Phylogenetic Differentiation of *Rickettsia parkeri* Reveals Broad Dispersal and Distinct Clustering within North American Strains

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ABSTRACT The tick-borne pathogen *Rickettsia parkeri* causes a mild rickettsiosis, with cases reported from several countries to its known distribution in the Americas. Molecular analyses have identified a clear distinction between strains of *R. parkeri sensu stricto* (s. s.) and *R. parkeri sensu lato* (s. l.) as well as separation between North American and South American *R. parkeri* s. s. strains. To expand on this previous work, we developed a multilocus sequence typing analysis with two aims: first, to investigate the genetic diversity within strains of North American *R. parkeri* s. s., and second, to further the understanding of the genetic relationships between *R. parkeri* s. s. and *R. parkeri* s. l. Sixty-four *R. parkeri* isolates and 12 *R. parkeri*-positive tick lysates were analyzed using a novel typing scheme consisting of four coding regions and two intergenic regions. A concatenated Bayesian phylogeny that identified eight clades was constructed: three represent the *R. parkeri* s. l. strains, and five represent the *R. parkeri* s. s. strains. The clades appear to be generally phylogeographically organized and associated with specific tick vectors. However, while one of the four *R. parkeri* s. s. North American clades appears to be limited to the southwestern United States, the other North American clades exhibit broad dispersal, most notably seen in the largest group, which includes representative samples extending from northern Mexico to Delaware. This work highlights the increasingly recognized geographic range of *R. parkeri* in the Americas and suggests a potential public health risk for these areas.

IMPORTANCE Since 1937, when *Rickettsia parkeri* was originally identified in *Amblyomma maculatum* group ticks, the recognized range and associated vectors for this pathogen have expanded significantly. In recent years, *R. parkeri* has been identified in 12 tick species from seven countries in the Americas. Herein, we provide evidence that the greatest genetic diversity within *R. parkeri* exists in North America, where one *R. parkeri sensu lato* and four *R. parkeri sensu stricto* genotypes are present. While one distinct *R. parkeri sensu stricto* genotype exists only in the southwestern United States, three genotypes are broadly distributed in the eastern United States, with the largest of these found across the known range of *R. parkeri* in North America. In contrast, the South American *R. parkeri sensu stricto* samples represent a single genotype and are completely clonal at the loci analyzed, irrespective of their country of origin.

KEYWORDS *Rickettsia parkeri*, Atlantic rainforest, *Amblyomma*, rickettsiosis, spotted fever group

The genus *Rickettsia* consists of obligately intracellular bacteria, many of which are human pathogens transmitted via arthropod vectors, including mites, fleas, and ticks (1). Until the early 2000s, *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), was the only tick-associated rickettsiosis recognized in the United States. However, in 2002, an illness caused by *Rickettsia parkeri* was confirmed in a patient from Virginia, thus establishing *R. parkeri* as the second tick-associated rickettsiosis in the United States (2). Human cases of *R. parkeri* rickettsiosis

resemble those of other spotted fever group rickettsioses but are characteristically less severe than RMSF; there have been no known fatalities associated with *R. parkeri* rickettsiosis. The incubation period ranges from 2 to 10 days post exposure, and the disease is most commonly characterized by an inoculation eschar at the site of tick attachment. Other symptoms in humans are less specific but often include a combination of fever, headache, malaise, and myalgia (3, 4). Since the initial case, more than 50 cases of *R. parkeri* rickettsiosis have been identified, all limited to the Americas (5–14).

The primary vectors of *R. parkeri* in North America are the ixodid ticks *Amblyomma maculatum* Koch *sensu stricto* (s. s.) in the eastern and Gulf Coast regions of the United States and *A. maculatum sensu lato* (s. l.) in southern Arizona (10–12, 14, 15). Recent field studies further identified *R. parkeri sensu stricto* (s. s.) in *A. maculatum* s. l. ticks in West Texas (16) and New Mexico (17), as well as in the northern state of Sonora (18) and the southeastern state of Tabasco, Mexico (19). In South America, *Amblyomma triste* Koch and *Amblyomma tigrinum* Koch, both members of the *Amblyomma maculatum* tick group (20), are the primary vectors of *R. parkeri* s. s. (5, 6, 21–23). Cases have been reported from Uruguay (7, 9, 13, 21) and Argentina (8, 23–26), where these ticks are prolific human biters.

Although ticks in the *A. maculatum* group are recognized as the primary vectors for *R. parkeri* s. s., other closely related strains of this pathogen have been described in other tick species. In South America, the Atlantic rainforest strains of *R. parkeri sensu lato* (s. l.) have caused four confirmed cases of human rickettsiosis in Brazil, with *Amblyomma ovale* Koch implicated as the responsible vector (27–30). A recent report further confirmed a case of human rickettsiosis caused by *R. parkeri* s. l. strain Atlantic rainforest from Colombia (31) in a region where an isolate of this pathogen was previously obtained from a questing *A. ovale* tick (32). An additional strain of *R. parkeri* s. l. Atlantic rainforest was isolated from *Amblyomma aureolatum* (Pallas) in southern Brazil in the state of Santa Catarina, where *R. parkeri* s. l. strain Atlantic rainforest had previously been detected in *A. ovale*, *A. aureolatum*, and *Rhipicephalus sanguineus* (Latreille) (33, 34).

Several strains of *R. parkeri* s. l. not associated with human illness have also been identified in the Americas. Rickettsial isolates closely related to *R. parkeri* s. l. Atlantic rainforest have been recovered from *Amblyomma nodosum* (Neumann) in Brazil (35) and *Amblyomma parvitarsum* Neumann (36) in Argentina and Chile. While *A. parvitarsum*, *A. nodosum*, *A. ovale*, and *A. aureolatum* are not known to exist in the United States, a recent study reported detection of a *Rickettsia* species most closely related to the Brazilian *R. parkeri* s. l. Atlantic rainforest strains recovered from *A. ovale* ticks in Veracruz, Mexico (37), confirming this pathogen's presence in North America. Additionally, the recently characterized *Rickettsia parkeri* s. l. strain Black Gap has been reported from the United States and northwestern Mexico in the tick *Dermacentor parumapertus* Neumann (38, 39). Strain Black Gap is most closely related to the South American Atlantic rainforest strains. While animal experimentation suggests that this strain could be a human pathogen, no human cases have been reported to date. However, coupled with the broad range of *D. parumapertus* in western North America, the close relationship between strain Black Gap and the *R. parkeri* s. l. Atlantic rainforest strains suggests a potential public health risk associated with this organism.

The public health implications of *R. parkeri* have expanded significantly since its initial isolation from *A. maculatum* in 1937 (40). As our knowledge of this emerging pathogen has progressed, so has our understanding of its relationship with its ixodid tick vectors. While the original isolates and human infections of *R. parkeri* were all associated with *A. maculatum* s. s., in recent years, this pathogen and several closely related strains have been detected in a wide range of confirmed and potential tick vectors. Recent genetic analyses based on both coding and noncoding regions identified South American and North American groupings of *R. parkeri* that could be subdivided based on their tick vectors (41). However, this study was based on relatively few *R. parkeri* s. s. strains and notably did not include any *A. maculatum* s. l. rickettsial strains

from the southwestern United States or Mexico. While this analysis clearly shows genetic separation between North and South American *R. parkeri* s. s. isolates, the 13 North American isolates included in the study represent only four U.S. states and exhibit very little genetic variation based on the eight molecular targets analyzed. The majority of human *R. parkeri* rickettsiosis cases have been reported from the United States; thus, to better characterize the genetic divergence within North American strains of *R. parkeri* s. s., we describe herein a comprehensive multilocus sequence typing analysis for an extensive panel of isolates of *R. parkeri* s. s. and *R. parkeri* s. l., with the inclusion of tick lysates for regions in which isolates are not available.

RESULTS

A total of 49 loci were assessed with the initial 10-isolate screening panel (see Table S1 in the supplemental material). Many of these loci clearly separated the *R. parkeri* s. l. strains from the *R. parkeri* s. s. strains. However, because this separation is not novel, loci that demonstrated polymorphisms within *R. parkeri* s. s. were considered for the final analysis, with priority given to those loci that exhibited differences within the North American *R. parkeri* s. s. strains. Of the 49 loci tested, six were selected for inclusion in the final analysis, including two intergenic regions (*R. parkeri* Portsmouth locus tags MC1_RS05545 to MC1_RS05550 and MC1_RS03940 to MC1_RS03945) and four genes (*R. parkeri* Portsmouth locus tags MC1_RS05545, MC1_RS06275, MC1_RS06395, and MC1_RS06595) (see Table 1). Partial sequences for these four genes and two intergenic regions were obtained for the 65 rickettsial isolates and 12 tick lysates shown in Table 2.

Of the 77 samples sequenced, 65 were strains of *R. parkeri* s. s., including 54 isolates and 11 tick lysates (Table 2). The remaining 11 *R. parkeri* samples were close relatives of *R. parkeri* s. s. and are designated the *R. parkeri* s. l. samples. These included the following 10 isolates: Atlantic rainforest strains Paty, Ao10, Ao240, Aa46, Adrianópolis, and Necocli_10_11, strain NOD Pantanal, strain Black Gap, strain Parvitarsum Ar, and strain Parvitarsum Ch. The single remaining tick is DP18-72, a lysate from a *D. parumapertus* tick infected with a rickettsial organism most closely related to strain Black Gap (Table 2). The final rickettsial isolate included in the analysis is *Rickettsia africae* Z9-Hu[T], used as an outgroup and to root the concatenated phylogeny.

Intergenic regions. (i) MC1_RS05545 to MC1_RS05550. Four genotypes exist at the MC1_RS05545 to MC1_RS05550 locus (Fig. S1) and are based on three single nucleotide polymorphism (SNPs) and two different insertion/deletion (indel) events. Compared to the reference genome Portsmouth (genotype I), all *R. parkeri* s. s. isolates and tick lysates are identical and comprise one genotype with the inclusion of strain NOD Pantanal. The second genotype for this locus (genotype II) consists of the Atlantic rainforest strains Aa46, Adrianópolis, Ao240, Necocli_10_11, and Ao10. These strains are identical to each other and have a four-base-pair insertion (TTAT) beginning at position 31 relative to the reference genome as well as two guanine-to-adenine transitions at sites 44 and 162 relative to the reference. Strain Black Gap and tick lysate DP18-72 comprise the third genotype (genotype III). Both of these samples have the same guanine-to-adenine transitions at positions 44 and 162 and an insertion at the same location in the consensus as the Atlantic rainforest strains; however, these two samples exhibit a smaller two-base-pair insertion (AT) beginning at position 31 of the consensus amplicon. Genotype IV for this locus consists of the *R. parkeri* s. l. strains Parvitarsum Ch and Parvitarsum Ar. These strains are identical to genotype III except they have a guanine-to-adenine transition at position 96 relative to the reference genome.

(ii) MC1_RS03940 to MC1_RS03945. The MC1_RS03940 to MC1_RS03945 locus has eight genotypes (Fig. S2). The first and second genotypes consist of the *R. parkeri* s. s. samples. North American strains Arabia Mountain, Ft. Story, Moe, and Tate's Hell (genotype II) have a nine-base-pair deletion at positions 13 to 21 (TCTTTTGTA) relative to the reference genome Portsmouth (genotype I) for this locus, while the remaining 56 North and South American *R. parkeri* s. s. samples (genotype I) are identical to strain

TABLE 1 Primers developed and used for amplification of coding or IGR for this analysis[a]

Locus tag (*R. parkeri* Portsmouth NC_017044)	Product	Forward primer	Reverse primer	Amplicon size (bp)	Annealing temp (°C)
MC1_RS05545–MC1_RS05550	IGR	GTGCAGTTCTGTTGTCATCC	TGCTTGAATGTACCGGAGAA	214	54
MC1_RS03940–MC1_RS03945	IGR	AGGTGTATACATAAAAGTCTCCA	CTTATCTCTCGCACCTTGGT	139	52
MC1_RS05545	Guanosine polyphosphate pyrophosphohydrolase	CTGGATCCCGTGGTCAAGTC	AGATGCCGAGCTTGGTAGAG	296	54
MC1_RS06275	Dihydrolipoyl dehydrogenase	TAACACCGCCTGCAAGCTTA	GTTATAGGCGGTGGTCCAGG	273	54
MC1_RS06395	Hypothetical protein	TTATTACCCGTGCCGGTTCC	TCTCCACTCCTTCCGGTTCT	321	54
MC1_RS06595	Alpha/beta hydrolase family protein	GGGGCTAGTAAACGGGTGGTG	AATATTGTAAGCCCGCCGCC	276	54

[a]IGR, intergenic region.

TABLE 2 Rickettsial samples used for analysis in this study

Country of origin	Sample[a]	Sample type	Specific location	Yr of isolation	Source material	Reference
Brazil	Agua Clara	Isolate	Água Clara, Mato Grosso do Sul	2008	*Amblyomma triste*	41
	At10	Isolate	Corumbá, Mato Grosso do Sul	2011	*Amblyomma triste*	Unpublished
	At24	Isolate	Paulicéia, São Paulo	2007	*Amblyomma triste*	35
	Pantanal At46	Isolate	Poconé, Mato Grosso do Sul	2012	*Amblyomma triste*	59
	NOD Pantanal	Isolate	Nhecolândia, Mato Grosso do Sul	2011	*Amblyomma nodosum*	Unpublished
	Atl. Rain. Paty	Isolate	Chapada Diamantina, Bahia	2014	*Amblyomma ovale*	60
	Atl. Rain. A010	Isolate	Peruíbe, São Paulo	2010	*Amblyomma ovale*	61
	Atl. Rain. Ao240	Isolate	Peruíbe, São Paulo	2010	*Amblyomma ovale*	61
	Atl. Rain. Adrianópolis	Isolate	Adrianópolis, Paraná	2014	*Amblyomma ovale*	60
	Atl. Rain. Aa46	Isolate	Blumenau, Santa Catarina	2011	*Amblyomma aureolatum*	34
Uruguay	At5URG	Isolate	Toledo, Chico, Canelones	2004	*Amblyomma triste*	21
Colombia	Atl. Rain. Necocli_10_11	Isolate	Necoclí	2010	*Amblyomma ovale*	32
Chile	Parvitarsum Ch	Isolate	Arica and Parinacota	2012	*Amblyomma parvitarsum*	62
Mexico	Am/MX 8M	Tick lysate	Yecora, Sonora	2016	*Amblyomma maculatum* s. l.	18
Argentina	AT-75	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	6
	AT-137	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	6
	AT-190	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	6
	AT-193	Tick lysate	Buenos Aires Province	2007	*Amblyomma triste*	6
	Parvitarsum Ar	Isolate	Salta	2013	*Amblyomma parvitarsum*	62
United States	Carr Canyon	Isolate	Cochise County, AZ	2018	*Amblyomma maculatum* s. l.	17
	Chiricahua	Isolate	Cochise County, AZ	2018	*Amblyomma maculatum* s. l.	17
	Cochise	Isolate	Cochise County, AZ	2016	*Amblyomma maculatum* s. l.	43
	Cottonwood Spring	Isolate	Cochise County, AZ	2018	*Amblyomma maculatum* s. l.	17
	Guindani Canyon	Isolate	Cochise County, AZ	2018	*Amblyomma maculatum* s. l.	17
	Portal	Isolate	Cochise County, AZ	2018	*Amblyomma maculatum* s. l.	17
	White Wing Spring	Isolate	Cochise County, AZ	2018	*Amblyomma maculatum* s. l.	17
	Arivaca Lake	Isolate	Pima County, AZ	2018	*Amblyomma maculatum* s. l.	17
	Atascosa Spring	Isolate	Santa Cruz County, AZ	2016	*Amblyomma maculatum* s. l.	43
	Cave Creek Canyon	Isolate	Santa Cruz County, AZ	2016	*Amblyomma maculatum* s. l.	43
	Pajarita	Isolate	Santa Cruz County, AZ	2017	*Amblyomma maculatum* s. l.	43
	Thumb Butte	Isolate	Yavapai County, AZ	2018	*Amblyomma maculatum* s. l.	17
	6W	Tick lysate	Sussex County, DE	2019	*Amblyomma maculatum* s. s.	63
	9A	Tick lysate	Kent County, DE	2019	*Amblyomma maculatum* s. s.	63
	10B	Tick lysate	Kent County, DE	2019	*Amblyomma maculatum* s. s.	63
	18A	Tick lysate	New Castle County, DE	2019	*Amblyomma maculatum* s. s.	63
	Apalachicola	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	Cash Bayou	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	Deep Creek	Isolate	Franklin County, FL	2012	*Amblyomma maculatum* s. s.	Unpublished
	High Bluff	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	Longleaf	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	Sandbank Creek	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	SR-65	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	Tate's Hell	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	TH07-94	Isolate	Franklin County, FL	2009	*Amblyomma maculatum* s. s.	64
	Kennesaw Mountain	Isolate	Cobb County, GA	2017	*Amblyomma maculatum* s. s.	65
	Coweta	Isolate	Coweta County, GA	2014	Human	12
	Arabia Mountain	Isolate	DeKalb County, GA	2017	*Amblyomma maculatum* s. s.	65
	Sweetwater	Isolate	Douglas County, GA	2017	*Amblyomma maculatum* s. s.	65
	Moe	Isolate	Rockdale County, GA	2016	*Amblyomma maculatum* s. s.	65
	110958_D	Tick lysate	Pulaski County, IL	2013	*Amblyomma maculatum* s. s.	50
	110954_A	Tick lysate	Jackson County, IL	2013	*Amblyomma maculatum* s. s.	50
	Bayou Heron	Isolate	Jackson County, MS	2009	*Amblyomma maculatum* s. s.	64
	Escatawpa	Isolate	Jackson County, MS	2009	*Amblyomma maculatum* s. s.	64
	Franklin Creek	Isolate	Jackson County, MS	2009	*Amblyomma maculatum* s. s.	64
	Grand Bay	Isolate	Jackson County, MS	2010	*Amblyomma maculatum* s. s.	64
	I-10	Isolate	Jackson County, MS	2009	*Amblyomma maculatum* s. s.	64
	Moss Point	Isolate	Jackson County, MS	2009	*Amblyomma maculatum* s. s.	64
	MS07-22	Isolate	Jackson County, MS	2009	*Amblyomma maculatum* s. s.	64
	MS07-44	Isolate	Jackson County, MS	2009	*Amblyomma maculatum* s. s.	64

TABLE 2 (Continued)

Country of origin	Sample[a]	Sample type	Specific location	Yr of isolation	Source material	Reference
	Oktibbeha	Isolate	Oktibbeha County, MS	2009	*Amblyomma maculatum* s. s.	64
	NIAID Maculatum 20[T]	Isolate	Mississippi	1948	*Amblyomma maculatum* s. s.	66
	Animas Creek	Isolate	Hidalgo County, NM	2018	*Amblyomma maculatum* s. l.	17
	DP 18-72	Tick lysate	Hidalgo County, NM	2018	*Dermacentor parumapertus*	Unpublished
	NC-15	Isolate	Mecklenburg County, NC	2010	*Amblyomma maculatum* s. s.	67
	NC-3	Isolate	Mecklenburg County, NC	2010	*Amblyomma maculatum* s. s.	67
	NC-8	Isolate	Mecklenburg County, NC	2010	*Amblyomma maculatum* s. s.	67
	Horry-SC2006	Isolate	Horry County, SC	2006	Human	3
	Black Gap	Isolate	Brewster County, TX	2015	*Dermacentor parumapertus*	39
	Windmill	Isolate	Jeff Davis County, TX	2019	*Amblyomma maculatum* s. l.	16
	Madera Canyon	Isolate	Jeff Davis County, TX	2019	*Amblyomma maculatum* s. l.	16
	Chisos Basin	Isolate	Brewster County, TX	2019	*Amblyomma maculatum* s. l.	16
	Ponderosa	Isolate	Jeff Davis County, TX	2019	*Amblyomma maculatum* s. l.	16
	Fairfax	Isolate	Fairfax County, VA	2010	*Amblyomma maculatum* s. l.	68
	I-66	Isolate	Fairfax County, VA	2010	*Amblyomma maculatum* s. s.	68
	Portsmouth	Isolate	Norfolk County, VA	2002	Human	2
	Ft. Story	Isolate	Princess Anne County, VA	2006	Human	69
Zimbabwe	*Rickettsia africae* Z9-Hu[T]	Isolate	Chiredzi, Masvingo	1992	Human	70

[a]Atl. Rain., Atlantic rainforest.

Portsmouth. The third genotype (genotype III) contains only strain NOD Pantanal, which has a seven-base-pair deletion at positions 26 to 32 (TGTATCT), a guanine-to-adenine transition at position 68, and an adenine insertion at position 73 relative to the reference genome. Genotype IV contains Atlantic rainforest strains Ao10, Ao240, and Adrianópolis. This genotype contains the same nine-base-pair deletion at positions 13 to 21 seen in genotype III; however, this genotype also contains an eight-base-pair insertion (TAAAAAAT) at positions 47 to 54, an adenine-to-cytosine transversion at position 69, and an 11-bp deletion (AATTATTAAAA) at positions 96 to 106 not seen in the third genotype. Genotype V contains only Atlantic rainforest strain Necocli_10_11 and is identical to genotype IV except it does not have the adenine-to-cytosine transversion at position 69. The sixth genotype (genotype VI) consists of the Atlantic rainforest strains Aa46 and Paty. Genotype VI is identical to genotype IV except it has a thymine-to-cytosine transition at position 14. Genotype VII consists of strain Black Gap, strain Parvitarsum Ch, and strain Parvitarsum Ar, and genotype VIII consists of tick lysate DP18-72. Genotype VII exhibits the nine-base-pair deletion at positions 13 to 21 seen in genotypes III to VI, but tick lysate DP18-72 (genotype VIII) does not exhibit this deletion. Both genotype VII and tick lysate DP18-72 (genotype VIII) have an 11-bp insertion (TAAAAAATTAT) at positions 47 to 57 and the same 11-bp deletion at positions 96 to 106 seen in genotypes IV to VI.

Coding regions. (i) MC1_RS05545. The MC1_RS05545 locus has six genotypes (Fig. S3). Genotype I consists of 42 of the 50 *R. parkeri* s. s. strains and tick lysates from North America, all identical to strain Portsmouth. Genotype II consists of strain NIAID Maculatum 20[T], which exhibits a thymine insertion at position 198 relative to the reference genome Portsmouth (genotype I). Genotype III contains strain Grand Bay, which exhibits a guanine-to-adenine transition at position 235 relative to genotype I. The fourth genotype (genotype IV) consists of only strain High Bluff, with a guanine-to-adenine transition at position 276 relative to genotype I. Genotype V contains the Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Paty, Ao240, and Ao10 as well as Parvitarsum Ar, Parvitarsum Ch, the North American strain Black Gap, and tick lysate DP18-72. Also included in genotype V are *R. parkeri* s. s. strains Agua Clara, At24, At5, At10, and At46 Pantanal as well as tick lysates AT-75, AT-137, AT-190, and AT-193. Finally, this group also contains a subset of the North American strains from southern Arizona and New Mexico: Animas Creek, Chiricahua, Cottonwood Spring, Guindani Canyon, Pajarita, Portal, and White Wing Spring, as well as tick lysate 10B from Delaware. The samples in genotype V all contain an adenine-to-cytosine transversion

at position 122 relative to genotype I. The sixth genotype for this locus (genotype VI) contains only strain NOD Pantanal, which contains the adenine-to-cytosine transversion at position 122 seen in genotype V as well as a thymine-to-cytosine transition at position 205 relative to genotype I.

(ii) MC1_RS06275. The MC1_RS06275 locus has three genotypes (Fig. S4). The first of these genotypes (genotype I) consists of the 50 North American *R. parkeri* s. s. strains and tick lysates, all identical to strain Portsmouth. Genotype II contains Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Paty, Ao240, and Ao10. When compared to the reference genome (genotype I), these strains have two SNPs: a guanine-to-thiamine transversion at position 43 and a thiamine-to-guanine transversion at position 215. The third genotype (genotype III) consists of strains Black Gap, NOD Pantanal, Agua Clara, At24, At5, At10, At46 Pantanal, Parvitarsum Ar, and Parvitarsum Ch as well as tick lysates DP18-72, AT-75, AT-137, AT-190, and AT-193, all of which exhibit the same transversion at position 43 as genotype II; however, they do not possess the SNP at position 215.

(iii) MC1_RS06395. The MC1_RS06395 locus consists of four genotypes (Fig. S5). The first genotype (genotype I) for this locus consists of 45 North American strains and tick lysates, all identical to strain Portsmouth. Genotype II for this locus consists of the South American *R. parkeri* s. s. strains Agua Clara, At24, At5, At10, and At46 Pantanal as well as the South American tick lysates AT-75, AT-137, AT-190, and AT-193. Also included in genotype II are a subset of the North American strains from southern Arizona and New Mexico: Animas Creek, Chiricahua, Cottonwood Spring, Guindani Canyon, Pajarita, Portal, and White Wing Spring. This second genotype exhibits an adenine-to-guanine transition at position 66, but no other polymorphisms separate these samples from reference genome Portsmouth (genotype I). Genotype III contains three SNPs relative to genotype I: the same adenine-to-guanine transition at position 66 seen in genotype II, an adenine-to-cytosine transversion at position 134, and a guanine-to-cytosine transversion at position 236. This genotype includes the South American Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Ao240, Paty, and Ao10, as well as *R. parkeri* s. l. strains Parvitarsum Ch, Parvitarsum Ar, and Black Gap and tick lysate DP18-72. The fourth genotype for this locus includes only strain NOD Pantanal, with four SNPs relative to genotype I; this strain exhibits the three SNPs present in genotype III but also includes a guanine-to-adenine transition at position 52 relative to genotype I.

(iv) MC1_RS06595. There are five genotypes in the MC1_RS06595 locus (Fig. S6). Genotype I for this locus consists of the 50 North American *R. parkeri* s. s. strains and tick lysates, all of which are identical to reference strain Portsmouth. Genotype II for this locus consists of the South American *R. parkeri* s. s. strains Agua Clara, At24, At5, At10, and At46 Pantanal as well as the South American tick lysates AT-75, AT-137, AT-190, and AT-193. This genotype exhibits an adenine-to-guanine transition at position 229 relative to the reference genome Portsmouth (genotype I). The third genotype (genotype III) for this locus contains the South American Atlantic rainforest strains Aa46, Adrianópolis, Necocli_10_11, Ao240, Paty, and Ao10 as well as *R. parkeri* s. l. strains Parvitarsum Ar and Parvitarsum Ch. This genotype contains the adenine-to-guanine transition at position 229 seen in the second genotype but also has another adenine-to-guanine transition at position 53 relative to genotype I. The fourth genotype (genotype IV) is composed of the North American strain Black Gap and tick lysate DP18-72 and contains the two adenine-to-guanine transitions at positions present in genotype III for this locus as well as a guanine-to-thymine transversion at position 121 relative to reference genome Portsmouth (genotype I). Strain NOD Pantanal is the only member of the fifth genotype (genotype V), with three SNPs relative to reference genome Portsmouth (genotype I): the same adenine-to-guanine transition at position 229 seen in the second genotype as well as an adenine-to-guanine transition at position 116 and a guanine-to-adenine transition at position 97 relative to reference genome Portsmouth (genotype I).

Concatenated phylogeny. DNA sequences for all 77 rickettsial samples for the six loci were concatenated and aligned for phylogenetic analysis; the concatenated final

alignment consists of 1,519 nucleotides. The loci were concatenated in the following order: MC1_RS05545, MC1_RS05545 to MC1_RS05550, MC1_RS06275, MC1_RS06595, MC1_RS03940 to MC1_RS03945, MC1_RS06395. The length of the amplicons for each individual locus is available in Table 1. After phylogenetic analysis, the *R. parkeri* s. s. and *R. parkeri* s. l. samples were well separated both from each other and from *R. africae* Z9-Hu[T] under high posterior probabilities (Fig. 1).

The *R. parkeri* s. l. group was subdivided into three clades, all under robust posterior probabilities. These include the Parvitarsum/Black Gap clade (clade 1), the Atlantic rainforest clade (clade 2), and the NOD clade (clade 3). Clade 1 includes *R. parkeri* s. l. strains Parvitarsum Ch and Parvitarsum Ar isolated from *A. parvitarsum* in Chile and Argentina, respectively. Strains Parvitarsum Ch and Parvitarsum Ar are completely clonal across all six loci. Also in this clade, separated from the Parvitarsum strains by strong posterior probability support, are strain Black Gap and tick lysate DP18-72, both from *D. parumapertus* in Texas and New Mexico, USA, respectively. Strain Black Gap and tick lysate DP18-72 are clonal at five of the six loci; however, tick lysate DP18-72 has a two-base-pair insertion as described above in intergenic region MC1_RS03940 to MC1_RS03945.

Clade 2 consists of the *R. parkeri* s. l. Atlantic rainforest strains Necocli_10_11 from *A. ovale* in Colombia and Ao10, Ao240, Adrianópolis, and Paty from *A. ovale* in Brazil. Also in this clade is *R. parkeri* s. l. Atlantic rainforest strain Aa46, isolated from *A. aureolatum* in Brazil. There is clear separation between the Colombian strain Necocli_10_11 and the Brazilian strains and strong support within this clade for the separation of Atlantic rainforest strains Paty and Aa46 from the rest of the group. Variation at each locus between the individual strains within this clade is described above.

Clade 3 contains the Brazilian isolate NOD Pantanal, the only strain in this study that was isolated from an *A. nodosum* tick. This clade has strong posterior probability support as a separate group from both the other two *R. parkeri* s. l. clades (clades 1 and 2) and the *R. parkeri* s. s. samples.

The *R. parkeri* s. s. samples are divided into four clades. The first of these (clade 4) consists of the South American *R. parkeri* s. s. isolates and tick lysates. All samples in this group originated from *A. triste* in Brazil, Uruguay, or Argentina (Table 2), and the five isolates and four tick lysates from this clade are completely clonal at all six loci.

Clade 5 consists of seven isolates from *A. maculatum* s. l. in the southwestern United States: six from southern Arizona (Chiricahua, Cottonwood Spring, Guindani Canyon, Pajarita, Portal, and White Wing Spring) and one from New Mexico (Animas Creek) (Table 2). There is robust posterior probability support to separate this group from both the South American *R. parkeri* s. s. samples (clade 4) and the rest of the North American *R. parkeri* s. s. samples. The seven isolates in clade 5 are identical to each other at all six loci analyzed and represent a mix of genotypes for the individual loci. For two of the four genes analyzed (MC1_RS05545 and MC1_RS06395), the isolates in this group are identical to the South American *R. parkeri* s. s. samples (clade 4). For the other two genes and two intergenic regions, the seven isolates in clade 5 are identical to the largest North American *R. parkeri* s. s. clade (clade 7).

Clade 6 contains only three samples from the United States: *A. maculatum* s. s tick lysate 10B from Delaware, isolate Oktibbeha from *A. maculatum* s. s. in Mississippi, and isolate Horry-SC2006 from a human case in South Carolina (Table 2). These three samples are completely clonal and are separated from the largest North American *R. parkeri* s. s. clade (clade 7) by one gene (MC1_RS05545), for which these three samples are identical to both clades 4 and 5, representing the South American *R. parkeri* s. s. samples and a subset of the isolates from the Southwestern United States.

The largest clade for the *R. parkeri* s. s. samples is clade 7, which contains 42 of the North American *R. parkeri* s. s. samples, including the reference genome strain Portsmouth and the type strain NIAID Maculatum 20[T] (Table 2). Nearly all of the 42 samples in clade 7 are clonal, but strains High Bluff and Grand Bay exhibit SNPs and NIAID Maculatum 20[T] exhibits a single-base-pair insertion in gene MC1_RS05545, as described above. The final
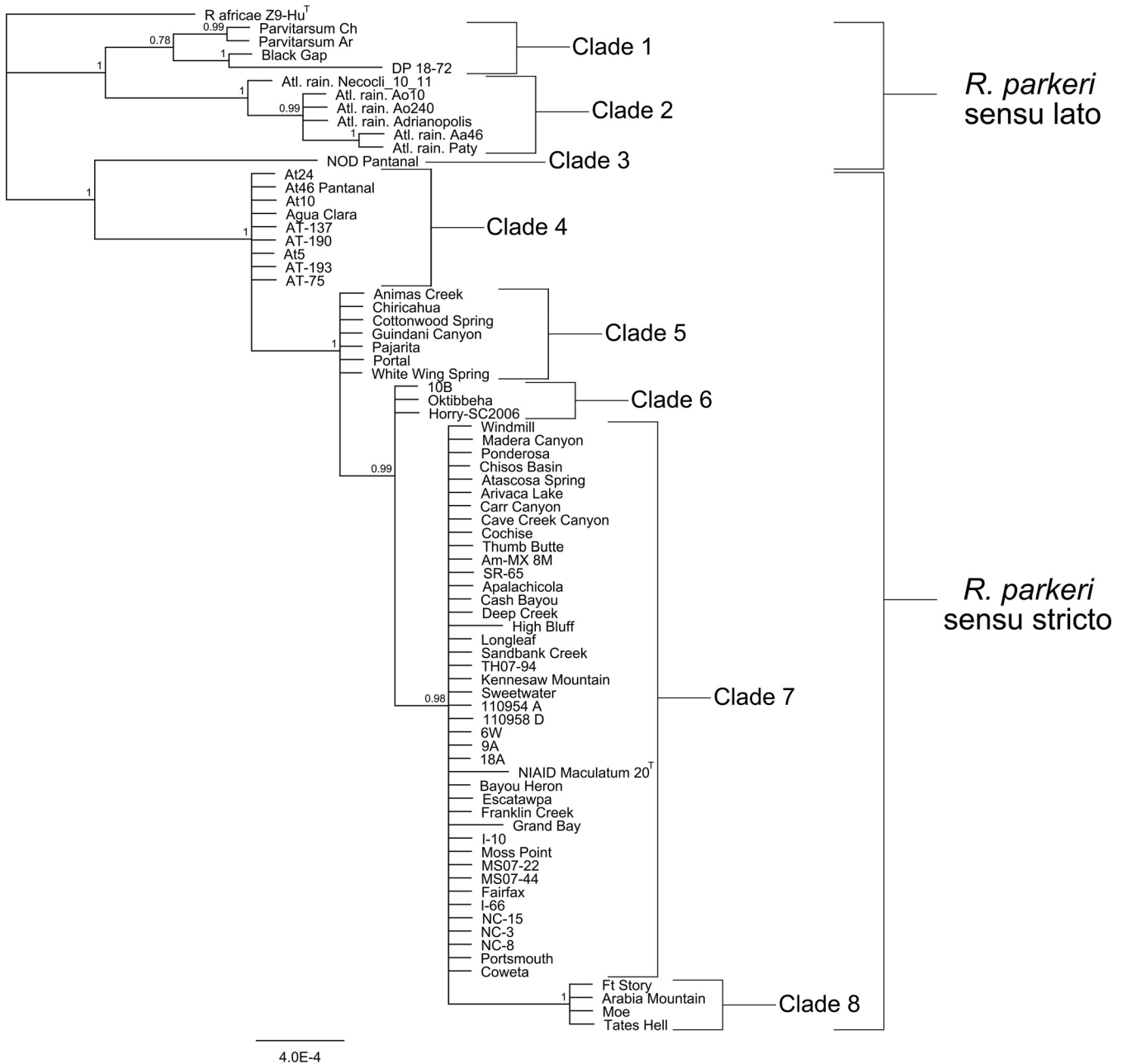
FIG 1 Concatenated phylogenetic analysis of *Rickettsia parkeri* s. s. and *R. parkeri* s. l. strains using *Rickettsia africae* Z9-Hu[T] as an outgroup. A total of 1,519 nucleotides representing 4 coding regions and 2 intergenic regions (Table 1) were concatenated and subjected to Bayesian analysis. Numbers at nodes are posterior probability support values, and clades are indicated by brackets and labeled.

clade (clade 8) nests within clade 7 and consists of three U.S. isolates: strains Moe and Arabia Mountain from *A. maculatum* s. s. from Georgia, strain Tate's Hell from *A. maculatum* s. s. from Florida, and strain Ft. Story from a human case in Virginia. These four isolates share a deletion in the MC1_RS03940 to MC1_RS03945 gene as described above.

DISCUSSION

The work shown here corroborates recent analyses identifying clear separation between strains of *R. parkeri* s. s. and *R. parkeri* s. l. as well as distinct separation between North American and South American strains of *R. parkeri* s. s. (41). In addition, results of the current investigation reveal four unique North American clades of *R. parkeri* s. s. (Fig. 1). Geographically, the most diversity within *R. parkeri* s. s. is seen in the eastern
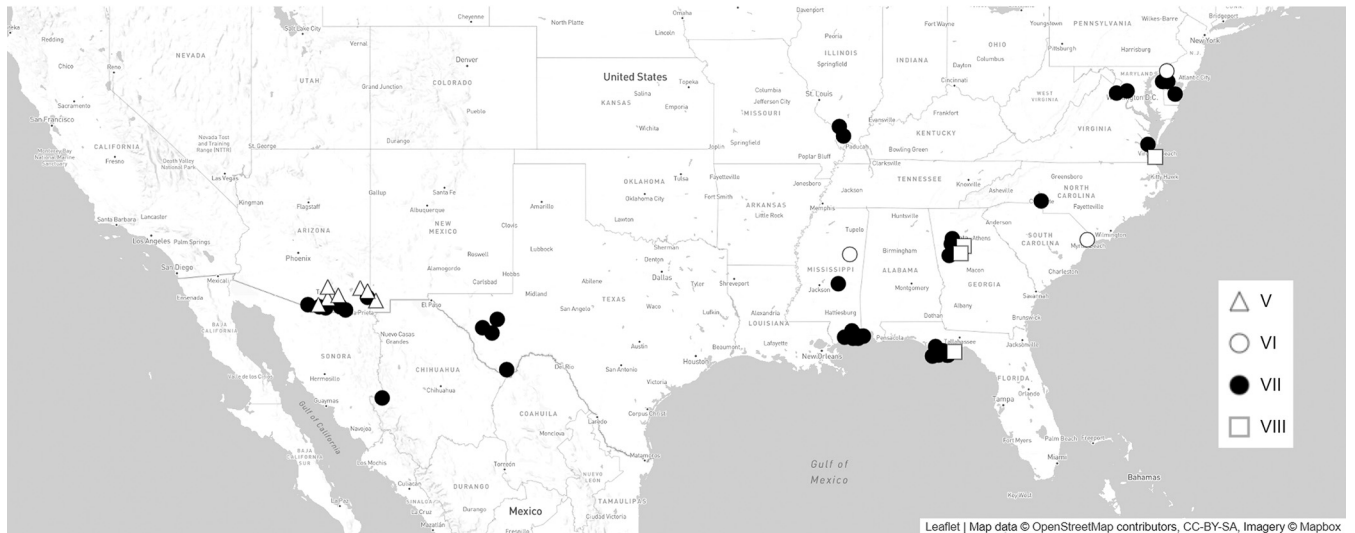
FIG 2 Map showing the distribution in North America of *R. parkeri* s. s., including clades 5 to 8. Clade 5 is indicated by white triangles, clade 6 by white circles, clade 7 by black circles, and clade 8 by white squares.

United States, where three separate clades are represented (Fig. 2). This is likely due to the more extensive availability of suitable habitat for *A. maculatum* in this region than in other areas where *R. parkeri* s. s. occurs in the United States. The long, humid summer season coupled with the wide availability of mammalian hosts in the Gulf Coast and eastern United States provides ideal conditions for this tick species and its pathogens to proliferate (10, 15). Found in these regions, the largest group of *R. parkeri* s. s. is clade 7, which is also the most geographically expansive; the range of samples in this clade extends from northern Mexico to the state of Delaware (Fig. 2), suggesting that this dominant strain represents the most widely dispersed and highly conserved strain in North America.

Conversely, clade 5 contains seven samples restricted to the southwestern United States that appear to represent an intermediate genotype between the South American *R. parkeri* s. s. samples (clade 4) and clade 7 (Fig. 1 and 2). The samples in clade 5 were all isolated from *A. maculatum* s. l. ticks within the Madrean Occidental Archipelago Sky Islands (42). These fractured riparian habitats are found in otherwise arid regions and likely depend on migratory birds, cattle, or other ungulates to disperse tick species to additional suitable habitats within the surrounding desert. There is no clear geographical or ecological distinction between the two genotypes from the southwestern United States; strains Pajarita (clade 5) and Atascosa Spring (clade 7) were collected on the same day in the Pajarito Mountains (43), the site of the first human cases in this state (11), thus confirming that at least two genotypes are circulating together among *A. maculatum* s. l. tick populations.

While there is significant geographical separation between the *R. parkeri* s. s. samples in clades 4 and 7, clade 1 is the only group with cross-continental representation, including *R. parkeri* s. l. strains from Chile, Argentina, and the United States (Fig. 3). The origin of the close genetic relationship between samples from North American *Dermacentor parumapertus* and South American *Amblyomma parvitarsum* is not immediately clear; *A. parvitarsum* is an ectoparasite of camelids in Argentina, Bolivia, Peru and Chile (44), while *D. parumapertus* is found on *Lepus californicus*, a common hare reported from the western United States and Mexico (39, 45). However, the hosts of the immature stages of *D. parumapertus* could offer some insight. In addition to parasitizing *L. californicus*, immature *D. parumapertus* ticks have been recovered from various small rodents, including several species whose ranges extend into central and eastern Mexico (46). A recent study of micromammals in Chile further identified *R. parkeri* s. l. strain Parvitarsum in 10 different flea species, including those collected from *Rattus rattus*, a pest with cosmopolitan distribution (47). To date, no immature *A. parvitarsum*

FIG 3 Map showing all eight clades of *R. parkeri* s. s. and *R. parkeri* s. l. and their respective distributions in North and South America. Clade 1 is represented by black squares, clade 2 by black triangles, clade 3 by a white star, clade 4 by black stars, clade 5 by white triangles, clade 6 by white circles, clade 7 by black circles, and clade 8 by white squares.

ticks have been collected from rodents; larval *A. parvitarsum* ticks are ectoparasites of lizards, and the nymphal hosts are not known (48). Nonetheless, it is clear that rodents within this tick's range are likely infected with strain Parvitarsum, suggesting a potential route of entry for this bacterium into North American mammalian and tick populations through either infected rodents or infected fleas. It is important, however, to note the small sample size within this clade. Analysis of additional strains could perhaps increase the resolution between these two groups of rickettsial organisms and offer further insights into the origin of their close genetic relationship.

Although the novel typing scheme developed here identifies clear geographical distinction between strains of *R. parkeri* s. s. and *R. parkeri* s. l. for most of the groupings, this is not a completely consistent finding. While the two South American *R. parkeri* s. l. Parvitarsum strains are clearly separate from the two North American *R. parkeri* s. l. Black Gap strains in clade 1, the Parvitarsum isolates share 100% sequence identity, though they originate from Chile and Argentina. Similarly, in clade 2, the single Colombian Atlantic rainforest strain (Necocli_10_11) separates from the Brazilian strains that comprise the rest of the clade; however, there is also strong support within this group for separation of the Brazilian Atlantic rainforest strains Paty and Aa46 from the rest of the clade (Fig. 1). While the biogeography of these samples does not clearly align, the tick vector from which these strains are isolated could potentially inform their phylogenetic differentiation, echoing the results from previous work (41). In clade 1, the *R. parkeri* s. l. strains can be separated based on their isolation from either *A. parvitarsum* (South American Parvitarsum strains) or *D. parumapertus* (North American Black Gap strains), and in clade 2, strain Aa46 is the only *R. parkeri* s. l. Atlantic rainforest strain isolated from *A. aureolatum*. While Atlantic rainforest strain Paty from *A. ovale* also clusters with strain Aa46, clade 2 has relatively few samples. It is possible that examining additional Atlantic rainforest strains from both *A. aureolatum* and other *Amblyomma* species in Brazil could elucidate the importance, if any, of the tick vector in relation to the rickettsial strain. Importantly, little is known about the reservoir hosts in nature for *R. parkeri*. It is possible that the genetic variation seen here could be partially attributed to habitat differences, and thus host differences, of the tick vectors from which the samples originated.

There have been confirmed human cases of *R. parkeri* rickettsiosis reported from Brazil, Colombia, Argentina, Uruguay, and the United States (5–12, 14, 49). In the United States, cases have been reported from 11 states: Arizona, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, Texas, and Virginia (10, 11, 14). However, these states represent a small segment of the total distribution of *A. maculatum* in the country. Since the initial description of *A. maculatum* in 1844, its known range has expanded significantly, from that of a coastal tick with limited range of 100 to 200 miles inland in the Gulf Coast region to now being established in many central U.S. states, such as Kansas, Oklahoma, and Illinois (10, 50). Recent studies have confirmed the northern expansion of *A. maculatum* s. s. and *R. parkeri* s. s. into Connecticut (51), representing the northernmost established populations of both tick and pathogen. This report by Molaei et al. highlights the continual expansion of *A. maculatum* s. s. into additional regions within North America and suggests an emerging public health risk for *R. parkeri* rickettsiosis in these areas.

While the typing scheme and resultant phylogenetic analyses presented here identify clear geographical separation between samples, the underlying causes for this separation are not immediately clear. Nonetheless, this work and that of others (41) reflect the increasingly recognized geographic range of *R. parkeri* in the Americas. This group of rickettsial organisms has been detected in 12 tick species from seven countries across two continents. It is reasonable to assume that these pathogens exist elsewhere in the Americas, as the recognized ranges of their tick vectors represent a more extensive distribution than the known range for *R. parkeri*. Future application of the multilocus sequence typing analysis presented here could serve as a tool to help inform the origins or clarify the taxonomic status of novel *R. parkeri* strains as they are discovered. Given the broad known distribution of *R. parkeri* s. s. and *R. parkeri* s. l. across North

and South America and their associations with many ixodid tick vectors of medical importance, this group of emerging human pathogens already presents a public health threat to most of the Western Hemisphere.

MATERIALS AND METHODS

All 50 of the North American *R. parkeri* s. s. and *R. parkeri* s. l. isolates used in this study are part of the Centers for Disease Control and Prevention's Rickettsial Isolate Reference Collection. The 14 South American isolates were kindly provided by David Walker (Necocli_10_11) and Marcelo Labruna (Agua Clara, At10, At24, Pantanal At46, At5URG, Parvitarsum Ar, Parvitarsum Ch, NOD Pantanal, and Atlantic rainforest strains Paty, Ao10, Ao2240, Adrianópolis, and Aa46). North American tick samples for the study were provided by Victoria Phillips (Illinois), Lauren Maestas and Michael Buoni (Delaware), and Jesus Delgado, David Delgado, and J. David Licona-Enriquez (Mexico). DNA extracts of *R. parkeri*-infected *A. triste* ticks were provided by Santiago Nava (Argentina).

Rickettsia parkeri s. s. and s. l. isolates (Table 2) were propagated in Vero E6 cells in minimum essential medium (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA). Cultures were maintained in a 5% atmospheric CO_2 incubator at 32°C until 90% infection was observed by visualization with acridine orange staining (BD, Franklin Lakes, NJ, USA). DNA was extracted from the propagated cultures using a KingFisher ML automated purification system (Thermo Scientific, Waltham, MA, USA). DNAs were eluted in 150 μl of KingFisher elution buffer and stored at 4°C prior to genetic analysis. Species verification was performed using a PCR assay targeting a portion of the rickettsial outer membrane protein *omp*A (52). PCR products were gel purified and bidirectionally sequenced on an Applied Biosystems 3500 genetic analyzer using a BigDye Terminator V3.1 kit (Applied Biosystems, Carlsbad, CA, USA). Resultant sequences were assembled in Geneious Prime 2019.1 (Geneious, Auckland, New Zealand) and compared to GenBank data using BLASTn analysis.

Genome alignments were performed using Geneious Prime. Whole genomes for *Rickettsia parkeri* strains Portsmouth (accession no. NC_017044), Grand Bay (NZ_LAOK01000001), Tate's Hell (NZ_LAOO01000001), AT#24 (NZ_LAOL01000001), and Atlantic Rainforest (CP040325) were aligned with a draft genome for *R. parkeri* strain Black Gap (data not shown). Thirty-two homologous intergenic regions and primer pairs previously used in genotyping analyses of *Rickettsia conorii* (53) and *R. rickettsii* (54) were located in the *R. parkeri* Portsmouth genome and compared to the other five aligned genomes to identify polymorphisms within these regions. Primers were modified when necessary for specificity with *R. parkeri* for any potentially informative intergenic regions. Additional intergenic regions and coding regions containing polymorphisms within the six aligned genomes were identified and selected for further analysis. Primers for these regions were designed using Geneious Prime.

PCR amplifications were conducted in 20-μl reaction mixtures, using 10 μl of *Taq* PCR master mix (Qiagen, Valencia, CA, USA), 1 μl each of the forward and reverse primers at 20 μM, 2 μl of DNA extract, and 6 μl of nuclease-free water. Reactions were run with an initial 5-min denaturation at 95°C, followed by 40 cycles of a 5-s 95°C denaturation, a 45-s annealing step (Table 1), and 1-min extension step at 72°C. The final step in the reaction was a single 10-min extension at 72°C. PCR products were gel purified and bidirectionally sequenced as described above. Resultant sequences were assembled using Geneious Prime, and alignment files were constructed in MEGA X (55).

An initial screening to check for polymorphisms in each locus was conducted using five *R. parkeri* strains that were chosen based on their geographical separation and variation in isolation source. These included strains NIAID Maculatum 20ᵀ (Mississippi, *A. maculatum* s. s.), Black Gap (Texas, *D. parumapertus*), Moe (Georgia, *A. maculatum* s. s.), Cochise (Arizona, *A. maculatum* s. l.), and Atlantic rainforest Aa46 (Brazil, *A. aureolatum*). The five sequences from this initial PCR screening were added to MEGA X alignments that included GenBank data from the five published *R. parkeri* genomes, creating initial alignments of 10 strains per locus. These alignments were used to identify genetic differences between these 10 strains to determine the potential for discrimination among a larger sample of strains.

For the final analysis, all isolates and tick lysates from Table 2 as well as an isolate of *Rickettsia africae* Z9-Huᵀ were PCR amplified and bidirectionally sequenced as described above according to the annealing temperatures from Table 1. *Rickettsia africae* was selected to be used as an outgroup for this work based on its close genetic relationship with *R. parkeri* (41). Primer sequences were identified and removed in MEGA X, and insertions and deletions were treated with the simple indel coding method (56). The resultant sequences were assembled, and alignments for each locus and the concatenated final analysis were generated with Geneious Prime.

Phylogenetic trees were inferred by the Bayesian method. Bayesian analyses were performed using the MrBayes 3.2.6 program within Geneious Prime (57). The general time reversible (GTR) model was utilized as the substitution model, and *R. africae* Z9-Huᵀ was designated as the outgroup. A gamma model of variable rates across sites was used, and 1,100,000 generations were employed with four range categories. Support values for branches are posterior probabilities obtained by MrBayes. Maps of the phylogenies were created using Microreact (https://microreact.org) (58).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.3 MB.

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