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Isolation of *Rickettsia parkeri* and Identification of a Novel Spotted Fever Group *Rickettsia* sp. from Gulf Coast Ticks (*Amblyomma maculatum*) in the United States[▽]

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Until recently, *Amblyomma maculatum* (the Gulf Coast tick) had garnered little attention compared to other species of human-biting ticks in the United States. *A. maculatum* is now recognized as the principal vector of *Rickettsia parkeri*, a pathogenic spotted fever group rickettsia (SFGR) that causes an eschar-associated illness in humans that resembles Rocky Mountain spotted fever. A novel SFGR, distinct from other recognized *Rickettsia* spp., has also been detected recently in *A. maculatum* specimens collected in several regions of the southeastern United States. In this study, 198 questing adult Gulf Coast ticks were collected at 4 locations in Florida and Mississippi; 28% of these ticks were infected with *R. parkeri*, and 2% of these were infected with a novel SFGR. Seventeen isolates of *R. parkeri* from individual specimens of *A. maculatum* were cultivated in Vero E6 cells; however, all attempts to isolate the novel SFGR were unsuccessful. Partial genetic characterization of the novel SFGR revealed identity with several recently described, incompletely characterized, and noncultivated SFGR, including "*Candidatus* Rickettsia andeanae" and *Rickettsia* sp. Argentina detected in several species of Neotropical ticks from Argentina and Peru. These findings suggest that each of these "novel" rickettsiae represent the same species. This study considerably expanded the number of low-passage, *A. maculatum*-derived isolates of *R. parkeri* and characterized a second, sympatric *Rickettsia* sp. found in Gulf Coast ticks.

Amblyomma maculatum (the Gulf Coast tick) is an aggressive, human- and animal-biting ixodid tick that is distributed widely across the southeastern United States (5, 19). Until recently, *A. maculatum* had never been associated directly with any known tick-borne infection of humans; however, *A. maculatum* is now recognized as a vector of *Rickettsia parkeri*, a spotted fever group rickettsia (SFGR) that causes a disease similar to, but milder than, Rocky Mountain spotted fever (RMSF). Since the recognition of the index patient in 2002 (37), more than 20 cases of *R. parkeri* rickettsiosis have been identified in Alabama, Florida, Kentucky, Maryland, Mississippi, North Carolina, South Carolina, Virginia, and Texas (12, 38, 57; CDC, unpublished data). In 1923, Cowdry (11) described minute intracellular bacteria, apparently rickettsiae, in the tissues and eggs of female *A. maculatum* ticks collected in Jackson County, MS. Parker et al. (41) subsequently isolated *R. parkeri* from Gulf Coast ticks collected in southeastern Texas in 1937. Since that report, spotted fever group (SFG) rickettsiae have been detected in *A. maculatum* specimens collected in at least 8 states (27, 42, 44, 55), but the only characterized *Rickettsia* sp. associated with the Gulf Coast tick is *R. parkeri* and the only extant strain of *R. parkeri* obtained from *A. maculatum* was isolated in 1948 (4).

A novel and incompletely characterized SFGR, distinct from other recognized *Rickettsia* spp., was detected recently in *A. maculatum* specimens collected in Georgia, Florida, and Mississippi (55). In this report, we describe further genetic characterization of this novel rickettsia and cultivation of 17 contemporary strains of *R. parkeri* obtained from Gulf Coast ticks collected in Florida and Mississippi.

MATERIALS AND METHODS

Tick collection and processing. Questing adult Gulf Coast ticks, collected from vegetation by using flannel cloth flags, were obtained from August 2005 to August 2007 from Tate's Hell State Forest (THSF), Franklin County, FL; Sopchoppy, Wakulla County, FL; Starkville, Oktibbeha County, MS; and Grand Bay National Wildlife Refuge (GBNWR), Jackson County, MS (see Table 1). The collection sites were characterized by palustrine flatwood or savannah habitats containing predominantly slash pine (*Pinus elliottii*), longleaf pine (*Pinus palustris*), saw palmetto (*Serenoa repens*), wiregrass (*Aristida stricta*), and mesic woody shrubs. Ticks were transported as live specimens to the laboratory and washed sequentially in 3 solutions, 2% Micro-Chem Plus (National Chemical Laboratories of PA, Inc., Philadelphia, PA), 10.5% sodium hypochlorite, and 3% hydrogen peroxide. The ticks were agitated gently in each solution for ~5 min and rinsed in sterile distilled water after the final wash. Individual specimens were blotted lightly on sterile filter paper and bisected longitudinally; one half of each tick was placed in 0.5 ml of Eagle's minimum essential medium with Earle's salts (MEM) and frozen at −80°C, and the other half was processed for DNA extraction.

Molecular detection of SFG rickettsiae in ticks. DNA was extracted from one half of each bisected tick, as described previously (31), and eluted in 100 μl (final volume). DNA extracts were screened by using a direct PCR assay with primers

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190-70 and 190-701 (47) to amplify a 632-bp segment of the rickettsial outer membrane protein A gene (*ompA*). All tick extracts that produced an amplicon of the expected size were tested subsequently by using a real-time SYBR green PCR assay designed to specifically amplify a 408-bp segment of the *ompA* gene of the novel SFGR. Five microliters of tick extract was combined with core reagents of a SYBR brilliant green kit (Stratagene, La Jolla, CA) in a 50-μl reaction mixture containing primers Rx-190-F (5′-GTGATGTTGCTGAGT TCG) and Rx-190-R (5′-TTATCTTTGCCGGGGTTA), each at a final concentration of 300 nM. The thermal cycler parameters were activation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 60 s, and 72°C for 90 s. The parameters for dissociation curves included denaturation at 95°C for 60 s, followed by 55°C for 30 s and then a ramp from 55°C to 95°C at a rate of 0.2°C/s with continuous collection of data. DNA extracts of previously identified, novel SFGR-infected *A. maculatum* ticks (55) were used as positive-control samples to calibrate the assay. The negative controls included DNA samples of *A. maculatum* ticks infected with *R. parkeri* and a Vero E6 cell culture infected with *R. rickettsii*.

Amplicons from *ompA*-positive ticks were also evaluated by using restriction fragment length polymorphism (RFLP) analysis (49). For each sample, 5.0 μl of PCR product obtained from the *ompA* screening assay was incubated with 5 U of AluI endonuclease (New England BioLabs, Inc., Ipswich, MA) and 1.0 μl of 10× enzyme buffer for 6 h at 37°C. Digested products were separated by using a 4% agarose gel in Tris-acetate-EDTA buffer and stained with ethidium bromide.

Cell culture isolation. All specimens that tested positive for the novel SFGR by the SYBR green assay and a subset of *R. parkeri*-positive ticks were evaluated by using cell culture. The remaining half of each identified tick was thawed and triturated with a sterile scalpel blade in 0.5 ml of MEM. Each triturate was inoculated onto a semiconfluent monolayer of Vero E6 cells in a T25 tissue culture flask containing 5 ml of MEM with 500 mmol L-glutamine, 5% tetracycline-free fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. Cell cultures were incubated at 34.5°C in an air atmosphere containing 5% CO_2. The medium and tick triturate were removed after ~24 h and replaced with 5.0 ml of fresh cell culture medium containing no antibiotics. The medium was changed approximately once a week thereafter. Cultures were monitored for evidence of infection by examining cytospin preparations of ~0.1 ml of cell culture medium fixed in absolute methanol and stained with 0.01% acridine orange. Cell cultures were examined weekly for a minimum of 4 weeks. The identity of each isolate was confirmed by *ompA* PCR and RFLP analysis or sequencing of the amplicon.

Electron microscopy of *R. parkeri*. Vero E6 cells infected with a tick-derived isolate of *R. parkeri* were scraped from a cell culture flask, pelleted by centrifugation, and fixed in 2.5% glutaraldehyde at room temperature for 1 h. The fixed pellet was embedded in 2% agarose and placed in buffered 1% osmium tetroxide at 4°C for 30 min. The specimen was then dehydrated using series of a graded ethanol concentrations and propylene oxide and embedded in an Epon substitute-Araldite mixture. Sections were stained with 4% uranyl acetate and Reynold's lead citrate.

Phylogenetic analysis of the novel SFGR. Additional genetic characterization of the novel SFGR was performed by using PCR assays targeting segments of multiple genes. A nested PCR assay was used to amplify a 535-bp segment of *ompA*, as described previously (55). An 811-bp segment of the *ompB* gene was amplified directly by using primers 120-2788 and 120-3599 (52). For the citrate synthase (*gltA*) gene, direct PCR amplification using three different primer sets was used to assemble a nearly complete, 1,191-bp sequence of the gene. Primers CS-78 and CS-323 (23) were used to amplify a 401-bp segment at the 5′ end; primers CS-239 and CS-1069 (24) were used to amplify 830 bp of sequence that overlapped the 3′ end of the leading segment and the 5′ end of the terminal segment; and primers Rp877p and CS1273f (50) were used to amplify a 476-bp segment at the 3′ end of *gltA*. Primers rpoB-Fav (5′-CGTGTTGAAGGCGGT AATT-3′) and rpoB-Rav (5′-AAGAAAGCCACAAGCACGTT-3′) were used to amplify directly a 512-bp segment of the RNA polymerase β-subunit (*rpoB*) gene. The 25-μl reaction mixtures for the *rpoB* assay contained primers at a final concentration of 1 μM, deoxynucleoside triphosphates (dNTPs) at a concentration of 200 μM, 1.5 mM $MgCl_2$, 0.625 U of GoTaq polymerase (Promega, Madison, WI), and 5 μl of template. The thermal cycler program for the *rpoB* assay consisted of an initial denaturing step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. The program ended with a 5-min elongation step at 72°C.

For each of these assays, distilled water was used as a negative-control sample, and DNA extracts of *R. parkeri*, *R. akari*, or *R. amblyommii* were used as positive-control samples. All amplified gene segments, excluding primers, were compared to sequences in the GenBank database by using the Basic Local Alignment Search Tool (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov). Gene sequences were aligned by using CLUSTAL W software (26), and phylogenetic relationships were inferred from alignments of *ompB* and *gltA* DNA sequences using MEGA 4 software (21) and the parsimony and neighbor-joining methods.

TABLE 1. Collection sites for questing adult Gulf Coast ticks (*A. maculatum*) obtained in Florida and Mississippi from 2005 to 2007 and percentages of ticks infected with spotted fever group rickettsiae

State	County	Year collected	No. of adult ticks tested		No. positive (% of total) for:	
			Males	Females	*R. parkeri*	Novel *Rickettsia* sp.[a]
Florida	Franklin	2005	11	16	3 (11)	1 (4)
		2007	45	47	24 (26)	1 (1)
	Wakulla	2006	8	1	1 (11)	0
Mississippi	Oktibbeha	2007	2	6	2 (25)	0
	Jackson	2007	28	34	25 (40)	3 (5)
Total			94	104	55 (28)	5 (2)

[a] Also designated "*Candidatus* Rickettsia andeanae" (6, 20) and *Rickettsia* sp. Argentina (36).

RESULTS

Frequency of SFGR infections in *A. maculatum*. Molecular evidence of infection with SFGR was obtained for 60 (30%) of 198 adult *A. maculatum* ticks collected at 4 sites in northwest Florida and southeast Mississippi in 2005 to 2007 (Table 1). A novel SFGR was detected using the SYBR green assay in 2 (2%) of 119 ticks collected at THSF and in 3 (5%) of 62 ticks collected at GBNWR (cycle threshold [C_T] values, 19.0 to 21.9). Three (60%) of the 5 ticks containing DNA of the novel SFGR were female. AluI digests of the novel SFGR *ompA* amplicons produced one large and slightly blurred band, consistent with the predicted RFLP pattern for this rickettsia, comprising three closely spaced fragments of 205, 210, and 214 bp.

DNA of *R. parkeri* was detected in each of the 55 remaining ticks that were negative as determined by the SYBR green assay (Table 1). AluI digests of the *ompA* amplicons consistently produced an RFLP pattern unique to *R. parkeri* comprising five distinct bands (55, 86, 124, 153, and 214 bp) (49). Three (11%) of 27 ticks collected at the High Bluff tract in THSF in 2005 and 24 (26%) of 92 ticks collected at the same site in 2007 were infected with *R. parkeri*; 13 (54%) of the infected ticks from this site were female. Twenty-five (40%) of 62 ticks collected at GBNWR in 2007 were infected with *R. parkeri*; 14 (56%) of the infected ticks from this site were female. *R. parkeri* was also detected in smaller collections of ticks from Sopchoppy, FL, in 2006 (11%) and Starkville, MS, in 2007 (25%). No RFLP pattern for the *R. parkeri*-positive or novel SFGR-positive samples suggested that there was simultaneous infection with both *Rickettsia* spp.

Cultivation of *R. parkeri* in Vero E6 cells. Seventeen stable isolates of *R. parkeri* were obtained from individual ticks collected in Florida (Tate's Hell, High Bluff, Sandbank Creek, Longleaf, SR-65, Apalachicola, Cash Bayou, and TH07-94) and Mississippi (Oktibbeha, Moss Point, Bayou Heron, MS07-

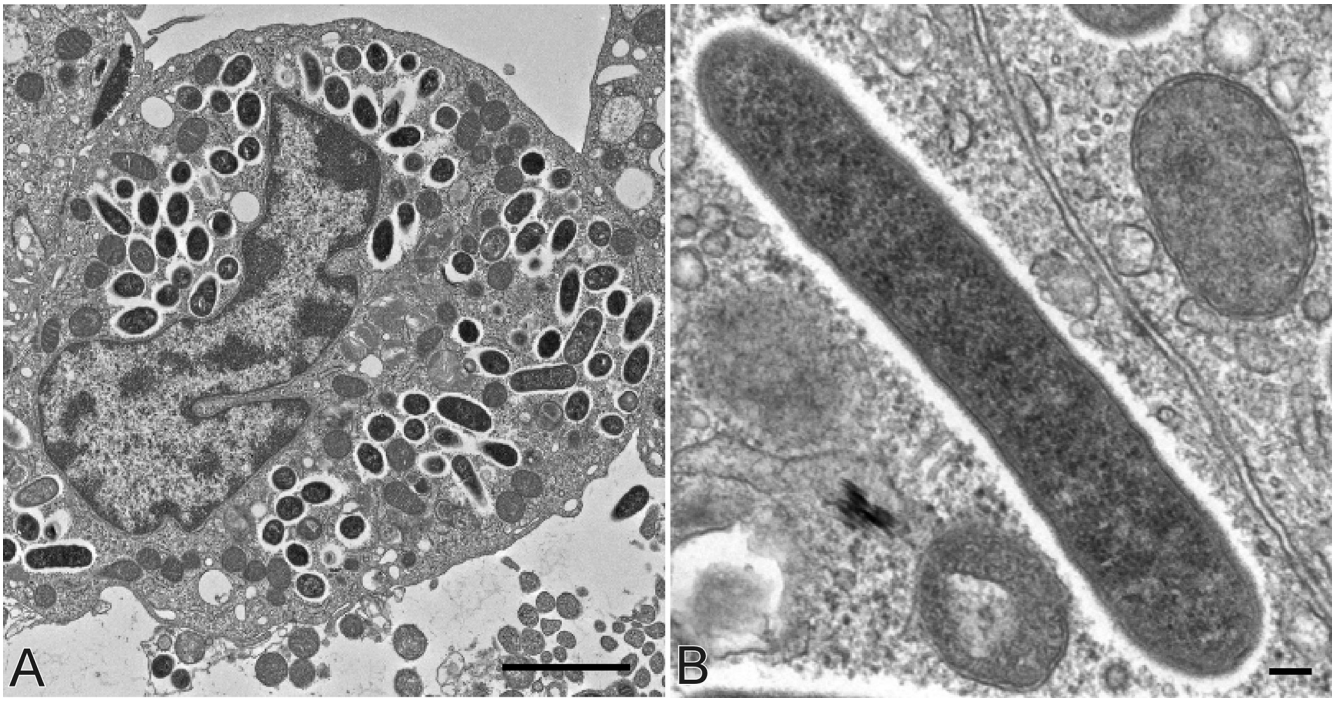

FIG. 1. Electron microscopy of the *R. parkeri* Moss Point isolate in Vero E6 cells stained with uranyl acetate and lead citrate. (A) Multiple intracellular rickettsiae free in the cytoplasm of an infected cell. Bar = 2 μm. (B) Bacillary form of *R. parkeri*, showing an outer electron-lucent halo or "slime" layer adjacent to the central beaded microcapsular layer and the internal trilaminar cell wall. Bar = 100 nm.

44, I-10, Grand Bay, Franklin Creek, Escatawpa, and MS07-22). In most of the primary cultures small rods were detected by acridine orange staining within 4 to 7 days after inoculation of the tick triturate. In cultured cells, *R. parkeri* rickettsiae were distributed freely in the cytoplasm and appeared to be coccoid or rod-shaped bacteria that were 1.0 μm to 2.7 μm long (mean, 1.5 μm; median, 1.4 μm) and 0.2 μm to 0.4 μm wide (mean and median, 0.4 μm) (Fig. 1A) and had an electron-lucent halo or "slime" layer adjacent to the central beaded microcapsular layer and an internal trilaminar cell wall (Fig. 1B). No intranuclear rickettsiae were identified in any of the >500 infected cells examined by electron microscopy.

All attempts to isolate the novel SFGR in Vero E6 cells incubated at 34.5°C were unsuccessful. Similar attempts to cultivate this *Rickettsia* sp. at 32°C using Vero E6 cells and the arthropod cell lines ISE6 and C6/36 were also unsuccessful (data not shown). No isolates of *R. parkeri* were obtained in any cell culture inoculated with a tick infected with the novel SFGR.

Phylogenetic analyses of the novel SFGR. Extracts of ticks containing DNA of the novel SFGR as determined by the SYBR green assay were tested by performing additional PCR assays. Partial sequences of each of the genes evaluated were identical for tick specimens collected in Florida and Mississippi. Complete identity was observed with segments of several other partial *ompA* sequences available from published sources, including sequences amplified from field-collected *A. maculatum* (GenBank accession no. EF372578) reported in our previous study (55), and with an overlapping 402-bp segment of the *Rickettsia* sp. Argentina sequence (GenBank accession no. EF451004) amplified from *Amblyomma parvum* ticks collected in Córdoba Province, Argentina (36). Complete identity was also observed with partial sequences from several unpublished sources, including sequences amplified from colony-reared *A. maculatum* (GenBank accession no. EF524203) and from a field-collected *A. maculatum* tick from Texas (GenBank accession no. EF689729), and with an overlapping 424-bp *ompA* sequence (GenBank accession no. EU826513) amplified from an *Amblyomma pseudoconcolor* specimen collected in northern Argentina. The highest levels of identity of the amplified segment of the *ompA* sequence with corresponding sequences of previously described species of SFG rickettsiae were <95%; these sequences included sequences of *R. massiliae* (GenBank accession no. DQ212707), *R. rhipicephali* (GenBank accession no. U43803), *R. raoultii* (GenBank accession no. DQ365801), *R. montanensis* (GenBank accession no. U43801), *R. aeschlimannii* (GenBank accession no. DQ235777), and "*Candidatus* Rickettsia amblyommii" (GenBank accession no. EF68973). Analysis of the partial *ompB* sequence (GenBank accession no. GU131157) revealed complete identity with the overlapping 725-bp segment of the "*Candidatus* Rickettsia andeanae" sequence (GenBank accession no. AY652981), a sequence detected previously in *A. maculatum* and *Ixodes boliviensis* ticks collected in Peru (6), and ~96% to 97% identity with the overlapping segments of the sequences of several other SFG rickettsiae, including *R. massiliae* (GenBank accession no. CP000683), *R. rhipicephali* (GenBank accession no. AF123719), *R. raoultii* (DQ365798), *R. aeschlimannii* (GenBank accession no. AF123705), and "*Candidatus* Rickettsia amblyommii" (GenBank accession no. FJ455415).

The concatenated sequence of the *gltA* gene (GenBank accession no. GU131156) showed complete identity with an overlap-

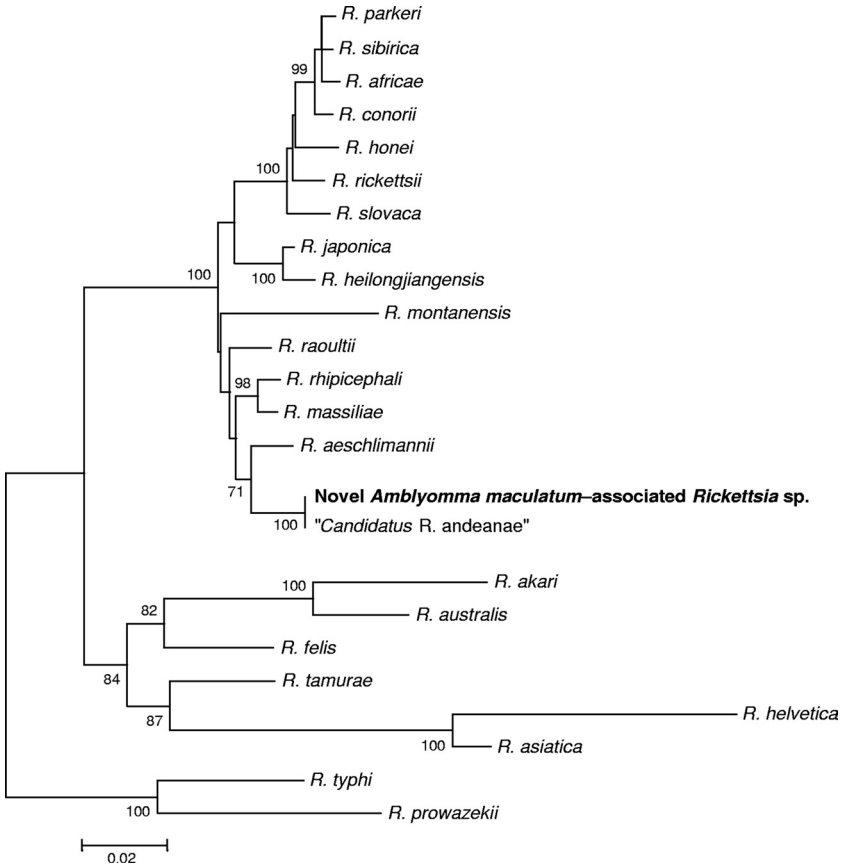

FIG. 2. Unrooted dendrogram showing the phylogenetic position of a novel spotted fever group *Rickettsia* sp. detected in *A. maculatum* ticks, inferred from comparison of 725 nucleotides in the outer membrane protein B gene (*ompB*) sequence aligned with complete or partial *ompB* sequences available in the GenBank database by using the neighbor-joining method. Bootstrap values of >70% for 1,000 replicates are indicated at the nodes. Bar = 2% nucleotide sequence divergence.

ping 1,064-bp segment of the *Rickettsia* sp. Argentina *gltA* sequence (GenBank accession no. EF451001) (36) and an overlapping 1,065-bp segment of the "*Candidatus* Rickettsia andeanae" *gltA* sequence (GenBank accession no. GU169050) and 99% identity with corresponding segments of the sequences of several other SFG rickettsiae, including *R. raoultii* (GenBank accession no. DQ365804), *R. aeschlimannii* (GenBank accession no. DQ235776), and *R. massiliae* (GenBank accession no. CP000683). A 427-bp segment of the *rpoB* gene (GenBank accession no. GU131158) showed 99% identity with overlapping segments of the *rpoB* sequences of many SFG rickettsiae, including *R. massiliae* (GenBank accession no. CP000683) and *R. rhipicephali* (GenBank accession no. AF440728).

Phylogenetic analyses of *ompB* (Fig. 2) and *gltA* (Fig. 3) sequences of the novel SFGR placed this bacterium in a clade of rifampin-resistant *Rickettsia* spp. comprising the *R. massiliae* subgroup (51). This new rickettsia appears to be a unique species (14) and to be identical to three other partially characterized SFG rickettsiae detected previously in at least 4 species of Neotropical ticks collected in Peru and Argentina (6, 20, 36).

DISCUSSION

In this investigation, we obtained 17 isolates of *R. parkeri* from Gulf Coast ticks collected in Florida and Mississippi and identified a second, phylogenetically distinct and as-yet-uncultivable SFGR that resides in *A. maculatum* ticks in the United States and in several other species of ixodid ticks in South America. From 1937 to 1974, investigators cultivated at least 4 stable isolates of *R. parkeri* from Gulf Coast ticks (strains 6-1, C, 62-♀1, and Maculatum 20), which were often obtained from pooled samples comprising multiple specimens collected in Alabama, Mississippi, and Texas (4, 43–45, 48); of these historical isolates, only the extensively passaged Maculatum 20 strain remains in reference collections around the world. The isolates of *R. parkeri* described in this report originated from individual specimens of *A. maculatum* and represent the first strains obtained from Gulf Coast ticks in >35 years; our study greatly increased the number of low-passage, *A. maculatum*-derived isolates of *R. parkeri* recovered from single tick specimens.

Some isolates of other *Rickettsia* species, following serial passage in cell culture or animals, exhibit changes in the genotype or virulence and differ markedly from the original wild-type SFGR (1, 2, 13, 15, 41). The availability of a large panel of minimally passaged isolates of *R. parkeri* that includes South American strains obtained from *Amblyomma triste* (35, 53) and *R. parkeri*-like rickettsiae obtained from *Amblyomma dubitatum* and *Amblyomma nodosum* (23, 34) provides investigators

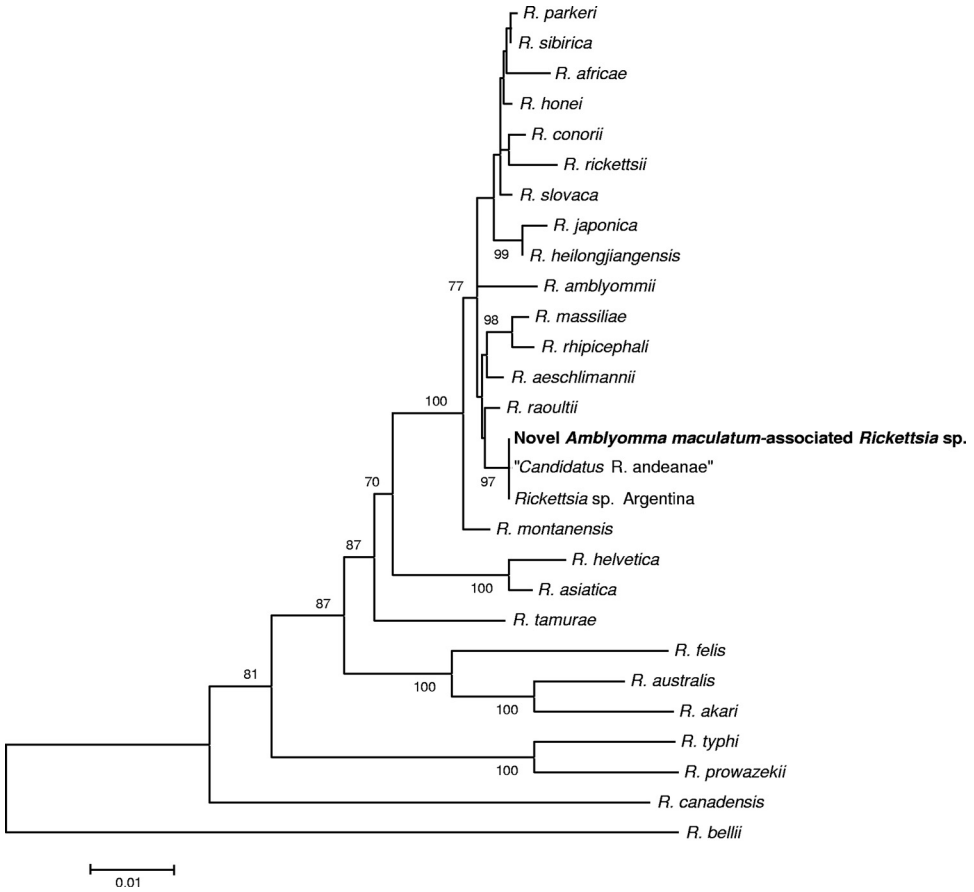

FIG. 3. Unrooted dendrogram showing the phylogenetic position of a novel spotted fever group *Rickettsia* sp. detected in *A. maculatum* ticks, inferred from comparison of 1,095 nucleotides of the citrate synthase gene (*gltA*) sequence aligned with complete or partial *gltA* sequences available in GenBank by using the neighbor-joining method. Bootstrap values of >70% for 1,000 replicates are indicated at the nodes. Bar = 1% nucleotide sequence divergence.

with opportunities to more accurately evaluate the molecular and phenotypic characteristics of this SFGR (2, 46).

The morphological appearance and intracellular location of the tick-derived Moss Point isolate of *R. parkeri* examined in this study were identical to the morphological appearance and intracellular location of the Portsmouth strain, which was isolated from a human skin biopsy specimen, determined previously by electron microscopy (37); however, in neither evaluation were rickettsiae identified in nuclei of Vero E6 cells, despite close inspection of hundreds of infected cells. Previously, investigators using light microscopy reported finding intranuclear *R. parkeri* rickettsiae in yolk sacs of infected chicken embryos (25); nonetheless, we have not found these rickettsiae in the nuclei of infected Vero E6 cells. The capacity of SFG rickettsiae to invade and replicate within host cell nuclei has been documented by electron microscopy for many species, including *R. rickettsii*, *R. japonica*, and *R. africae* (7, 16, 56), and this is considered a defining feature of this group; however, electron microscopy studies of cultured cells infected with other SFG rickettsiae, including "*Candidatus* Rickettsia amblyommii," *R. peacockii*, and *R. raoultii*, have not found intranuclear localization of these rickettsiae (29, 30, 54). The ability of SFG rickettsiae to directionally polymerize host cell actin has been suggested to be a mechanism that is used by these bacteria to penetrate host cell nuclei (18). Nonetheless, *R. parkeri* directionally polymerizes F-actin in Vero cells (18) and exhibits robust intracellular motility in several different cell lines (M. Welch and G. Baldridge, personal communications). Invasion of host cell nuclei by SFG rickettsiae might also depend on the stage of infection or the type of cell infected (3, 30). Additional studies that examine the kinetics and physiology of host cell infection by *R. parkeri* are needed to resolve this paradox.

We identified infections with *R. parkeri* in approximately 10% to 40% of questing adult *A. maculatum* ticks collected at 4 locations in 2 states during a 3-year period. Although precise estimates of the prevalence of infection cannot be obtained from the data, it is evident from this investigation and from our previous survey (55) that *R. parkeri* occurs commonly in widely separated populations of Gulf Coast ticks. Indeed, the consistently high frequency with which this agent is detected in *A. maculatum* ticks is remarkable when it is compared with the occurrence of other pathogenic SFG rickettsiae found in the United States. For example, *R. rickettsii* (the agent of RMSF) is rarely found in >1% of ticks surveyed for this rickettsia, and generally the prevalence is considerably lower (33, 39). In

1955, Philip and White (44) were surprised to discover that no isolates of *R. rickettsii* were obtained from any of >3,200 *Amblyomma*, *Dermacentor*, and *Ixodes* spp. tick specimens collected in Jackson County, MS, despite multiple reports of serologically confirmed "RMSF" in this county during the previous 10 years. In contrast, these investigators repeatedly isolated *R. parker*i from pooled specimens of *A. maculatum* collected at several sites in the same county.

During a previous survey (55), we detected a unique *ompA* sequence in specimens of *A. maculatum* collected in several southeastern states that suggested that there is a *Rickettsia* species that was not recognized previously in the United States. During the present investigation, we identified the same unique *ompA* sequence in Gulf Coast tick specimens collected at additional sites, and analyses of other genes of this *Rickettsia* sp. revealed identity to several recently deposited sequences amplified from various *Amblyomma* tick species in South America, including sequences of "*Candidatus* Rickettsia andeanae" from *A. maculatum* (6, 20), *Rickettsia* sp. Argentina from *A. parvum* (36), and an unidentified SFGR from *A. pseudoconcolor*. By evaluating multiple gene targets and comparing identical regions of the gene of interest, identities could be established in order to collectively link these sequences to a common SFGR; however, full species status for this novel SFGR requires cultivation and more thorough genomic characterization.

Other *Rickettsia* spp. and rickettsia-like bacteria detected in ticks have proven to be difficult to cultivate. For many years, attempts to isolate *R. peacockii*, an endosymbiont associated with *Dermacentor andersoni*, were unsuccessful; these attempts included inoculation of guinea pigs, meadow voles, mice, embryonated chicken eggs, and at least 7 different cell lines (8, 32). *R. peacockii* was eventually cultivated when investigators established a cell line from embryonic tissues of laboratory-reared *D. andersoni* (DAE100) that was persistently infected with *R. peacockii* (54). Cell-free *R. peacockii* obtained from persistently infected DAE100 cell cultures can be propagated in many other cell lines of arthropod origin (22); however, a primary isolate of *R. peacockii* has not been obtained by using any cell line other than DAE100. Our efforts to isolate the novel SFGR in broadly permissive cell lines of primate, mosquito, or tick origin were unsuccessful; cultivation of this novel SFGR may eventually require a strategy similar to that used to obtain *R. peacockii* in continuous culture. It is also possible that the disinfectants used to treat the tick exoskeleton penetrated the integument and inactivated the novel SFGR. The disinfection protocol was required to prevent fungal overgrowth, which consistently hampered our previous attempts to isolate SFG rickettsiae from *A. maculatum* ticks when lower concentrations of the antimicrobial solutions were used. Nonetheless, we were able to obtain 17 consecutive isolates of *R. parkeri* from other ticks prepared in the same way, suggesting that our disinfectant protocol did not affect the viability of rickettsiae within the tissues of these ticks.

R. parkeri and the novel SFGR occur sympatrically in populations of Gulf Coast ticks in the southeastern United States (55; this study); however, we did not detect simultaneous infections with both of these rickettsiae in any of ~200 ticks evaluated in our study. Naturally acquired coinfection of ticks with multiple *Rickettsia* spp. has been described infrequently (9, 58). Laboratory studies have suggested that *Dermacentor variabilis* and *D. andersoni* ticks are unable to transovarially maintain infections with more than one SFGR; i.e., ovarian infection with the primary infecting species blocks subsequent vertical transmission of a second infecting *Rickettsia* sp. (8, 28). However, it may be possible for a transovarially infected tick to acquire a different *Rickettsia* sp. during feeding and maintain both agents horizontally through the nymphal and adult stages (8). These data and the apparent infrequency with which the novel SFGR occurs in *A. maculatum* specimens in several southeastern states compared to the frequency of infection with *R. parkeri* (55; this study) suggest that *R. parkeri* establishes residence in Gulf Coast ticks more successfully and may limit the number of infections with the novel SFGR by transovarial interference; nonetheless, there are currently no experimental data that demonstrate that either *Rickettsia* sp. is able to exclude infection by the other organism. Similarly, nothing is known about the impact of either *Rickettsia* sp. on the fitness of infected *A. maculatum* ticks. Considerable work to examine these interactions and the pathogenic potential, if any, of the novel SFGR remains.

For almost 100 years, *A. maculatum* has been recognized as an aggressive human-biting tick (19). Surprisingly, the only direct association between Gulf Coast ticks and human health prior to 2004 was an isolated case of tick paralysis (40). It now appears that *R. parkeri* and a newly recognized SFGR are distributed widely in *A. maculatum* and many other *Amblyomma* sp. ticks in the Western Hemisphere (6, 10, 17, 20, 31, 35, 36, 53, 55). Studies that better characterize the phenotypic characteristics, microbiology, and ecology of *R. parkeri* and this novel SFGR will undoubtedly provide greater perspective to these initial discoveries.

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