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Phylogenetic Evidence for the Existence of Multiple Strains of *Rickettsia parkeri* in the New World

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ABSTRACT The bacterium *Rickettsia parkeri* has been reported to infect ticks of the "*Amblyomma maculatum* species complex" in the New World, where it causes spotted fever illness in humans. In South America, three additional rickettsial strains, namely, Atlantic rainforest, NOD, and Parvitarsum, have been isolated from the ticks *Amblyomma ovale*, *Amblyomma nodosum*, and *Amblyomma parvitarsum*, respectively. These three strains are phylogenetically closely related to *R. parkeri*, *Rickettsia africae*, and *Rickettsia sibirica*. Herein, we performed a robust phylogenetic analysis encompassing 5 genes (*gltA*, *ompA*, *virB4*, *dnaA*, and *dnaK*) and 3 intergenic spacers (*mppE-pur*, *rrl-rrf*-ITS, and *rpmE*-tRNA^{fMet}) from 41 rickettsial isolates, including different isolates of *R. parkeri*, *R. africae*, *R. sibirica*, *Rickettsia conorii*, and strains Atlantic rainforest, NOD, and Parvitarsum. In our phylogenetic analyses, all New World isolates grouped in a major clade distinct from the Old World *Rickettsia* species (*R. conorii*, *R. sibirica*, and *R. africae*). This New World clade was subdivided into the following 4 clades: the *R. parkeri sensu stricto* clade, comprising the type strain Maculatum 20 and all other isolates of *R. parkeri* from North and South America, associated with ticks of the *A. maculatum* species complex; the strain NOD clade, comprising two South American isolates from *A. nodosum* ticks; the Parvitarsum clade, comprising two South American isolates from *A. parvitarsum* ticks; and the strain Atlantic rainforest clade, comprising six South American isolates from the *A. ovale* species complex (*A. ovale* or *Amblyomma aureolatum*). Under such evidences, we propose that strains Atlantic rainforest, NOD, and Parvitarsum are South American strains of *R. parkeri*.

IMPORTANCE Since the description of *Rickettsia parkeri* infecting ticks of the "*Amblyomma maculatum* species complex" and humans in the New World, three novel phylogenetic close-related rickettsial isolates were reported in South America. Herein, we provide genetic evidence that these novel isolates, namely, strains Atlantic rainforest, NOD, and Parvitarsum, are South American strains of *R. parkeri*. Interestingly, each of these *R. parkeri* strains seems to be primarily associated with a tick species group, namely, *R. parkeri sensu stricto* with the "*Amblyomma maculatum* species group," *R. parkeri* strain NOD with *Amblyomma nodosum*, *R. parkeri* strain Parvitarsum with *Amblyomma parvitarsum*, and *R. parkeri* strain Atlantic rainforest with the "*Amblyomma ovale* species group." Such rickettsial strain-tick species specificity suggests a coevolution of each tick-strain association. Finally, because *R. parkeri sensu stricto* and *R. parkeri* strain Atlantic rainforest are human pathogens, the potential of *R. parkeri* strains NOD and Parvitarsum to be human pathogens cannot be discarded.

KEYWORDS *Amblyomma*, New World, *Rickettsia parkeri*, spotted fever group, tick

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During the first half of the 20th century, a novel bacterial agent of the spotted fever group was isolated from *Amblyomma maculatum* ticks in the southern United States (1). The agent, shown to be mildly pathogenic for guinea pigs (2), was later described as *Rickettsia parkeri* (3). After almost 6 decades in which *R. parkeri* was known only from ticks, in 2004, there was the first description of a spotted fever clinical case in a human in the United States (4). This first case has been followed by a growing number of *R. parkeri* rickettsiosis cases in the United States, all linked to the transmission by *Amblyomma maculatum* (5, 6). More recently in Arizona, *R. parkeri* was reported to have infected *Amblyomma triste* ticks, which were the likely vector of the infection for two human clinical cases (7).

In South America, the first report of *R. parkeri* dates from 2004, when the agent was found to have infected *A. triste* ticks in southern Uruguay (8), an area where clinical cases of a tick-borne spotted fever clinically similar to Mediterranean spotted fever had been reported (9, 10). A subsequent study provided serological evidence for *R. parkeri* as the etiological agent of the Uruguayan spotted fever (11). In Argentina, *R. parkeri* was reported to have infected *A. triste* ticks in 2008 (12) and later shown to be the etiological agent of clinical cases of spotted fever (13). Yet, during the 21st century, *R. parkeri* was reported to have infected *A. triste* ticks in Brazil (14) and *A. maculatum* ticks in Peru (15), although the diseases caused by *R. parkeri* have never been confirmed in these two countries. Additional records of *R. parkeri* include the infection of *Amblyomma tigrinum* ticks in Uruguay (16), Bolivia (17), Argentina (18), and Brazil (19). In the latter two countries, *A. tigrinum* was epidemiologically associated with human clinical cases of spotted fever rickettsiosis, confirmed to be caused by *R. parkeri* at least in Argentina (18). *Amblyomma maculatum*, *A. triste*, and *A. tigrinum* are morphologically and genetically closely related tick species, forming the "*A. maculatum* species complex" (20). The above reports of *R. parkeri* indicate that *R. parkeri sensu stricto* is primarily associated with ticks of the *A. maculatum* species complex in the New World.

From 2010 to 2016, three clinical cases of spotted fever rickettsiosis were reported in Brazil (21–23). The cases were shown to be caused by a novel agent, named strain Atlantic rainforest, phylogenetically related to *R. parkeri*, *Rickettsia africae*, and *Rickettsia sibirica* (21). Subsequent studies showed that these clinical cases were epidemiologically associated with the tick *Amblyomma ovale* (24, 25), and also with *Amblyomma aureolatum* (26). These two tick species form the "*A. ovale* species complex" (27). A laboratory study showed that *A. ovale* is a competent vector of strain Atlantic rainforest (28). Additional studies reported strain Atlantic rainforest-infected *A. ovale* ticks in Colombia (29) and Belize (30). Recently, a unique North American strain of *R. parkeri* isolated from *Dermacentor parumapertus* ticks collected in Texas was determined genetically as nearly identical to *Rickettsia* sp. strain Atlantic rainforest, further supporting the relatedness of these taxa (31).

In 2009, a novel spotted fever group agent (strain NOD) was isolated from *Amblyomma nodosum* ticks in Brazil (32). More recently, another spotted fever group agent, named strain Parvitarsum, was isolated from *Amblyomma parvitarsum* ticks in Argentina and Chile (33). These two novel agents, known only from ticks, were shown to be phylogenetically related to *R. parkeri*, *R. africae*, and *R. sibirica*. While the distribution of *R. parkeri* in association with the *A. maculatum* species complex was shown to encompass North and South America, the taxonomic status of strains Atlantic rainforest, NOD, and Parvitarsum remains unresolved. Herein, we provide phylogenetic evidence for the classification of these strains as belonging to the species *R. parkeri*.

RESULTS

Partial sequences of 5 genes (*gltA*, *ompA*, *virB4*, *dnaA*, and *dnaK*) and 3 intergenic spacers (*mppE-pur*, *rrl-rrf*-ITS, and *rpmE*-tRNA^{fMet}) were obtained for the 39 rickettsial isolates listed in Table 1 and used for alignment with corresponding sequences of *R. africae* strain ESF and *R. sibirica sibirica* strain 246 from GenBank. The maximum parsimony (MP) analyses revealed the segregation of *Rickettsia* species into three groups for the *gltA* gene, seven groups for the *ompA* gene, seven groups for the *dnaA*

TABLE 1 Rickettsial isolates used for DNA amplification in the present study

No.	*Rickettsia* species or strain	Code	Source	Geographical locality	Country	Rickettsial collection[a]	Reference
1	*Rickettsia parkeri*	Maculatum 20^T	*Amblyomma maculatum*	Liberty County, Texas	United States	CDC	2
2	*R. parkeri*	Tate's Hell	*A. maculatum*	Franklin County, Florida	United States	CDC	51
3	*R. parkeri*	Cash Bayou	*A. maculatum*	Franklin County, Florida	United States	CDC	51
4	*R. parkeri*	Oktibbeha	*A. maculatum*	Oktibbeha County, Mississippi	United States	CDC	51
5	*R. parkeri*	Moss Point	*A. maculatum*	Jackson County, Mississippi	United States	CDC	51
6	*R. parkeri*	Escatawpa	*A. maculatum*	Jackson County, Mississippi	United States	CDC	51
7	*R. parkeri*	NC-3	*A. maculatum*	Mecklenburg County, North Carolina	United States	CDC	52
8	*R. parkeri*	NC-8	*A. maculatum*	Mecklenburg County, North Carolina	United States	CDC	52
9	*R. parkeri*	NC-15	*A. maculatum*	Mecklenburg County, North Carolina	United States	CDC	52
10	*R. parkeri*	Portsmouth	Human	Norfolk County, Virginia	United States	CDC	4
11	*R. parkeri*	Ft. Story	Human	Virginia Beach County, Virginia	United States	CDC	53
12	*R. parkeri*	Fairfax	*A. maculatum*	Fairfax County, Virginia	United States	CDC	54
13	*R. parkeri*	I-66	*A. maculatum*	Fairfax County, Virginia	United States	CDC	54
14	*R. parkeri*	45	*Amblyomma triste*	Delta do Paraná, Buenos Aires Province	Argentina	From tick DNA	12
15	*R. parkeri*	132	*A. triste*	Delta do Paraná, Buenos Aires Province	Argentina	From tick DNA	12
16	*R. parkeri*	136	*A. triste*	Delta do Paraná, Buenos Aires Province	Argentina	From tick DNA	12
17	*R. parkeri*	34	*A. triste*	Delta do Paraná, Buenos Aires Province	Argentina	From tick DNA	12
18	*R. parkeri*	218	*A. triste*	Delta do Paraná, Buenos Aires Province	Argentina	From tick DNA	12
19	*R. parkeri*	At24	*A. triste*	Paulicéia, São Paulo	Brazil	FMVZ/USP	14
20	*R. parkeri*	Corumbá	*A. triste*	Corumbá, Mato Grosso do Sul	Brazil	FMVZ/USP	Unpublished
21	*R. parkeri*	Água Clara	*A. triste*	Água Clara, Mato Grosso do Sul	Brazil	FMVZ/USP	55
22	*R. parkeri*	Pantanal At46	*A. triste*	Poconé, Mato Grosso	Brazil	FMVZ/USP	56
23	*R. parkeri*	At5URG	*A. triste*	Toledo Chico, Canelones	Uruguay	FMVZ/USP	57
24	Strain Atlantic rainforest	P-240	*Amblyomma ovale*	Peruíbe, São Paulo	Brazil	FMVZ/USP	24
25	Strain Atlantic rainforest	P-51	*A. ovale*	Peruíbe, São Paulo	Brazil	FMVZ/USP	24
26	Strain Atlantic rainforest	Adrianópolis	*A. ovale*	Adrianópolis, Paraná	Brazil	FMVZ/USP	25
27	Strain Atlantic rainforest	Paty	*A. ovale*	Chapada Diamantina, Bahia	Brazil	FMVZ/USP	25
28	Strain Atlantic rainforest	Aa47	*Amblyomma aureolatum*	Blumenau, Santa Catarina	Brazil	FMVZ/USP	26
29	Strain Atlantic rainforest	Aa46	*A. aureolatum*	Blumenau, Santa Catarina	Brazil	FMVZ/USP	26
30	Strain NOD	NOD	*Amblyomma nodosum*	Pontal do Paranapanema	Brazil	FMVZ/USP	32
31	Strain NOD	Pantanal	*A. nodosum*	Nhecolândia, Mato Grosso do Sul	Brazil	FMVZ/USP	Unpublished
32	Strain Parvitarsum	Argentina	*Amblyomma parvitarsum*	Salta	Argentina	FMVZ/USP	33
33	Strain Parvitarsum	Chile	*A. parvitarsum*	Arica and Parinacota	Chile	FMVZ/USP	33
34	*Rickettsia africae*	Z8-Ah	*Amblyomma hebraeum*	South of the country	Zimbabwe	UTMB	58
35	*R. africae*	RaPele	Human	Hluhluwe-Imfolozi Park	South Africa	FMVZ/USP	Unpublished
36	*Rickettsia conorii* Israeli	PoHu16026	Human	Beja, Alentejo region	Portugal	INSA	59
37	*R. conorii* Malish	PoHu10908	Human	Faro, Algarve region	Portugal	INSA	59
38	*R. conorii* Malish	PoHu17458	Human	Faro, Algarve region	Portugal	INSA	59
39	*R. sibirica mongolitimonae*	PoHu10991	Human	Évora, Alentejo region	Portugal	INSA	60

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gene, four groups for the *dnaK* gene, nine groups for the *virB4* gene, three groups for the *mppA-purC* intergenic spacer, five groups for the *rrl-rrf*-ITS intergenic spacer, and eight groups for the *rpmE*-tRNA^{fMet} intergenic spacer (see Fig. S1 to S8 in the supplemental material). The divergence values were calculated for each of the eight molecular markers. The highest divergence was found for the *ompA* gene (1.61%) followed by the intergenic spacer *rpmE*-tRNA^{fMet} (1.21%). The lowest values were for *gltA* (0.14%) and *dnaA* (0.31%) genes.

In both the *gltA* and the *mppA-purC* trees, all New World isolates formed a single group with *R. sibirica*, which was separated from *R. africae* and *R. conorii* isolates (Fig. S1 and S6). In the *dnaA* tree, the *A. maculatum-R. parkeri* isolates (North America) formed a group separated from the *A. triste-R. parkeri* isolates (South America), which were separated from the remaining South American and Old World isolates (Fig. S4). In the *ompA*, *virB4*, *dnaK*, *rrl-rrf*-ITS, and *rpmE*-tRNA^{fMet} trees, the 23 *R. parkeri* isolates from *A. maculatum* (North America) or *A. triste* (South America) formed a group separate from the remaining groups (Fig. S2, S3, S5, S7, and S8); in the case of the *dnaK* tree, this single group also included the *R. sibirica* isolates and the two isolates of strain NOD (NOD and Pantanal) (Fig. S5). In the *ompA*, *virB4*, *dnaA*, and *rpmE*-tRNA^{fMet} trees, the six isolates of strain Atlantic rainforest formed a group with isolates of strain Parvitarsum (Fig. S2 to S4 and S8); in the *dnaA* tree, this group also included *R. sibirica sibirica* (Fig. S4). In the *rrl-rrf*-ITS tree, the six isolates of strain Atlantic rainforest formed a separate group (Fig. S7). The two isolates of strain NOD formed a single group in the *ompA*, *virB4*,

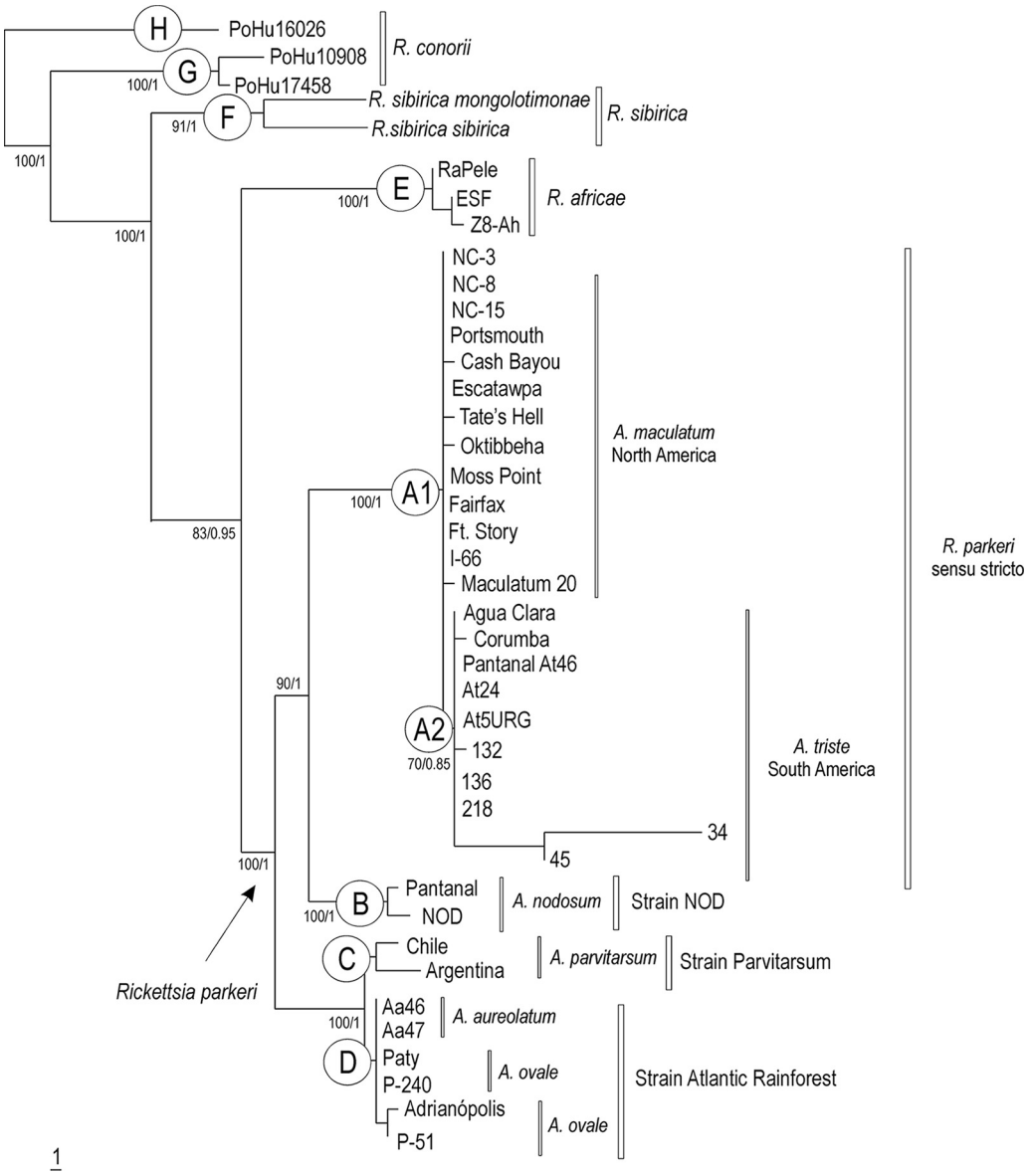

FIG 1 Molecular phylogenetic analysis of New World isolates of *Rickettsia parkeri sensu stricto*, strains Atlantic rainforest, NOD, and Parvitarsum, in relation to Old World isolates of *Rickettsia africae*, *Rickettsia sibirica*, and *Rickettsia conorii*. A total of 3,603 aligned nucleotide sites of 5 protein-coding genes (*gltA*, *ompA*, *virB4*, *dnaA*, and *dnaK*) and 3 intergenic spacers (*mppE-pur*, *rrl-rrf*-ITS, *rpmE*-tRNA^{fMet}) were concatenated and subjected to Bayesian analysis. Numbers at nodes are support values derived from posterior probability. Scale bar, units of expected substitutions per site. Each main clade is indicated by a capital letter (A to H) shown inside a circle.

dnaA, and *rpmE*-tRNA^{fMet} trees (Fig. S2 to S4 and S8); in the *rrl-rrf*-ITS tree, these isolates grouped with isolates of strain Parvitarsum and *R. sibirica* (Fig. S7). All New World isolates (*R. parkeri*, strain Atlantic rainforest, strain NOD, and strain Parvitarsum) regardless of separation, remained sisters to each other, well separated from the clade containing strains of *R. conorii* and, most of the time, from the different strains of *R. africae* and *R. sibirica*.

DNA sequences of each of the eight molecular markers were concatenated for each isolate and aligned to be used in the phylogenetic analysis. The final alignment with the 41 rickettsial isolates included 3,603 nucleotides, with 57 informative sites. Under high bootstrap support (MP analysis) or high posterior probabilities (Bayesian [B] analysis), all New World isolates were well separated from the Old World *Rickettsia* species (*R. conorii*, *R. sibirica*, and *R. africae*) (Fig. 1). The large New World clade was subdivided into

TABLE 2 Matrix of the divergence of the *Rickettsia* isolates used in the present study[a]

	Clade								
Clade[b]	A_1	A_2	B	C	D	E	F	G	H
A_1	0.12								
A_2	0.19	0.21							
B	0.74	0.87	0.27						
C	0.93	0.80	0.77	0.24					
D	0.85	0.93	0.85	0.23	0.08				
E	1.02	1.15	0.95	0.83	0.85	0,03			
F	0.83	0.97	0.80	0.64	0.66	0.86	0,13		
G	1.62	1.74	1.55	1.33	1.35	1.61	1.37	0.10	
H	1.61	1.75	1.57	1.45	1.47	1.68	1.42	1.23	0.00

[a]Based on an alignment of a concatenated sequence of 3,579 nucleotides (nt), composed of the genes *gltA* (257 nt), *ompA* (490 nt), *virB4* (684 nt), *dnaA* (663 nt), and *dnaK* (615 nt) and the intergenic spacers *mppE-pur* (197 nt), *rrl-rrf*-ITS (330 nt), and *rpmE*-$tRNA^{fMet}$ (343 nt).

[b]Each letter represents a clade in Fig. 1 as follows: A_1, *Rickettsia parkeri* isolates from North America; A_2, *R. parkeri* isolates from South America; B, strain NOD isolates; C, strain Parvitarsum isolates; D, strain Atlantic rainforest isolates; E, *R. africae* isolates; F, *R. sibirica sibirica*; G, *R. conorii* Malish isolates; H, *R. conorii* Israeli.

the following 4 major clades, all under high bootstrap support or posterior probabilities: the *R. parkeri sensu stricto* clade (clade A), comprising the type strain Maculatum 20 and all other isolates of *R. parkeri* from North and South America, associated with ticks of the *A. maculatum* species complex; the strain NOD clade (clade B), comprising two South American isolates from *A. nodosum* ticks; the Parvitarsum clade (clade C), comprising two South American isolates from *A. parvitarsum* ticks; and the strain Atlantic rainforest clade (clade D), comprising six South American isolates from the *A. ovale* species complex (*A. ovale* or *A. aureolatum*). The *R. parkeri sensu stricto* clade was subdivided clearly into two large clades, one containing all *R. parkeri sensu stricto* isolates from North America, associated with *A. maculatum* ticks (clade A_1), and one containing all *R. parkeri sensu stricto* isolates from South America, associated with *A. triste* (A_2). The tree topology shown in Fig. 1 was generally the same for MP and B analyses; the only difference was that the B analysis did not separate North American isolates from the South American isolates of *R. parkeri sensu stricto*.

The overall divergence values of the concatenated sequences were 0.64 to 1.75% between American (clades A to D) and European/Asian (clades F to H) isolates and 0.83 to 1.15% between American and African (clade E) isolates (Table 2). The divergence between European/Asian and African isolates was 0.86 to 1.68%. The divergence values among the American clades (A to D) were generally lower, between 0.19 and 0.93%. Within-clade divergence values were even lower, ranging from 0.0 to 0.27%.

DISCUSSION

Since the initial molecular characterization of *R. parkeri sensu stricto* from *A. maculatum* ticks and human patients in the United States (4), this *Rickettsia* species has also been reported in South America, where it infected *A. triste* (8, 12, 14) and, subsequently, human patients (13, 18). These molecular characterizations were based on the most commonly used molecular markers (portions of the *gltA*, *ompA*, and *ompB* genes), which showed no polymorphism among North and South American isolates. Interestingly, until some decades ago, the taxa *A. maculatum* and *A. triste* represented the same tick species (*A. maculatum*). In a morphological study, Kohls (34) proposed *A. triste* as a valid species, which had been accepted until the present (35). On the other hand, because of the high morphological similarities between *A. maculatum* and *A. triste*, associated with low genetic polymorphism between North American populations of *A. maculatum* and South American populations of *A. triste* (20), the possibility of conspecificity of these ticks has not been discarded, and further studies are needed to evaluate this hypothesis (36). Presently, *A. maculatum* and *A. triste*, together with *A. tigrinum*, form the *A. maculatum* species complex (20), with which *R. parkeri sensu stricto* has been associated. Our phylogenetic analysis corroborates such an assumption by showing all *R. parkeri sensu stricto* isolates from *A. maculatum* and *A. triste* in a single

large clade (clade A). On the other hand, the separation of this clade into two subgroups, clade A_1 containing North American isolates and clade A_2 with South American isolates, could be a result of the geographical distance of the isolates, the host tick species, or a combination of both. Further studies employing South American isolates of *R. parkeri sensu stricto* from *A. maculatum*, as well as from *A. tigrinum*, would help to elucidate these subgroups.

In the original reports of the strains Atlantic rainforest, NOD, and Parvitarsum in South America, the limited phylogenetic analysis provided enough data to only demonstrate a close relatedness to *R. parkeri*, *R. africae*, and *R. sibirica* (21, 32, 33). Herein, we present a robust phylogenetic analysis with strong statistical support to demonstrate a monophyletic group formed by strains Atlantic rainforest, NOD, and Parvitarsum and isolates of *R. parkeri sensu stricto* from North and South America. In addition, the genetic divergence values between New World isolates were generally $\leq$1.00, whereas values between New World isolates and Old World isolates (*R. africae*, *R. sibirica*, and *R. conorii*) were generally $\geq$1.00 (Table 2). On the basis of this evidence, we propose that Atlantic rainforest, NOD, and Parvitarsum are South American strains of *R. parkeri*. In fact, Paddock et al. (31) recently provided molecular evidence to classify *Rickettsia* sp. strain Atlantic rainforest as a distinct strain of *R. parkeri*. Interestingly, each of these *R. parkeri* strains seems to be primarily associated with a tick species or a tick species group, namely, *R. parkeri sensu stricto* with the *A. maculatum* species group (includes *A. triste*), *R. parkeri* strain NOD with *A. nodosum*, *R. parkeri* strain Parvitarsum with *A. parvitarsum*, and *R. parkeri* strain Atlantic rainforest with the *A. ovale* species group (includes *A. aureolatum*). Such rickettsial strain-tick species specificity suggests a coevolution of each tick-strain association.

Our study evaluated multiple isolates of a strain of *R. parkeri* from North America (*R. parkeri sensu stricto*) and four distinct strains from South America (*R. parkeri sensu stricto*, *R. parkeri* strain Atlantic rainforest, *R. parkeri* strain NOD, and *R. parkeri* strain Parvitarsum). During the course of the present study, another strain of *R. parkeri* was reported from the United States, namely, *R. parkeri* strain Black Gap, isolated recently from *D. parumapertus* in the United States, and showed to be nearly identical to *R. parkeri* strain Atlantic rainforest (31). In addition to these established strains, other unique *R. parkeri*-like genotypes have been characterized genetically from South American ticks. These include *Rickettsia* sp. strain Cooperi in *Amblyomma dubitatum* (37), *Rickettsia* sp. strain ApPR in *Amblyomma parkeri* (38), *Rickettsia* sp. strain PA in *Amblyomma naponense* (39), all from Brazil, and *Rickettsia* sp. strain tuberculatum in *Amblyomma tuberculatum* from the United States (40). Collectively, these data reveal that North American strains of *R. parkeri* are thus far associated predominantly with 3 species of ticks (*A. maculatum*, *D. parumapertus*, and *A. tuberculatum*), and the South American strains of *R. parkeri* are associated predominantly with at least 7 species of South American ticks (*A. triste*, *A. ovale*, *A. nodosum*, *A. parvitarsum*, *A. dubitatum*, *A. parkeri*, and *A. naponense*). It also seems likely that additional strains of *R. parkeri* will be discovered in the Americas in the years to come.

Nonetheless, the greater diversity of *R. parkeri* in South America, associated with the genus *Amblyomma*, suggests that this species radiated first in this continent and thereafter entered into North America. This could have occurred during the great American biotic interchange ca. 3 million years ago, when the formation of the Isthmus of Panama was completed (41). This period coincides with the most likely introduction of the genus *Amblyomma* into North America (42, 43). Therefore, it is possible that *R. parkeri* radiated with the genus *Amblyomma* within South America and thereafter entered with this tick genus into North America, where the bacterium subsequently adapted to other tick genera, such as *Dermacentor*.

Rickettsia parkeri sensu stricto and *R. parkeri* strain Atlantic rainforest are emerging agents of tick-borne spotted fever rickettsiosis in the New World, where they cause acute febrile illness characterized by fever, rash, inoculation eschar, and lymphadenopathy, but no deaths so far (6, 13, 18, 44). In the New World, Rocky Mountain spotted fever (also known as Brazilian spotted fever), caused by *Rickettsia rickettsii*, is the most

TABLE 3 Primer pairs used for amplification of rickettsial genes or intergenic regions in the present study

Primer pair	Target	Primer sequence (5′ to 3′)		Amplicon size (nt)[a]	Reference
		Forward	Reverse		
1	*gltA*	GCAAGTATCGGTGAGGATGTAAT	GCTTCCTTAAAATTCAATAAATCAGGAT	401	37
2	*ompA*	ATGGCGAATATTTCTCCAAAA	GTTCCGTTAATGGCAGCATCT	632	61
3	*virB4*	TCTATAGTACATGATTCTGCT	TGATTACCGAGTGTAGTATTATG	840	62
4	*dnaA*	CTTTACAATCATTACGGTG	GCAACTAAGCCCCATCC	788	62
5	*dnaK*	GCATTCTAGTCATACCGCC	CAAAAAATGAAAGAAACTGCTGA	650	62
6	*mppA-purC*	GCAATTATCGGTCCGAATG	TTTCATTTATTTGTCTCAAAATTCA	160	63
7	*rpmE*-tRNA^{fMet}	TTCCGGAAATGTAGTAAATCAATC	TCAGGTTATGAGCCTGACGA	144	63
8	*rrl-rrf*-ITS	GCAACTAAGCCCCATCC	GATAGGTCGGGTGTGGAAG	350	62

[a]nt, nucleotides.

commonly reported tick-borne spotted fever, which is characterized by more severe symptoms, including high fatality rates in some areas (44). Because the usual serological tests for the diagnosis of spotted fever are not able to distinguish between the infections caused by spotted fever group agents (45), it is possible that many spotted fever cases in the New World could be caused by *R. parkeri sensu lato* agents. Such an assumption was recently corroborated in the United States, where human cases previously assigned as Rocky Mountain spotted fever were in fact shown to be caused by *R. parkeri* (46). This scenario becomes even more unresolved if we consider that spotted fever is considered to be highly unreported in Latin America.

MATERIALS AND METHODS

A total of 34 rickettsial isolates, mostly from ticks and a few from humans, were used in this study. The origin of each isolate, as well as the rickettsial collection that provided it for the present study, is described in Table 1. All isolates were grown in Vero cells by using the standard techniques of each laboratory (described in the references cited in Table 1). When >90% of the cells were infected, the monolayer was harvested and subjected to DNA extraction using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. In addition, we also processed DNA samples from 5 *A. triste* ticks from the study by Nava et al. (12), who showed that these 5 tick samples were infected by *R. parkeri*. For the 39 rickettsia samples (34 isolates and 5 tick samples), the amplification of fragments of five rickettsial genes and three intergenic spacers was attempted with the primer pairs listed in Table 3. DNA fragments amplified by PCR were separated by 1.5% agarose gel electrophoresis, stained with Sybr Safe (Thermo Fisher Scientific, Waltham, MA), and visualized in a photo gel documentation system (AlphaImager HP system, San Jose, CA). Amplicons were purified with ExoSap (USB Corporation, Cleveland, OH) and DNA sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), in an ABI automated sequencer (model ABI 3500 genetic analyzer; Applied Biosystems/Thermo Fisher Scientific, Foster City, CA) according to the manufacturer's specifications.

DNA sequences of the different target genes or intergenic spacers were edited for the removal of primer sequences by using the SeqMan software (DNAStar, Inc., Madison, WI). The sequences were subjected to multiple alignments by using the program Clustal X (47) by changing the parameters related to the insertion of indels (insertion weight, 1; extension, 1) and then manually adjusted by using GeneDoc v. 2.6.01 (48). The genome sequences of *R. africae* strain ESF (accession number NC_012633.1) and *R. sibirica sibirica* strain 246 (accession number AABW01000001.1) were downloaded from the GenBank database. The fragments of the five rickettsial genes and three intergenic spacers listed in Table 3 were saved and included in our alignments, which included a total of 41 rickettsial isolates. Phylogenetic trees were inferred by Bayesian (B) and maximum parsimony (MP) methods. The concatenated alignment of all markers (*gltA*, *ompA*, *virB4*, *dnaA*, *dnaK*, *mppE-pur*, *rrl-rrf*-ITS, and *rpmE*-tRNA^{fMet}) was analyzed by B and MP methods. The markers were analyzed individually only by the MP method. MP trees were constructed using the PAUP* v4.0b10 program (49) via a heuristic search with 100 replicates of random additions of the terminals followed by branching (RAS-TBR branch-breaking). Bootstrap support analyses were performed on 100 replicates with the same parameters used in the search. Bayesian analyses were performed in the MrBayes v.3.1.2 program (50); 1,000,000 generations were employed using GTR as a substitution model and four range categories plus an invariant proportion of sites. For the verification of support of branches in the Bayesian analyses, the "posteriori" probability values obtained using the MrBayes program were used. Similarity matrices (based on uncorrected *p*-distance) were constructed using the Pontos program provided by J. M. Alves at http://sourceforge.net/projects/pontos/.

Accession number(s). The GenBank accession numbers for the DNA sequences generated in this study for the 39 rickettsial isolates shown in Table 1 are the following: *gltA* gene, MF737524 to MF737556, MF737558 to MF737562, and MF737564; *ompA* gene, MF737606 to MF737643; *virB4* gene, MF925495 to MF925531, MF925534, and MF925699; *dnaA* gene, MF737565 to MF737578, MF737580 to MF737602, MF737604, and MF737605; *dnaK* gene, MF925658 to MF925689, MF925691 to MF925696, and MF925698; *mppA-purC* intergenic spacer, MF925535 to MF925568, MF925570 to MF925573, and MF925575;

rpmE-tRNA^{fMet} intergenic spacer, MF925576 to MF925608, MF925610 to MF925614, and MF925616; and *rrl-rrf*-ITS intergenic spacer, MF925617 to MF925649, MF925651 to MF925655, and MF925657.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02872-17.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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