdorfer, 1988; Labruna, 2009; Polo et al., 2017). This is of interest from an evolutive perspective. If the AG (root) group of rickettsial organisms is a symbiont of ticks, evolution did play a major role when *Rickettsia* “found a way” (other than the tick transovarial-transmission) to perpetuate in permanent cycles. Interestingly, the finding of *Rickettsia* species in phylogenetically distant tick species suggests horizontal transmission during the tick-rickettsia evolving process. Such transmission could also be via tick parasitoids (e.g., *Ixodiphagus* spp.) or hyperparasitism (a tick feeding on another tick specimen), as demonstrated for other tick-associated bacteria (Plantard et al., 2012; Bohacsova et al., 2016; Williamson and Schwan, 2018), in addition to amplifying vertebrate hosts.

Until the end of the 20th century, only three *Rickettsia* species were known to occur in the Neotropical region: the SFG *R. rickettsii* and the TG agents, *R. prowazekii* and *R. typhi*. During the first two decades of the 21st century, rickettsiology underwent a tremendous advancement, and currently more than 15 *Rickettsia* species - in addition to several other uncharacterized agents of this bacterial genus - are known to occur in the Neotropical region, mostly associated with ticks (Labruna et al., 2011b; Parola et al., 2013; Bermúdez and Troyo, 2018; Sánchez-Montes et al., 2020). Such advancement has been accompanied by the emergence and re-emergence of several tick-borne rickettsial diseases in this region of the world; however, rickettsioses remain underreported or neglected in most of the Latin American countries (Parola et al., 2009; Bermúdez and Troyo, 2018; Minervino et al., 2020; Sánchez-Montes et al., 2020).

This study aims to summarize the current knowledge of Neotropical *Rickettsia* and the ticks to which they are associated approaching both phylogenetic and ecological points of view. It explicitly focuses on demonstrating that (i) *Rickettsia* is associated with lineages of ticks that are phylogenetically close, (ii) phylogenetically distant lineages of ticks can share species of *Rickettsia* because environmental niche sharing, and (iii) several species of *Rickettsia* and/or ‘*Candidates*’ are restricted to only one species of tick, demonstrating either a tight parallelism in tick and *Rickettsia* evolution, or a fundamental lack of data. Our study aims to demonstrate that in addition to the close phylogenetic clustering of some *Rickettsia*-ticks in the Neotropics, the concept of environmental niche sharing by ticks may shape the tick-bacteria relationships observed in some biomes.

## 2. Methods

### 2.1. Purpose and bibliographical revision

We focused on the associations of *Rickettsia* spp. in the Neotropical region with ticks and vertebrates. For this we performed a bibliographical analysis, adhering to the concepts of PRISMA (Moher et al., 2015). This study intends to address the evolutionary relationships between ticks and *Rickettsia* in the target region, considering that a presence/absence perspective, as proposed here, does not reflect either the prevalence of *Rickettsia* spp. in questing ticks, landscape features

governing the patterns of tick distribution, social habits driving infection rates in humans, or competence phenomena among species of *Rickettsia* in ticks. All these traits may bias our inferences. We intend to present this approach as a proof-of-concept, setting some basic ecological concepts that could be applied to other biogeographical regions.

A data search was conducted on PubMed using the terms “tick(s)” and “*Rickettsia*”, together with the names of the countries in the Neotropics, or with explicit reference to the Neotropical region. All the studies published as original articles in English, Spanish or Portuguese up to March 2020 were selected. Reviews and studies dealing with clinical cases were excluded; serological studies on hosts were also removed, since our approach is based on the detection of the bacteria in ticks. The list of papers was manually checked screening the title and abstract of each reference as a preliminary filtering removing papers not dealing with the topic. After reading the full texts, all articles that reported the direct detection of *Rickettsia* spp. in ticks in the Neotropical region were selected. We extracted from each paper the details regarding the species of *Rickettsia*, the tick species, the hosts(s) in which ticks were collected, the positivity or negativity of the tick to *Rickettsia*, and the site of collection with coordinates. In this regard, all direct detections were confirmed by molecular analyses; the only exception were the studies of the 20th century, in which only *R. rickettsii* was detected in ticks, and in all cases, confirmed by isolation of high virulent strains through guinea pig infestation or inoculation. Supplementary Material 1 includes the set of data, as well as the DOI (Digital Object Information) used to permanently identify a document. The DOI was not available for every paper included in the meta-analysis, since some studies were from mid 20th Century.

A “record” is considered in this study as the finding of a species of *Rickettsia*, in a species of Ixodidae, in a locality, with independence of the number of tick pools analyzed, or the total number of ticks processed for DNA extraction. Some studies processed ticks in pools, while others tested them individually because a smaller sample size; by a variety of reasons, it is not possible to determine a mean proportion of ticks in which the DNA of a given *Rickettsia* was detected.

### 2.2. Phylogenetic relationships among vertebrates, ticks, and *Rickettsia* spp

Since most of the available studies did report species of *Rickettsia* in feeding ticks, we first aimed at evaluating if the set of ticks positive for species of *Rickettsia* (R+) was associated with a set of vertebrates phylogenetically different from those on which ticks negative to *Rickettsia* (R-) were found. This would be an indication that vertebrates feeding positive ticks are different from those feeding negative ticks, and therefore that vertebrates play a role in the associations rickettsiae-ticks. The high number of species of vertebrates recorded in the final dataset may potentially introduce noise elucidating the phylogenetic relationships of R+ or R- ticks and vertebrates; therefore, we collapsed the records to the generic name of the vertebrate. To prepare a phylogenetic tree of the genera of vertebrates on which R+ or R- ticks were collected, we used the package “rotl” (Michonneau et al., 2016) for R (R Core Team, 2020) to query the website “Open Tree of Life” (OTL at <http://tree.opentreeoflife.org>, accessed May 2020) and download the tree of the involved vertebrates genera. The complete list of hosts genera gathered from published reports was not available in OTL and some of the genera were not included in further calculations.

We calculated the Rao’s quadratic entropy (Rao, 2010) to check for significant phylogenetic differences between sets of vertebrates in which R+ or R- ticks have been reported. The purpose is to evaluate if both sets of ticks are associated with groups of vertebrates that are phylogenetically similar. The hypothesis is that *Rickettsia* is associated with ticks: therefore, both sets of vertebrates should be phylogenetically similar. If a significant value appears in this test, it means that associations of *Rickettsia* spp. with ticks could be at least partially driven by the host on which they fed. The phylogenetic information contained in the tree

obtained from OTL was used as input for the test, using the package “picante” (Kembel et al., 2010) for R.

The phylogenetic tree of ticks was prepared with sequences of a 410 bp fragment of the mitochondrial 16S rRNA gene. Sequences were aligned with BioEdit Sequence Alignment Editor (Hall, 1999) with the CLUSTAL W program (Thompson et al., 1994), with manual edition when necessary. Phylogenetic relationships were assessed in terms of maximum likelihood method (ML). Best fitting substitution model was determined with the Bayesian Information Criterion using the ML model test implemented in MEGA 5 (Tamura et al., 2011). The tree made with 16S DNA sequences was generated with the GTR model and a discrete Gamma-distribution (+G).

We measured the phylogenetic linking of each species of rickettsiae to the ticks using the mean pairwise distance (MPD) as the measure of phylogenetic clustering among species of the bacteria and the ticks. In this context, the mean phylogenetic distance is the phylogeny-based diversity of ticks for each species of rickettsia. The index is thus an estimation of the clustering of species of *Rickettsia* to specific clades of the tick phylogenetic tree. The purpose of calculating MPD between the tick species and *Rickettsia* is to demonstrate the hypothesis of segregation of *Rickettsia* along clades of ticks: a significant association with a portion of the tick’s phylogenetic tree would mean for a strict association tick-rickettsia, once the independence with the set of vertebrate hosts is demonstrated.

### 2.3. Networks of ticks-hosts-rickettsiae

We wanted to assess the relationships in the Neotropical region among vertebrates, ticks, and *Rickettsia* for the compiled set of literature reports. We built networks of paired relationships, formed by sets of nodes (species of ticks and *Rickettsia*) that have relationships displayed as links. Each link between interacting nodes represents the weighted number of times a couple of nodes has been reported. The purpose of the networks is the delineation of clusters: these are sets of nodes that tend to co-occur together more frequently than with other nodes of the network. This is an important feature of networks because it explains groups of statistically associated nodes, that observe high relationships among them. Ticks collected on domestic animals were not considered because they distort the topology of the network.

There are several terms regarding networks that indicate the importance of each node. As mentioned, nodes are either ticks or rickettsiae “interacting” with a variable strength defined by the weighted number of records. This is called ‘weighted degree’, because it is weighted according to the total number of references. From this simple measure, the ‘betweenness centrality’ is calculated. The index measures the importance of each node in the context of the network. It is also possible to compute the modularity, also explained as the clustering of the nodes of the network. Clusters (or modules) are sets of nodes (organisms) that interact significantly more among them than with the rest of nodes in different clusters; thus, clusters indicate organisms that are more or less independent to organisms in other clusters. All the calculations were done in Gephi 0.92 (Bastian et al., 2009) using the algorithms included in the software.

### 2.4. The environmental niche of ticks as driver of “spillover” of *Rickettsia* among tick species

In addition to the clustering of the rickettsiae with the tick’s phylogenetic clades, we evaluated the extent at which ticks share portions of its environmental niche. This part of the study is aimed to evaluate the environmental traits that may affect the circulation of the rickettsiae among clusters of ticks that are otherwise phylogenetically unrelated, because they overlap in an environmental niche, where some vertebrates feed these ticks. The ascertainment of the environmental niche of a species requires the use of geo-referenced records. We used the largest dataset of ticks in the Neotropics, recently compiled and made public

(Estrada-Peña et al., 2020) that contains the coordinates of about 13,000 *bona fide* records of ticks in the target region. These geo-referenced records were used to train models of environmental habitat suitability. The purpose is not to draw maps of predicted distribution of ticks but to know the range of environmental variables to which each tick is associated.

The environmental variables were obtained from the TerraClimate repository (<http://www.climatologylab.org/terraclimate.html>), accessed May 2019, using monthly values between the years 1958 and 2019 of maximum and minimum temperature, rain, and water vapor deficit, covering the target territory. Maxent v 3.3.3 was used for running models, using default parameters (Merow et al., 2013). We calculated the portion of the environmental niche that is shared by ticks using the packages “raster” (Hijmans, 2020) and “phyloclim” (Heibl and Calenge, 2018) for R, calculating Schoener’s D distance as the index of environmental sharing.

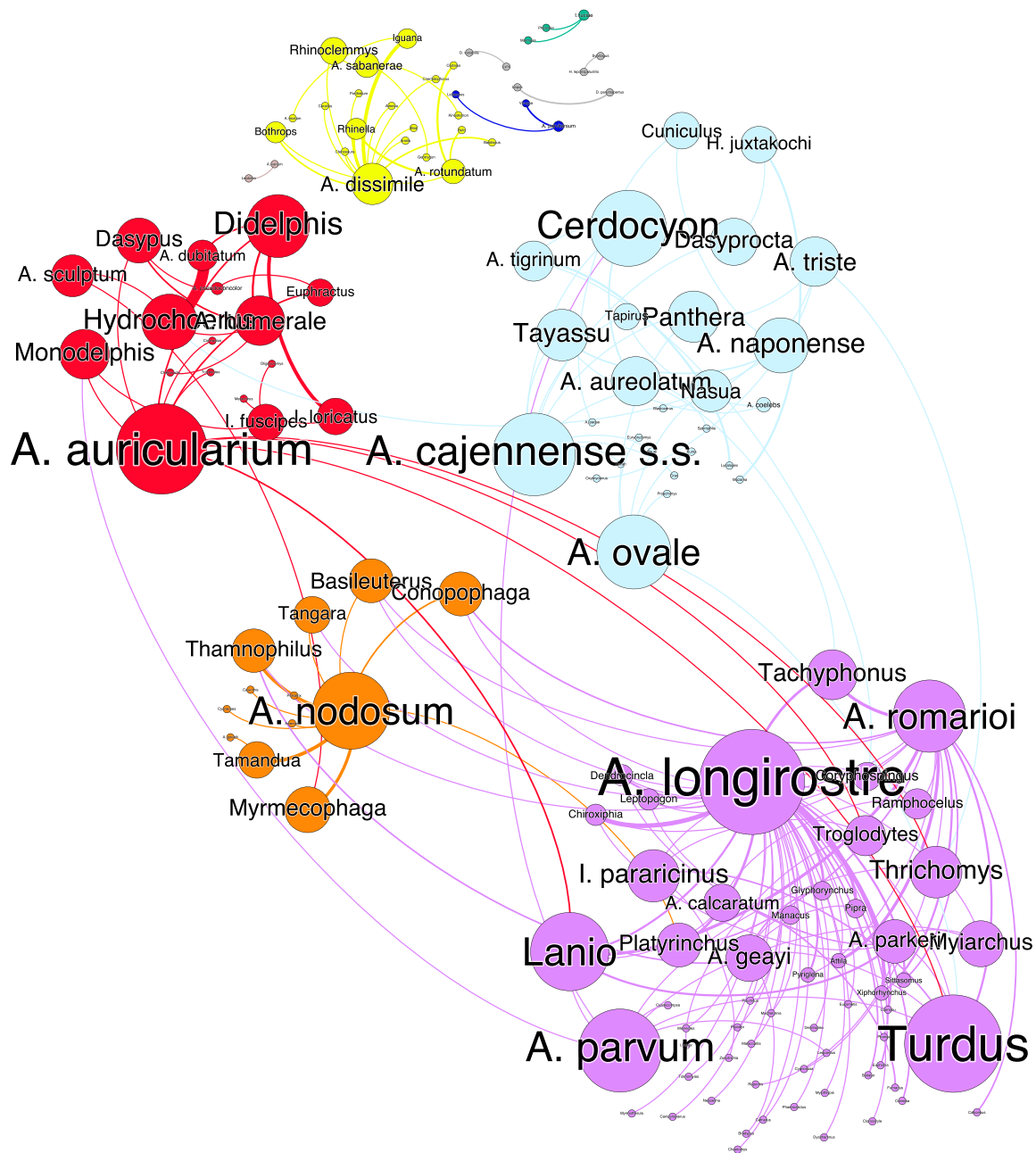
## 3. Results

### 3.1. General results

This study included 219 published reports on *Rickettsia* in ticks in the Neotropical region with 599 records, about which complete details are provided in Supplementary Material 2. These reports included 31 species of *Rickettsia* (including several ‘*Candidatus*’ and yet unnamed species) recorded in 50 species of Ixodidae. Two species of *Dermacentor* were removed because they exist most in southern Nearctic, i.e., northern Mexico, therefore providing an incomplete view of their tick-vertebrates relationships. Ticks in which rickettsial DNA (R+) was detected were collected on 210 species of hosts (111 genera), while ticks negative to rickettsial DNA (R-) were collected on 217 species of hosts (98 genera). Fig. 1 displays the information of the records of tick species on wild hosts, removing the data on domestic animals, hosts reported without a name (i.e., “small mammals”) or ticks not identified at species level. Each color in the Fig. 1 represents a different community (cluster) of co-occurring ticks and vertebrates, which reflects the affinities of each tick towards its hosts. The figure is an overview of the clusters observed among ticks and hosts. The size of each node is proportional to its relative importance in the network (the betweenness centrality). In example, *Amblyomma longirostre* has a high importance in the network because it has been collected on a large variety of hosts, and acts as a “bridge” among several clusters. In the same way, the bird genus *Turdus* is a very central one because several species of ticks have been reported on it. Therefore, the size of each node does *not* indicate the total number of records: betweenness centrality emanates from the topology of the network.

### 3.2. The detection of *Rickettsia* in ticks is independent of the host(s) feeding ticks

We first ascertained if the tick-vertebrates associations could have any influence on the results obtained in the literature compilation. We used the phylogenetic information contained from the tree of the vertebrates, to assess if R+ ticks were collected on a phylogenetically different set of hosts compared with R- ticks. The Rao’s quadratic entropy measures the similarities of the two sets of hosts on which R+ or R- ticks were collected, regarding its position in the phylogenetic tree. Value for hosts of R+ ticks (0.38) is similar to the value for the hosts of ticks reported as R- (0.35), without significant differences ( $p = 0.456$ ). Therefore, the phylogenetic arrangement of the vertebrates on which R+ or R- ticks were found is similar. This result supports the hypothesis that both R+ and R- ticks were obtained from phylogenetic similar sets of hosts and that the finding of R+ ticks is not related to the host on which ticks were feeding.



**Fig. 1.** The network of relationships among ticks and hosts as reported in 219 publications regarding *Rickettsia* spp. in ticks in the Neotropics. Data on domestic animals have been excluded, because these data bias the ticks-hosts relationships. Hosts are included at the generic level to improve readability. The size of each node is proportional to its betweenness centrality, a property explaining how important the node in the network is. The size of the labels is proportional to the weighted number of records. The width of the link between nodes is proportional to the records of each species of ticks on each genus of host. Colors mean for communities of organisms that interact more frequently among them than with the rest of organisms (i.e., clusters). Any generalist information on hosts as “birds” or “carnivores” has been excluded from the calculations and the figure.

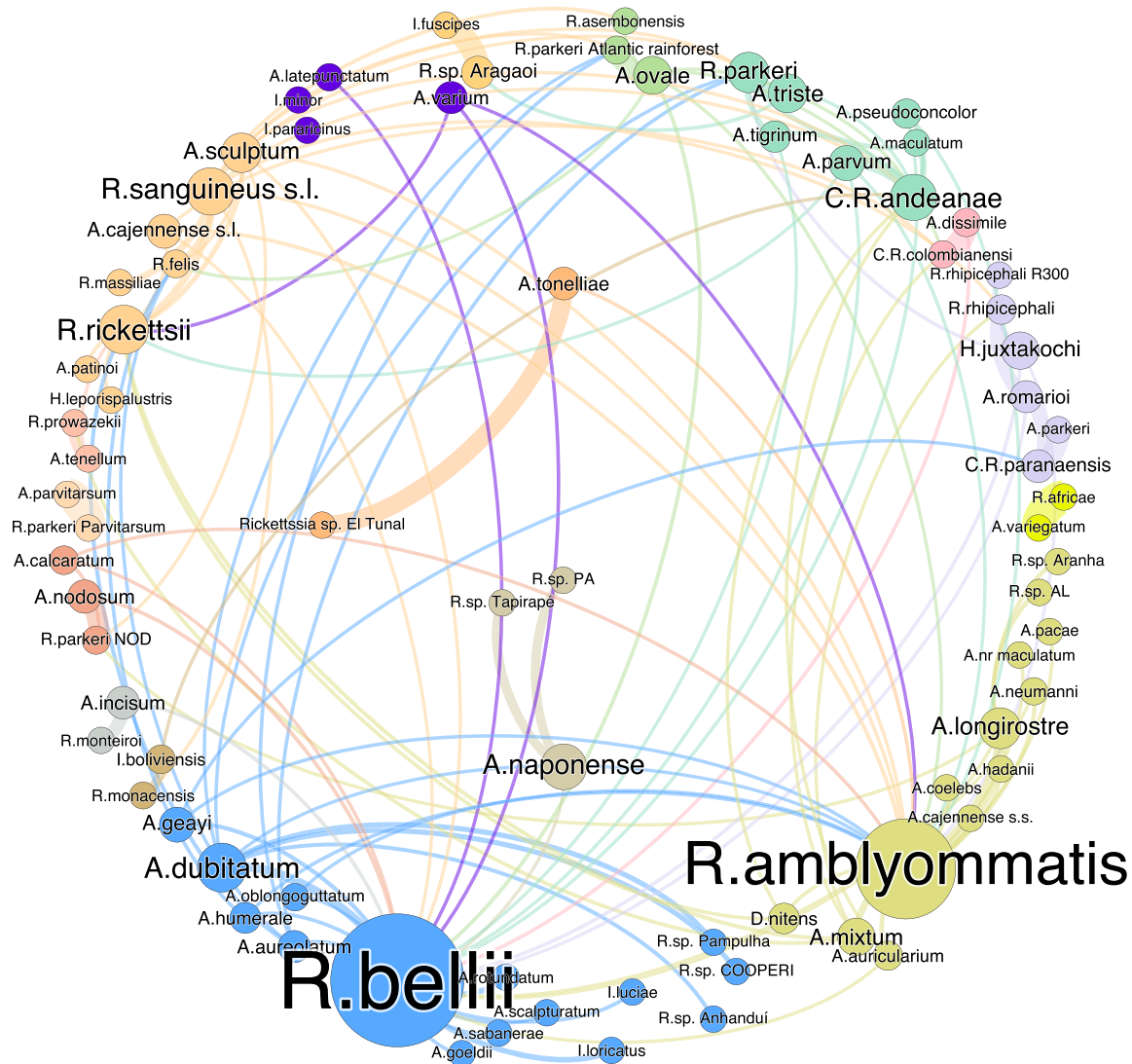
### 3.3. The recorded rickettsial organisms in the Neotropics

We summarized the reports of *Rickettsia* in ticks in the Neotropics in the Fig. 2. The figure is arranged in clusters (colors) that define the sets of more frequently co-occurring organisms; to note that the figure also includes *Rhipicephalus sanguineus* s.l. and *Amblyomma variegatum* that were later removed from further analyses, or *Amblyomma cajennense* s.l. that is known to consist of six different species. *Rickettsia africana* has been reported only in the imported tick *A. variegatum* and therefore both organisms are detected in an isolated cluster; similarly, *Rickettsia monteiroi* has been detected only in *Amblyomma incisum*; therefore the clustering algorithm detects these as a separate cluster. Similar

comments could be drawn for the association *R. parkeri* strain *Parvitarsum* and *Amblyomma parvitarsum*. Both *R. bellii* and *Rickettsia amblyommatis* are the most important bacteria in the network, because they have been detected in several species of ticks. The rest of the network displays the clusters of ticks and *Rickettsia*, some species of ticks being infected with several species of bacteria, or several species of *Rickettsia* detected in several species of ticks.

The Table 1 summarizes the results of the phylogenetic diversity of rickettsiae recorded in ticks. We detected four groups of rickettsiae circulating in Neotropical ticks regarding its association with ticks, which are further outlined in the Fig. 3. Some rickettsiae have been recorded only in one species of ticks and therefore could be presumed





**Fig. 2.** The network of relationships among ticks and rickettsiae as reported in 219 publications regarding *Rickettsia* spp. in ticks in the Neotropics. The size of each node is proportional to its betweenness centrality, a property explaining how important the node in the network is. The size of the labels is proportional to the weighted number of records. The width of the link between nodes is proportional to the records of each species of ticks on each genus of host. Colors mean for communities of organisms that interact more frequently among them than with the rest of organisms (i.e. clusters). This does not mean that a specific rickettsial organism exists only in the community it has been detected (see the links) but that it statistically “tends to appear more frequently” in these ticks. The network includes information on species that are not commonly associated with ticks (i.e. *Rickettsia felis*) or about tick species that were not reliably identified (i.e. *Amblyomma cajennense* s.l.) aiming to provide with the complete information.

either to colonize only that tick species, or results produced by the paucity of data. Most of these bacterial organisms are recently recognized species and could therefore be found in other species of ticks in future surveys. We include in this group yet unnamed species, like the strains Tapirapé, PA, AL, Anhanduí, Aragoi, Aranha, Cooperi, Pampulha and El Tunal. DNA of each of these strains has been detected so far in only one species of ticks; interestingly, two of these yet unnamed species have been recorded in both *Amblyomma dubitatum* and *A. longirostre*. The phylogenetic link among rickettsiae and ticks could not be calculated for all these organisms recorded in single species of ticks (Table 1) since the phylogenetic diversity of ticks is null.

On the other side of the spectrum, there are species of *Rickettsia* that have been recorded in almost every species of tick in the Neotropics, therefore associated with a high phylogenetic diversity of ticks and low values of “p” in Table 1. The two species in this category are *R. amblyommatis* and *R. bellii*. Both have been recorded in the largest number of tick species (see Figs. 2 and 3). A third group of rickettsiae have a high phylogenetic linkage to ticks (see Table 1 and Figs. 2 and 3).

This is interpreted as a true association of these rickettsiae with specific clusters of the tick’s phylogeny. The paradigm is *R. rickettsii*, found in ticks of the *A. cajennense* s.l. group, and its sister clade of *Amblyomma parvum* (but only one record in the later tick). The bacterium is also found in *Amblyomma aureolatum* and *Amblyomma varium*. Considering the data of these rickettsiae, we conclude that *R. rickettsii* in the Neotropics might be mainly associated with the tick clade including the former *A. cajennense* s.l. group of species. Similar results were observed for ‘*Candidatus Rickettsia paranaensis*’, that has a high phylogenetic affinity for ticks of the group including *Amblyomma parkeri*, *Amblyomma geayi* and *Amblyomma romarioi*: this rickettsial organism is linked to these ticks, all of them belonging to the same phylogenetic clade.

### 3.4. Some Neotropical *Rickettsia* segregate along gradients of temperature shared by ticks

There is a fourth group of Neotropical rickettsiae for which results of phylogenetic clustering shows a relative linkage to definite clusters of

**Table 1**

The mean phylogenetic diversity (mpd) of the ticks in which every group of *Rickettsia* spp. was recorded in feeding ticks. The method calculates the mean pairwise distance (MPD) between all species in each community (here, the complete tree of ticks). “mpd.obs” is the observed “mpd” of the community; “mpd.rand.mean” is the mean mid in null communities. These null models include randomizations of the tip labels of the phylogeny, and various community randomizations that can hold community species richness and/or species occurrence frequency constant; “mpd.obs.z” is the standardized effect size of mpd versus null communities and “mpd.obs.p” is the value P-value (quantile) of observed mpd versus null communities. Positive mpd.obs.z > 0 values and high quantiles (mpd.obs.p > 0.95) indicate phylogenetic evenness, or a greater phylogenetic distance among co-occurring species than expected. Negative values and low quantiles (mpd.obs.p < 0.05) indicate phylogenetic clustering, or small phylogenetic distances among co-occurring species than expected. Significant values of p are in bold typeface. Both *R. felis* and *R. africae* have been removed from the table as well as other species for which enough data were not available. Supplementary Material 2 contains all the relationships recorded. *Ixodes minor* and *Ixodes parvicinus* are omitted because only *Rickettsia* sp. has been reported in these species.

Group/Species of <i>Rickettsia</i> spp.	mpd. obs	mpd.rand. mean	mpd.obs. z	mpd. obs.p
Candidatus <i>Rickettsia andeanae</i>	1.314	1.315	-0.314	0.36
Candidatus <i>Rickettsia colombianensi</i>	1.253	1.345	-0.144	0.43
Candidatus <i>Rickettsia paranaensis</i>	0.909	1.380	-7.420	0.04
<i>Rickettsia amblyommatis</i>	1.819	1.912	-0.159	0.45
<i>Rickettsia bellii</i>	1.914	1.999	-0.115	0.66
<i>Rickettsia parkeri</i>	0.814	1.224	-6.921	0.03
<i>Rickettsia parkeri</i> ARF	0.722	1.328	-4.221	0.09
<i>Rickettsia parkeri</i> NOD	1.244	1.346	-0.809	0.18
<i>Rickettsia rhipicephali</i>	1.892	1.677	-2.657	0.26
<i>Rickettsia rickettsii</i>	0.912	1.351	-6.211	0.02
<i>Rickettsia parkeri</i> Parvitarsum	Not calculated because records in one only tick species of tick exist (therefore, there is not phylogenetic variability). This can be interpreted as a complete total speciation of the rickettsia in that tick species or the need of a more extensive surveillance.			
<i>Rickettsia monteiroi</i>				
<i>Rickettsia asemoensis</i>				
<i>Rickettsia</i> sp. Tapirapé				
<i>Rickettsia</i> sp. PA				
<i>Rickettsia</i> sp. AL				
<i>Rickettsia</i> sp. Anhanduí				
<i>Rickettsia</i> sp. Aragaói				
<i>Rickettsia</i> sp. Aranha				
<i>Rickettsia</i> sp. Cooperi				
<i>Rickettsia</i> sp. Pampulha				
<i>Rickettsia</i> sp. El Tunal				

ticks. However, this is difficult to interpret only on phylogenetic premises since the clusters of ticks associated with these bacteria display a high phylogenetic distance among them (Fig. 3). We aimed to demonstrate that, in addition to clustering with tick phylogeny, some species of Neotropical *Rickettsia* are evolving in phylogenetically separated groups of ticks because there is an environmental segregation. We tested this hypothesis using the *R. parkeri* group of strains and ‘Candidatus *Rickettsia andeanae*’, modeling the environmental suitability of the ticks involved and calculating how the ticks segregate along environmental gradients.

We modeled the distribution of 13 species of ticks involved in the circulation of *R. parkeri* and ‘*Ca. R. andeanae*’ using a previously released dataset of geo-referenced tick records. All the models accounted for a high accuracy (AUC > 0.8) and therefore were interpreted as adequately capturing the environmental niche of the modelled ticks. All the tick species displayed preferences for portions of a gradient of temperature; however, these preferences were not significant for soil humidity or atmospheric water vapour. We plotted the expected niche occupancy of each species in the gradient of temperature and calculated the habitat overlap among them. The tick species maintaining the circulation of *R. parkeri* s.l. are clearly separated along the gradient of maximum annual temperature (Fig. 4A). The tick species involved in the circulation of *R. parkeri* s.s. use intermediate positions of the gradient of

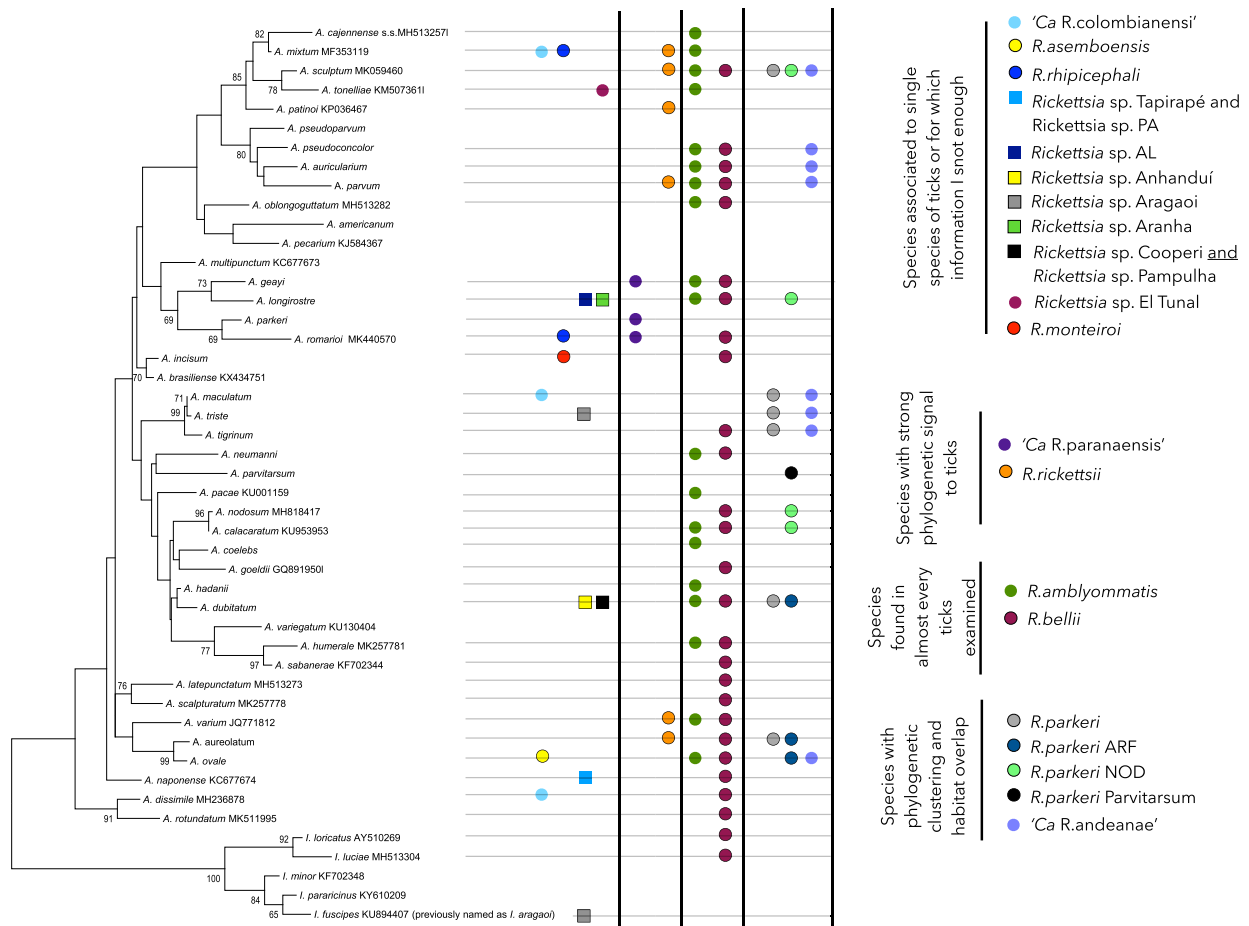
temperature, ticks circulating strain ARF are in intermediate-coldest portions of this gradient, and the ticks circulating strain NOD are in the warmest portion of the temperature gradient. Table 2 displays the Schoener’s D distance among the tick species involved in the circulation of *R. parkeri* s.l. indicating the amount of habitat overlap in the temperature gradient (values in the range 0–1). The overlap in the temperature range of phylogenetically unrelated tick species would facilitate the circulation of *R. parkeri* s.l. Opposing results have been obtained for the ticks circulating ‘*Ca. R. andeanae*’, for which only one strain is currently recognized. The eight tick species supporting the circulation of ‘*Ca. R. andeanae*’ share a similar temperature range (Fig. 4B, Table 3).

#### 4. Discussion

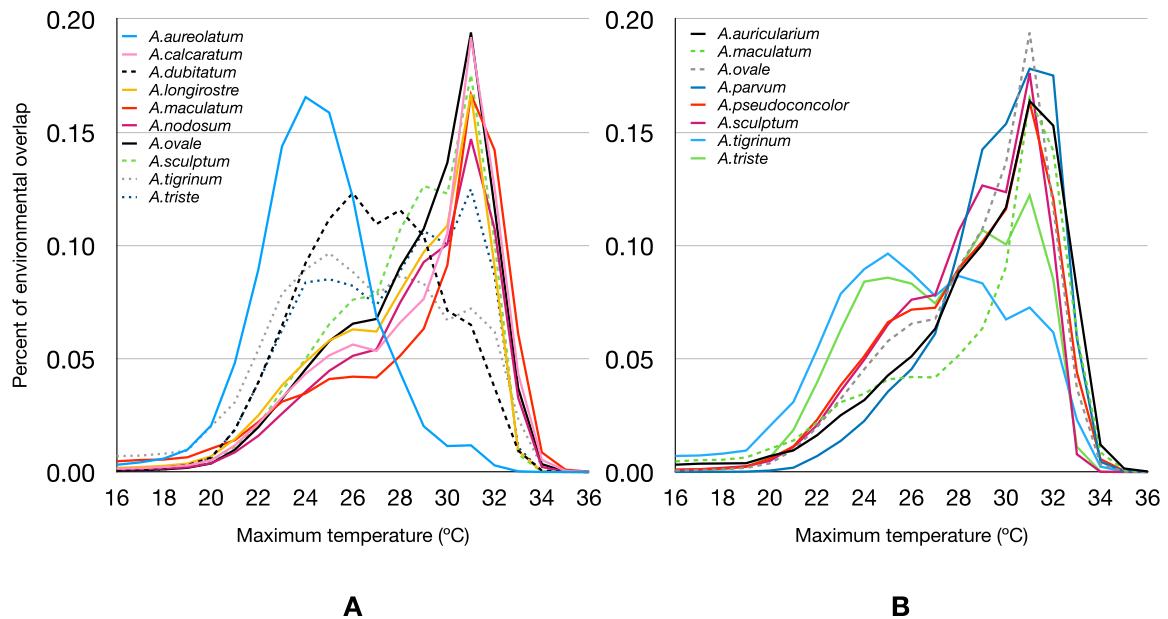
We carried out the first meta-analysis of the relationships among *Rickettsia* spp., ticks, and vertebrates in the Neotropical region, using published data since around the year 1930. We acknowledge that old reports may not reflect the variety of species and strains as known today. Our revision has been focused on the Neotropical region, an area that underwent a complete revolution in the past few years regarding the description of new species of Ixodidae, the compilation of its distribution and hosts relationships, and the recording of new *Rickettsia* (Labruna et al., 2011b; Parola et al., 2013; Nava et al., 2017; Bermúdez and Troyo, 2018; Sánchez-Montes et al., 2020). Existing reports describe local or regional associations of ticks and *Rickettsia*. Our meta-analysis approach, connecting methods from different fields of science, and addressing the first network analysis of its class, aims to understand the phylogenetic and environmental pressures shaping the evolution of *Rickettsia*. Such an approach has been never carried out in any other region of the world.

We recognize limitations in this study. Our analysis could be limited by an incomplete search of “grey literature”. However, considering the size of our dataset (219 reports, with 599 records, 31 species of *Rickettsia* recorded in 50 species of Ixodidae and 217 species of vertebrates in 120 genera) the probable absence of a few communications could hardly change the results. However, the novelty of the topic and the large areas still to be surveyed will probably introduce changes in the future. This study is qualitative, based on pairs of tick-rickettsial associations, ignoring the prevalence of *Rickettsia* in each tick species. Prevalence cannot be calculated from ticks collected while feeding, because it is not possible to know if the detected DNA was already in the tick or transferred with the blood meal (Estrada-Peña et al., 2013). Moreover, the different processing pipelines to detect rickettsial DNA in ticks (using individual specimens, pools, etc.) make impossible to establish a common framework to summarize these data. It is known that some *Rickettsia* may interact among them in the tick and promote antagonistic mechanisms that act regulating the presence of other *Rickettsia* (Macaluso et al., 2002). For example, both *Amblyomma sculptum* and *A. dubitatum* are commonly found on the same hosts. *Amblyomma sculptum* rarely has *R. bellii* and it is the main vector of *R. rickettsii*; however, *R. bellii* is dominant in *A. dubitatum*, in which *R. rickettsii* has been not recorded, even if both species share the host (Luz et al., 2019). Moreover, under laboratory conditions, a *R. bellii*-infected *A. dubitatum* colony was successful in maintaining *R. bellii*, but not *R. rickettsii*, by transovarial transmission, suggesting some ‘interference’ of *R. bellii* (Sakai et al., 2014).

Other aspects could bias the relationships outlined in this study. One is the collecting success of a given tick species in the field. For example, up to 25% of *Ixodes fuscipes* (previously known as *Ixodes aragaói*) have been found to be infected with *Rickettsia* sp. Aragaói in only one survey (Blanco et al., 2017). However, *I. fuscipes* is very difficult to collect, biasing the perception of the distribution or the relative importance of *Rickettsia* sp. Aragaói. Further on this, the detection threshold of the pathogen in the tick may play an important role in the reported rates of *Rickettsia*. It is known that *R. rickettsii* is hard to detect in *A. sculptum*



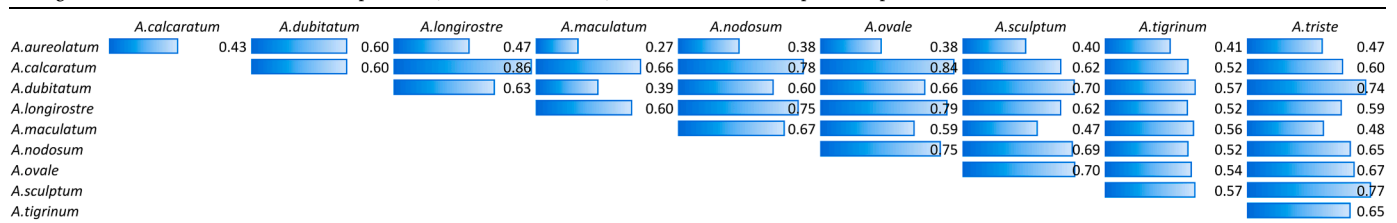
**Fig. 3.** The complete set of relationships between the ticks and rickettsiae in the Neotropics. Each plot and color mean for a species as explained in the legend that has been recorded in feeding ticks. At left, a phylogenetic tree of ticks, based on 16S rRNA gene, shows the relationships of the arthropods. *Haemaphysalis juxtakochi* and *Haemaphysalis leporispalustris*, and some *Ixodes* sp., which have been found infected in a few instances, have been omitted to improve clarity of the phylogenetic tree. *Ixodes minor* and *I. parvicinus* are included in the tree to show phylogenetic relationships with other ticks, but only *Rickettsia* sp. has been reported in these tick species. Only bootstrap values higher than 65 are shown.



**Fig. 4.** The sharing of environmental weather variables by the ticks in which *Rickettsia parkeri* s.l. has been recorded (A) and those in which 'Candidatus R. andeanae' has been recorded (B). The chart shows the sharing for maximum annual temperature that resulted to be the best discriminating variable delineating habitat sharing among these species.

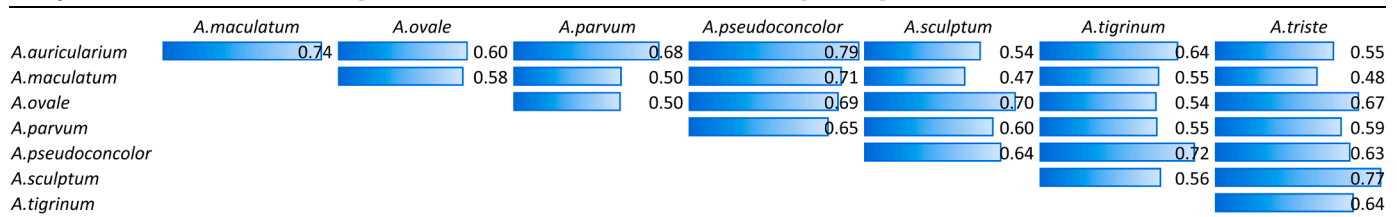
**Table 2**

The Schoener's D value of habitat overlapping of the species of ticks in which *R. parkeri* s.l. has been recorded. The D value is calculated for habitat sharing as the average for maximum and minimum temperatures, the annual rainfall, and the annual atmospheric vapor value.



**Table 3**

The Schoener's D value of habitat overlapping of the species of ticks in which '*Ca R. andeanae*' has been recorded. The D value is calculated for habitat sharing as the average for maximum and minimum temperatures, the annual rainfall, and the annual atmospheric vapor value.



(Luz et al., 2019). Therefore, some species may be easily detected (and over-reported) but the DNA of others may be arduous to detect, leading to an under-reporting. All these factors may affect a bibliographical meta-analysis of *Rickettsia* but, at the best of our knowledge, this is the best way to proceed and analyze the existing data.

We found 31 species of *Rickettsia* in the target region, excluding *R. africae*, which is associated only to *A. variegatum*, an imported tick, and that has been recorded only in sites of the Caribbean region where the tick spread in the past. *Rickettsia africae* did not evolve along the New World ticks and is therefore not further elaborated in our study. Many rickettsiae are endosymbionts and the level of their dependence in the tick is variable. An obligate symbiont should be present in most individuals of the tick, and thus co-cladogenesis between symbiont and ticks should be expected. However, many species of *Rickettsia* in the world have an impact on human health, and the Neotropical region is not an exception: both *R. rickettsii* and *R. parkeri* s.l. are considered serious concerns for human health. While reports of new species of *Rickettsia* accumulate around the world, complete genomic sequences and annotations are available for only a few species (Diop et al., 2019).

We first did check for independence of R+ or R- ticks regarding the host communities on which ticks were collected. This is an important point to demonstrate that the evolution of *Rickettsia* is not linked with the vertebrates but to the ticks (supported by further tests). The Rao's entropy test (Rao, 2010) allows to compare how different are both sets of ticks (R+ or R-) in terms of the phylogenetic information of shared vertebrates. A significant difference would preclude any further analysis since the rickettsial fauna found in ticks could be explained by the host phylogenetic composition. Results demonstrated that both R+ and R-ticks have been "extracted" from phylogenetically similar groups of hosts, and therefore, any differences in rickettsial composition of ticks do not depend only on the vertebrate's blood-source. This is the first time this result is demonstrated, and it confirms the dependence of the speciation of *Rickettsia* along two not mutually exclusive events: the strict association tick-*Rickettsia* or the circulation of *Rickettsia* in species of ticks that exploit a diversity of hosts and that could result infected in the period of rickettsemia.

We could not elaborate about the rickettsiae that have been recorded only in one tick species, but we can foresee this as a fertile field of research. Future surveys will demonstrate if these rickettsiae are actually linked to more tick species, but it seems, with the currently available data, that many species of *Rickettsia* are evolving linked to one species of

tick, supporting the hypothesis of a probable coevolution. Future research will expand our knowledge capturing the complete relationships of these (yet) unnamed species and the ticks involved, underlining the importance of specific tick endosymbionts in the studies of the tick's microbiomes (Binetruy et al., 2019).

Our study demonstrated that Neotropical rickettsial organisms may be tightly associated with phylogenetically close clusters of ticks and secondarily, and probably only in a few cases, along a gradient of environmental traits in which ticks overlap. A paradigm of the first could be the presumed association of *R. rickettsii* with the ticks of the *A. cajennense* complex. However, this result could be also biased, since ticks from the *A. cajennense* complex are usual parasites of humans, and thus over-reported as the principal vector of *R. rickettsii*, while other tick species associated with this bacterium could be neglected. The under or over-reporting of the disease in humans may lead researchers to sample more ticks in areas where human clinical cases are over-reported. This overlooks other associations where the disease in humans is under-reported (or does not exist at all, rickettsiae circulating in "silent" foci in which tick species scarcely biting humans are under-reported). The importance of environmental features driving the distribution of ticks circulating *Rickettsia* has never been addressed; we, however, think that it has deep importance in the segregation of these bacteria. This finding opens a potential research field regarding the joint distribution of these ticks and their hosts, and how changes of environmental traits could affect the currently known distribution of both ticks and vertebrates, therefore modifying the circulation of the bacteria in natural foci (see i. e., Fernández-Ruiz and Estrada-Peña, 2020). The concept of an environmental continuum in which several species of ticks may overlap and share hosts is directly related to the systemic transmission of rickettsiae. It is known that it occurs when uninfected and infected ticks feed simultaneously on susceptible animals, also called amplifying hosts, developing rickettsemia (Moraes-Filho et al., 2018). Susceptible vertebrates may feed phylogenetically unrelated ticks, because they overlap in portions of the environmental niche, "sharing" rickettsiae. Actually, the ticks in which *R. parkeri* s.l. has been recorded also share a large number of hosts (Estrada-Peña et al., 2020). The findings of this study show that the ticks in which *R. parkeri* s.l. has been detected, share about 70%–80% of the recorded temperature gradient in the region. This finding allows us to hypothesize that strains of *R. parkeri* s.l. may probably circulate among all these species, as they "specialize" to different tick species. In other words, the habitat sharing would



contribute to the circulation of ancestral forms, and then the tick would select for strains. The hypothesis of segregation along environmental gradients is supported by the finding of different “populations” of *Amblyomma ovale* associated with different biomes in Brazil (Biten-courth et al., 2019) as well as the phylogenetic divergence of strains of *R. amblyommatis* from Argentina, associated with different species of ticks that do not share habitat (Sebastian et al., 2020).

A different result has been observed regarding ‘*Ca. R. andeanae*’. This species is not recognized to segregate in strains, and the same species has been recorded in several clades of *Amblyomma* ticks that share environmental niche, spatial range, and hosts. The environmental overlap of the *Amblyomma* species sharing ‘*Ca. R. andeanae*’ is higher than for tick species circulating *R. parkeri* s.l. We hypothesize that this could be the reason why only one strain of ‘*Ca. R. andeanae*’ is currently recognized: it is evolving in 8 species of ticks that have phylogenetic relationships and share similar environmental niches. The ticks have similar physiological requirements modulated by the weather and hosts, and therefore the same rickettsial species can contribute to the metabolism of the ticks in the same way, without the need of segregation into strains.

An interesting finding of this study is that some species of *Rickettsia* are widespread in many species of ticks, namely *R. amblyommatis* and *R. bellii*. This is a scenario that deserves further exploration, since molecular differentiation of strains of *R. amblyommatis* has been reported (Sebastian et al., 2020). These authors elaborated about the detection of different haplotypes of *R. amblyommatis* in phylogenetically unrelated species of *Amblyomma*, but distributed along a continuum of ecological conditions, supporting a possible separation of the rickettsia according to tick species that colonize different environmental niches. Notably, *R. amblyommatis* is also widespread in parts of USA, mainly linked to *Amblyomma americanum* (Karpathy et al., 2016). The role of tick parasites of migratory birds, like *A. longirostre*, has been proposed as one of the main routes of dispersal of these bacteria, since immatures of *A. longirostre* are commonly transported from Neotropics to the Nearctic region (Mukherjee et al., 2014).

*Rickettsia bellii* is the most ancient known divergent known species of *Rickettsia* and has the widest tick species range in the Neotropics, being reported associated with 27 tick species, in a diversity of lineages (Krawczak et al., 2018). The findings reported by de Abreu et al. (2019) indicated that the distribution of *R. bellii* in Brazilian tick populations, particularly as a co-infecting species, is most likely underestimated. Data acquired from observation of naturally infected colonies of *A. dubitatum* (Sakai et al., 2014) and *Ixodes loricatus* (Horta et al., 2006), demonstrated highly efficient transstadial survival and transovarian transmission, suggesting that *R. bellii* is most likely maintained in nature via those mechanisms.

It is noteworthy that ‘*Ca. R. andeanae*’ is common in ticks belonging to the *Amblyomma maculatum* complex, and therefore has been also reported outside the target region. A high prevalence of ‘*Ca. R. andeanae*’ was reported in *A. maculatum* from USA (Kansas and Oklahoma), whereas *R. parkeri* s.s. was absent (Paddock et al., 2015). Coinfections of *A. maculatum* with *R. parkeri* and ‘*Ca. R. andeanae*’ are not common but have been reported (Varela-Stokes et al., 2011; Ferrari et al., 2012; Budachetri et al., 2014). Geographical differences in infection rates, particularly the absence of *R. parkeri* and the overwhelming presence of ‘*Ca. R. andeanae*’ in *A. maculatum* suggest that rickettsial exclusion by transovarial interference may be occurring on a broader population scale (Paddock et al., 2015). Infrequent evidence of ‘*Ca. R. andeanae*’ in *A. maculatum* has been reported where *R. parkeri* is frequently found in this tick vector (Nadolny et al., 2014; Pagac et al., 2014). The interaction among different species of *Rickettsia* was proposed by Burgdorfer et al. (1981) with the term “rickettsial exclusion”. The concept is based on epidemiological observations and experimental evaluation of transovarian transmission, describing a phenomenon whereby primary infection by one rickettsial agent impeded ovarian infection by a second species. This phenomenon has also been suggested as the most plausible explanation for the apparent exclusion of *R. parkeri* s.s. in populations of

adult *A. maculatum* ticks in some states of the USA with a high prevalence of ‘*Ca. R. andeanae*’ (Lee et al., 2017; Paddock et al., 2015). In contrast, the absence of such exclusion has been demonstrated in a study by Levin et al. (2018). Field results also failed to demonstrate this exclusion reporting relatively equal ratios of both *R. parkeri* and ‘*Ca. R. andeanae*’ in the same populations of ticks. Other results from the Neotropics (de Abreu et al., 2019) demonstrated that both *A. longirostre* and *Amblyomma calcaratum* ticks can be naturally co-infected with *R. amblyommatis* and *R. bellii* and that *A. romarioi* can simultaneously harbour both ‘*Ca. R. paranaensis*’ and *R. bellii*.

It has been demonstrated that the four strains of *R. parkeri* have a common ancestor (Nieri-Bastos et al., 2018). However, the link between strains of *R. parkeri* and phylogenetically close groups of ticks is not strict, therefore supporting the environmental explanation. Interestingly, different ticks like *Amblyomma tigrinum*, *Amblyomma triste* or *A. dubitatum* could act as “bridges” between the extremes of the temperature range, linking parts of the distribution of the most cold-tolerating species and those that prefer the warmest extreme of the range. This hypothesis explicitly states that the *R. parkeri* s.l. strains are evolving separately in different clusters of ticks, but its circulation among them is supported by the environmental overlap of ticks. The record of *R. parkeri* strain Parvitarsum in *A. parvitarsum*, a tick that does not overlap its distribution range with the other 10 tick species is not easy to fit with this hypothesis. Most likely, this is a case of segregation of the tick after the *Rickettsia* species was adapted to the tick.

We propose several ways for the evolution and circulation of *Rickettsia* in the Neotropics. The first is the strict association of a *Rickettsia* to single species of ticks; the second is the association between phylogenetically close clusters of ticks and species of *Rickettsia*; the third considers a scenario of habitat (and host) sharing that would allow the circulation of these bacteria through unrelated clades of ticks. The finding of *Rickettsia* in ticks of the genus *Ixodes* introduces an interesting question. It is not yet possible to know if these bacteria are common in *Ixodes* ticks, but *Rickettsia* sp. Aragoi has been recorded exclusively in *I. fuscipes* suggesting that speciation processes are under way also in the genus *Ixodes*. Other species, like *I. loricatus* and *Ixodes luciae* have been also found infected with the widespread *R. amblyommatis* and *R. bellii*. These, however, could be cases of “spillover” from the infected vertebrates. Immatures of *I. luciae* and *I. loricatus* have been found on Didelphidae and Cricetidae, which are also hosts for some species of *Amblyomma*. However, the adult stage of both *I. luciae* and *I. loricatus* are considered to be highly specific to Didelphidae (Guglielmo et al., 2011; Saraiva et al., 2012).

The first meta-analysis of the phylogenetic and environmental relationships of *Rickettsia* in the Neotropics opens several interesting questions. The network constructs revealed important associations among the bacteria and the tick hosts, and we encourage their use in future prospects on the topic. We advocate a major effort for completing the overview of relationships among ticks and *Rickettsia*, either form feeding or questing ticks. Also, prevalence of *Rickettsia* species in questing ticks should be analysed to obtain meaningful details of the complete range of relationships (i.e., quantitative versus qualitative) that could explain better these complex relationships. Maps of the predicted distribution of ticks, with special emphasis on environmental sharing should be a priority to better capture the impacts of the environment on the distribution of the major vectors of pathogenic *Rickettsia*.

To conclude, we propose that Neotropical *Rickettsia* are mainly associated with well delineated phylogenetic clades of ticks, but this is not the only pressure driving its circulation in permanent foci. Some ticks share portions of the environmental niche, and in some cases share species of *Rickettsia*, most probably because they share hosts that occupy portions of the niche gradient. Other *Rickettsia* species are associated with almost every surveyed species of ticks in the Neotropics, a fact in which we could foresee an obligate symbiotic association. Last, but not least, many species of *Rickettsia* have been recorded only in one single

tick species, suggesting either a true adaptation to the tick or a lack of adequate surveys.

## Authors statement

All the authors read and approved this version of the manuscript, the cover letter and the response to the Reviewers. All funding sources have been acknowledged in the manuscript. No previous versions of the manuscript have been published, and the Supplementary Materials have been purposely prepared for this manuscript.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tbd.2021.101754.

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