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Original article

Multi-locus phylogenetic analysis groups the New World bacterium *Rickettsia* sp. strain ApPR with the Old World species *R. africae*; proposal of "*Candidatus* Rickettsia paranaensis"

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ABSTRACT

Rickettsia parkeri sensu stricto (s.s.) is an emerging human pathogen in the Americas. Comprehension of the etiology of *R. parkeri* infections in South America is complicated by the existence of genetic variants (Atlantic rainforest, NOD and Parvitarsum) of this species that are associated with specific groups of *Amblyomma* ticks. The rickettsial bacterium strain ApPR was first reported in *Amblyomma parkeri* ticks in Southern Brazil in 2012 and was considered, based on sequencing of fragments of the *gltA, htrA*, *ompA* and *ompB* genes, to represent yet another genetic variant of *R. parkeri*. In the current work, a multi-locus phylogenetic analysis employing additional genes and intragenic regions was performed using DNA extracted from (a) larvae of *A. parkeri* and *Amblyomma* species haplotype Nazaré ticks collected from wild birds, (b) a nymph of *Amblyomma* sp. haplotype Nazaré recovered from a monkey (*Callicebus nigrifons*), representing the first report of that tick parasitizing a non-human primate and (c) from a cultured isolate of ApPR, isolated from colony-reared adults of *Amblyomma geayi*. Phylogenetic inference performed using Maximum-likelihood (ML), Maximum Parsimony (MP) and Bayesian (B) methods, consistently placed strain ApPR outside the New World *R. parkeri* complex and instead grouped it in proximity to the Old World species *Rickettsia africae* and *Rickettsia sibirica*. Estimates of evolutionary divergence provided additional support for the inferred phylogenetic relationship. Given the clear evolutionary distance between strain ApPR and *R. parkeri* we propose the recognition of "*Candidatus* Rickettsia paranaensis".

1. Introduction

Rickettsia parkeri, a member of the spotted fever group (SFG) of *Rickettsia* (Parker et al., 1939), is considered to represent an emerging pathogen in the New World, specifically in both North and South America (Paddock et al., 2010; Herrick et al., 2016; Romer et al., 2011; Spolidorio et al., 2010; Krawczak et al., 2016a). In addition, genetically related species including *R. africae* and *R. sibirica* are recognized as agents of rickettsial disease in humans in regions of the Old World including Africa, Europe, and Asia (de Sousa et al., 2006; Kelly et al., 1994; Parola et al., 2013). Moreover, the presence of *R africae* was reported on multiple islands of the Caribbean (Kelly et al., 2010). Infection with *R. parkeri*, is associated with a milder form of rickettsial spotted fever than that caused by *Rickettsia rickettsii*, being characterized by the development of an eschar at the site of the tick bite, followed by the appearance of a rash (sparse maculopapular or papulovesicular eruptions on the trunk and extremities) and fever that may reach 40 °C (Paddock et al., 2004; Silva et al., 2011; Faccini-Martínez et al., 2014). Diagnosis of infections with such eschar-forming rickettsiae is commonly achieved by employing molecular biology-based analyses, specifically polymerase chain reaction (PCR) and nucleotide sequencing of DNA extracted from cutaneous swabs and/or biopsy material collected from the eschar (Paddock et al., 2004; Bechah et al., 2011; Silva et al., 2011; Romer et al., 2014).

In North America, the tick *Amblyomma maculatum* is recognized as the principal vector of *R. parkeri* sensu stricto (s.s.) (Paddock et al.,

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2010; Herrick et al., 2016). However, in South America the etiology of *R. parkeri* infections of humans is more complex and involves a variety of tick species including, *Amblyomma ovale* and possibly *Amblyomma aureolatum* as vectors of *R. parkeri* strain Atlantic rainforest in Brazil and Argentina (Szabó et al., 2013; Barbieri et al., 2014; Nieri-Bastos et al., 2016; Lamattina et al., 2018), together with *Amblyomma triste* and *Amblyomma tigrinum* (both members of the *A. maculatum* complex), as vectors of *R. parkeri* s.s. in Argentina and Uruguay (Venzal et al., 2004; Pacheco et al., 2006; Nava et al., 2008; Lado et al., 2014; Romer et al., 2014). In Brazil, *R. parkeri* s.s. has been associated principally with *A. triste* populations sustained by marsh deer but has not been linked to any confirmed cases of human disease in that country (Nieri-Bastos et al., 2018).

Until recently, the taxonomic status of the Atlantic rainforest strain was undetermined as were those of some additional New World *R. parkeri*-like bacteria (strain NOD, strain ApPR and strain Parvitarsum), that were characterized as such based upon sequencing of a limited number of gene fragments and which were reported as infecting various species of *Amblyomma* ticks. Specifically, strain NOD was detected principally in *A. nodosum* in Brazil (Ogrzewalska et al., 2009; Witter et al., 2016), while strain ApPR was detected in *Amblyomma parkeri* and an uncharacterized species of *Amblyomma* (*Amblyomma* sp. haplotype Nazaré), also in Brazil (Pacheco et al., 2012; Ogrzewalska et al., 2012; Zeringóta et al., 2017; Luz et al., 2017). The Parvitarsum strain, has never been recorded in Brazil but was reported in *Amblyomma parvitarsum* ticks collected in Chile, Argentina and Peru (Ogrzewalska et al., 2016; Muñoz-Leal et al., 2017). To resolve this lacuna, a multi-sequence phylogenetic analysis was undertaken to determine the relationships between numerous representatives of Old World and New World *Rickettsia* within the *R. parkeri* complex, with the notable exception of the ApPR strain (Nieri-Bastos et al., 2018). The results of that analysis revealed a clear distinction between the Old World species and the New World members of the *R. parkeri* complex and confirmed that Atlantic rainforest, NOD and Parvitarsum are indeed South American strains of *R. parkeri*. The omission of the ApPR strain from the phylogenetic analysis was motivated principally by the lack of a cell-culture derived isolate of the bacterium. That situation was resolved by the isolation in cultured Vero cells, as reported in the current study, of a representative of the strain ApPR from the tick *Amblyomma geayi*. Interestingly, *A. geayi* is a member of the *Amblyomma longirostre* and *A. parkeri* species group (Labruna et al., 2009; Martins et al., 2013), which most likely also includes *Amblyomma* sp. haplotype Nazaré (Ogrzewalska et al., 2012; Zeringóta et al., 2017).

Herein, we report the execution of a multi-locus, phylogenetic analysis similar to that reported by Nieri-Bastos et al. (2018) but with the inclusion of sequences derived from the *A. geayi* isolate of *Rickettsia* sp. strain ApPR and of PCR amplicons generated from homogenated ticks (*A. parkeri* and *Amblyomma* sp. haplotype Nazaré), collected from birds and a non-human primate (*Callicebus nigrifons*), in southeastern Brazil. The results of the analysis demonstrated that, contrary to previous assertions, the ApPR bacterium is not a strain of *R. parkeri* and that instead it grouped with the Old World species clade, comprising *R. africae* and *R. sibirica*. The significance of this novel finding is discussed and the name "*Candidatus* Rickettsia paranaensis" is proposed herein to clearly distinguish strain ApPR from *R. parkeri*.

2. Materials and methods

2.1. Tick collection and identification

Two females of *A. geayi*, identified morphologically using the methods reported by Barros-Battesti et al. (2006), were collected live from a specimen of the sloth (*Bradypus variegatus*) in the municipality of Belém (1° 45′ 23″ S – 48° 19′ 43″/altitude 50 m), in the state of Pará, eastern Brazilian Amazon, in 2015. The ticks were transported to the Department of Parasitology (DPA), at the Federal Rural University of Rio de Janeiro (UFRRJ), where they were placed into 10 ml plastic vials plugged with cotton wool and maintained in a Biological Oxygen Demand (BOD) incubator at 27 ± 1 °C and with 85% relative humidity (RH). Larvae and nymphs derived from the females were fed on tick-naïve New Zealand rabbits (*Oryctolagus cuniculus*) and subsequently maintained under the same conditions as described above. Four unfed first-generation nymphs were tested for molecular detection of rickettsia, as described below. A total of five females and four males obtained from the second generation of the laboratory colony were transported live to the Department of Preventative Veterinary Medicine and Animal Health at the University of São Paulo (USP) in July of 2016, where they were frozen at −80 °C for subsequent attempts at isolation of rickettsial agents, as described below.

A larva of *A. parkeri* (sample N24) was collected from a passerine bird *Turdus albicollis* in the Itatiaia National Park (INP) in 2015 and two larvae of *Amblyomma* sp. haplotype Nazaré (samples S56 and S72) were collected from the avian species *Xiphorynchus fuscus* and *Sittasomus griseicapillus* in the INP in 2016 and 2017, respectively. Details of the capture methods were as reported by Luz et al. (2017). Eight nymphs of *Amblyomma* sp. haplotype Nazaré were collected from a monkey (*Callicebus nigrifrons*), captured by staff of The Wildlife Rescue Center (IEF-MG/IBAMA), in the municipality of Barbacena, Minas Gerais state in 2014, DNA extracted from one of the nymphs was used in this study. All ticks were identified to the species level through a combination of morphological and molecular methods (sequencing of fragments of the genes encoding 16S and 12S mitochondrial ribosomal RNA), as detailed in Luz et al. (2017).

2.2. Isolation of Rickettsia in Vero cells

Unfed, frozen adults of *A. geayi* were thawed and processed by the shell-vial technique, as previously described (Labruna et al., 2004). Briefly, cultures of Vero cells were inoculated with tick-body homogenates, centrifuged, and incubated at 28 °C. The percentage of Vero cells infected with *Rickettsia* was monitored periodically by Giménez staining of cells scraped from each inoculated monolayer. Following the establishment of the isolate in the laboratory (i.e. at least 3 cell passages, with the prevalence of infected cells exceeding 90%), rickettsial DNA was extracted from the infected cells and used for molecular characterization, as described below.

2.3. DNA extraction, PCR amplification and sequencing of Rickettsia gene fragments

DNA was extracted from ticks using the bead-beater phenol chloroform method reported by Santolin et al. (2013), while DNA from the cultured bacteria was extracted using the guanidine isothiocyanate phenol technique (Sangioni et al., 2005). Purified DNA was initially examined using PCR assays targeting fragments of the rickettsial genes *gltA*, *htrA*, *ompA* and *ompB*, using the primers and cycling conditions reported in Zeringóta et al. (2017). DNA derived from *R. parkeri* (strain Atlantic rainforest-A010), was used as positive amplification control in all assays. Negative controls, comprised reactions where molecular biology grade water was included as template. Sequencing of amplicons, followed by submission to the BLAST nucleotide search algorithm, confirmed that the ticks and the cell cultures were infected with stain ApPR. Thereafter, the DNA extracts were used as template in the PCR assays reported by Nieri-Bastos et al. (2018), for the targets *gltA*, *ompA*, *virB4*, *dnaA*, *dnaK*, *rpmE-tRNA*, *rrl-rrs-ITS* and *mppA-purC* with some modifications. Firstly, new primers were designed for the amplification of the *rrl-rrs-ITS* and *mppA-purC* genomic regions (Table 1), to overcome inconsistent results with those targets for most of the tick homogenates. Secondly, a touch-down approach was used to resolve problems of non-specific amplification, noted for some homogenates, in the assays targeting *dnaK* and *rrs-rrf-ITS*. The cycling conditions employed therein, were as follows: initial denaturation and polymerase activation at 94 °C

Table 1

Primers designed in this study for the amplification of the *rrl-rrs-ITS* and *mppA-purC* genomic regions using DNA recovered from tick homogenates as templates.

Primer name	Target	Expected product length (bp)	Nucleotide sequence (5'–3')
rrs-rrf-ITS F	23S-5S ribosomal RNA intergenic spacer	554	5’-GGTAGCTAAGTACGGAAGGG-3’
rrs-rrf-ITS R			5’-CAGCGACTTACTCTCCCATG-3'
mppA F	mppA-purC intergenic spacer	551	5'-CAAATGGCTCAAGAGAAAAA-3'
mppA R			5'-TTTTCAATGCCGATCATTTC-3'

for 5 min, followed by 10 cycles at 94 °C for 20 s, 56 °C for 20 s and 72 °C for 30 s, with an additional 30 cycles at 94 °C for 20 s, 52 °C for 20 s and 72 °C for 30 s with a final extension at 72 °C for 5 min. The PCR mixtures (12.5 μL) contained 1.25 μL of Platinum Taq DNA polymerase buffer (Thermo Fisher Scientific, Brazil) $MgCl_2$ (final concentration 2.5 mM), 200 μM dNTPs, 10 *p*moles of each primer, 0.5 units of Platinum Taq DNA polymerase, and 2 μL of DNA template.

2.4. Sequencing and phylogenetic analyses

Nucleotide sequencing of PCR products, in both directions, was performed employing the amplification primers and the BigDye Ready Reaction mix (ABI Corp). The reaction products were analysed on a 3500-automated genetic analyzer (ABI Corp). Sequence alignments were performed using Sequencher (Version 5.4.4, Genecodes Corporation, CA). Aligned sequences were entered into the BLAST search algorithm (Altschul et al., 1990) and the NCBI nucleotide database to determine gene identity.

A multi-locus sequence analysis (MLSA) was performed using a concatenated approach with the gene fragments described above to determine phylogenetic relationships, as reported by Nieri-Bastos et al. (2018), albeit with the data set updated to include *Rickettsia* sp. strain ApPR. Two strategies were employed to concatenate the different gene fragments. In the manual approach, each fragment was aligned separately and then, after trimming, gene fragments were assembled to create the concatenated sequence. In the second strategy, concatenation was performed automatically using the software Mesquite 2.75 (Maddison and Maddison, 2018). The representatives of the *Rickettsia* sp. strain ApPR selected for use in the phylogenetic analysis were (1) the ApPR isolate cultured from *A. geayi*, (2) strain ApPR detected in *A. parkeri* (homogenate N24) and (3) strain ApPR detected in *Amblyomma* sp. haplotype Nazaré (homogenate S56).

Phylogenetic inference was performed sequentially beginning with Maximum Parsimony (MP), then Maximum-likelihood (ML), and terminating in the use of Bayesian (B) methods with sequences produced using both concatenation strategies. The robustness of the nodes in the ML and MP trees was estimated through Bootstrap analyses of 1000 replicates employing the MEGA software version 6.0 (Tamura et al., 2013). Divergence between and within groups was calculated based on p-distance, employing the MEGA software version 6.0. Bayesian analysis was performed using the MrBayes v.3.1.2 software (Ronquist and Huelsenbeck, 2003) with 1,000,000 generations, using GTR as a substitution model.

3. Results

The adult ticks collected from the sloth (*B. variegatus*) were identified as *A. gaeyi* based on morphological characters. Sequencing of fragments of 16S and 12S rRNA from the larva (N24), identified it as a specimen of *A. parkeri* based on a level of 100% nucleotide identity (410/410 bases), to the partial 16S sequence JN573300 and the same level of identity (314/314 bases) to the partial 12S sequence EU805551 deposited in the GenBank as pertaining to *A. parkeri* collected in Brazil. The nucleotide sequences of the 12S and 16S rRNA gene fragments amplified from the larvae S56 and S72, were identical to those generated from the nymph parasitizing the monkey *C. nigrifrons* and showed 100% identity to the sequences KU953954 (16S rDNA) and KU953956 (12S rDNA), deposited in the GenBank as derived from larvae of *Amblyomma* sp. haplotype Nazaré collected from wild birds in Minas Gerais state, Brazil.

Sequencing of PCR amplicons (*gltA*, *htrA*, *ompA* and *ompB*) generated from 4 colony-reared nymphs of *A. gaeyi*, originating from the sloth-associated females used to establish our laboratory colony, demonstrated that they were infected with the *R. parkeri*-like bacterium, strain ApPR. The same observation was made for the *A. parkeri* and *Amblyomma* sp. haplotype Nazaré larvae collected from wild birds and for the *Amblyomma* sp. haplotype Nazaré nymph collected from the non-human primate *C. nigrifrons*. The nymph was one of eight specimens recovered from *C. nigrifrons*. Six of the eight nymphs were examined for the presence of rickettsial DNA and all were found to be positive, the remaining two nymphs were left unprocessed. Sequencing of fragments of the genes *gltA, ompA* and *ompB* revealed 3/6 ticks to be infected with the ApPR strain and the other 3 ticks to be infected with *R. rhipicephali*. In all cases, the novel sequences showed 100% identity to the same fragments reported in strain ApPR and *R. rhiphicephali* detected by PCR in haplotype Nazaré ticks recovered from birds in the states of Minas Gerais and Rio de Janeiro, southeastern Brazil (Zeringóta et al., 2017; Luz et al., 2017).

Vero cells were successfully infected using the shell vial technique, employing the ApPR bacterium present in homogenates of second-generation adults of *A. geayi* as the inoculum. Sequence analysis of PCR-generated fragments of *gltA*, *htrA*, *ompA* and *ompB,* showed the cultured isolate to be identical to the bacterium present in the previously examined *A. geayi* nymphs.

The design of modified primers and PCR protocols, as described in the Material and methods section, provided solutions for problems of non-specific amplification encountered when using tick homogenates as template and permitted the successful amplification of the target sequences required to perform the phylogenetic analysis. Sequences obtained from the strain ApPR isolate recovered from *A. geayi* were deposited in the GenBank with the following accession numbers: MK166034 (*gltA*), MK166035 (*ompA*), MK166036 (*virB4*), MK166037 (*dnaA*), MK166038 (*dnaK*), MK166039 (*mppA-purC), *MK166040 (*rrl-rrs-ITS)* and MK166041 (*rpmE-tRNA*) and were used as the basis for analysis of inter-isolate variation. The sequences of the cultured isolate were identical to those derived from amplicons generated using DNA from the homogenized *A. geayi* F_1 nymphs as template. Sequences obtained from the bacteria present in the N24 tick homogenate (*A. parkeri*) were identical to the sequences from the *A. geayi* isolate, with the exception of a single nucleotide polymorphism (SNP) at nucleotide 629 (G substituted with A), in the fragment of the *dnaA* gene that resulted in a non-synonymous mutation, whereby the altered codon would be translated into methionine in place of isoleucine. The sequences produced using DNA extracted from the three distinct *Amblyomma* sp. haplotype Nazaré ticks were identical and the SNP identified in N24 was not found in those DNA’s. However, a SNP (G to A; resulting in a synonymous mutation) was recorded in the *ompA* fragment at nucleotide position 158. In addition, a substitution of C for T was recorded at nucleotide position 229 in the *rrl-rrs-ITS* intergenic sequence. The polymorphic sequences were deposited in the GenBank with the following accession numbers; MK166033 (*dnaA)*, MK253743 (*ompA*) and MK166032 (*rrl-rrs-ITS*).

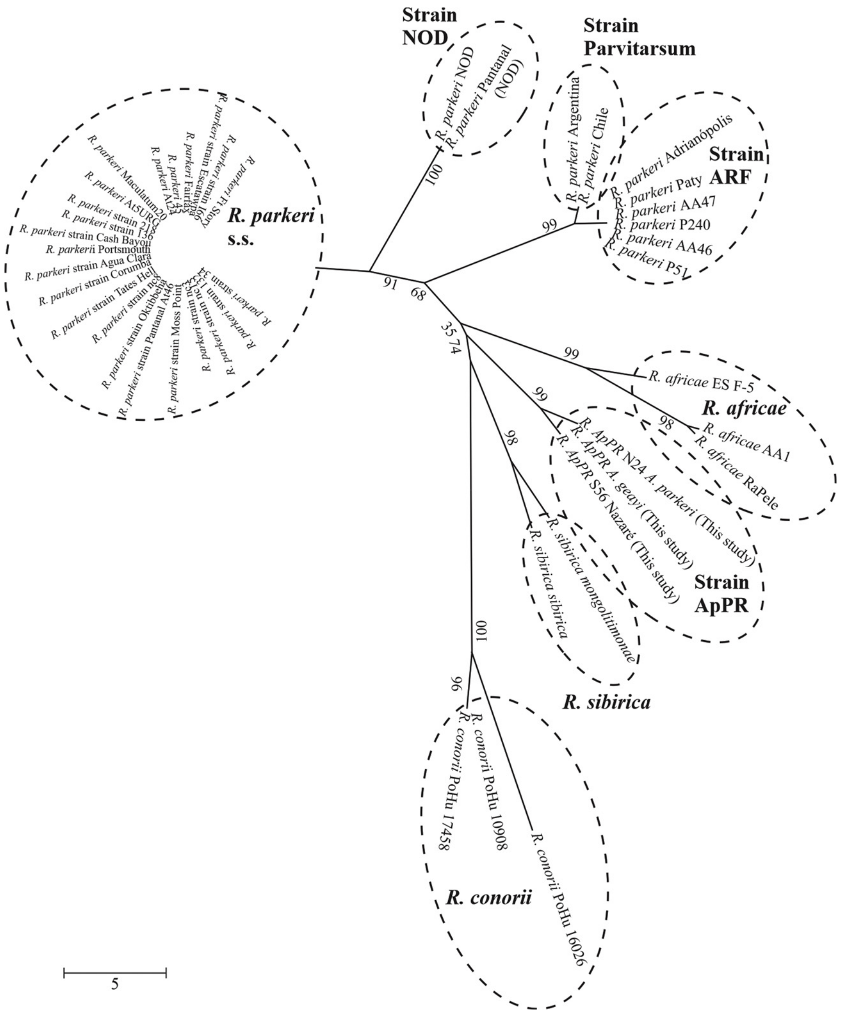

Fig. 1. Phylogenetic tree, presented in the radiation format and produced using the Maximum Parsimony method, employing concatenated sequences of 3589 aligned nucleotide composed of fragments of the *gltA, ompA, virB4, dnaA, dnaK, mppA-purC, rrl-rrs-ITS* and *rpmE-tRNA* PCR gene fragments. Tree #1 out of 8 most parsimonious trees (length = 114) is shown. The consistency index is (0.846154), the retention index is (0.966981), and the composite index is 0.848229 (0.818215) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm, with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in units of the number of changes over the whole sequence.

Phylogenetic analysis, using three distinct approaches, consistently grouped the ApPR isolates as a single cluster positioned between the two Old World species *R. africae* and *R. sibirica*, within a major cluster with *R. conorii* strains. No differences were observed between the trees obtained using sequences that were manually or automatically concatenated. The existence of the inferred relationship between ApPR, *R. africae* and *R. sibirica* was initially noted in the maximum parsimony (MP) tree (Fig. 1), albeit with limited support based upon low bootstrap values. The data from maximum likelihood (ML) analysis (Fig. 2), afforded increased confidence in the robustness of the observed groups, with the nodes supported by superior bootstrap values. Conclusive evidence for the inferred relationship was provided using Bayesian analysis (Fig. 3), wherein support values of 100 (derived from posterior probability), placed each taxonomic entity -*R. sibirica, R. africae, R. conorii* and ApPR- within its own separate clade. The validity of our findings was further supported by the placement of the New World *R. parkeri* (strains Atlantic rainforest, NOD, Parvitarsum, and *R. parkeri* s.s from South and North America) into the same branchs as were reported by Nieri-Bastos et al. (2018).

Analysis of genetic divergence was conducted as an adjunct to the phylogenetic analyses. The divergence values calculated when the concatenated sequences of ApPR were compared to the Old World species and to the New World variants of *R. parkeri* are presented in Table 2. The lowest divergence value (0.49%) was recorded for strain NOD. The other two South American variants of *R. parkeri* (Atlantic rainforest and Parvitarsum) showed the same divergence value (0.56%), which was similar to those recorded for the Old World species *R. africae* (0.54%) and *R. sibirica* (0.51%). A greater degree of divergence (0.63%) was noted between ApPR and the *R. parkeri* s.s. isolates from North and South America (Table 2). In concordance with the findings of the phylogenetic analyses, the divergence values recorded between the New World *R. parkeri* isolates and the Old World species *R. africae* (range 0.74%–0.66%) and *R. sibirica* (range 0.71%–0.62%) were larger than the values observed for ApPR (0.51%–0.54%). The divergence values recorded between *R. conorii* and the other rickettsiae were significantly higher, ranging from a minimum of 0.90% for strain ApPR, through to a maximum of 1.13% in comparison to the *R. parkeri* s.s. isolates (Table 2).

4. Discussion

Previous molecular detection and characterization of the ApPR strain was reported using homogenates of *A. parkeri* and *Amblyomma* sp. haplotype Nazaré larvae collected from passerine birds in the states of Paraná (Pacheco et al., 2012), São Paulo (Ogrzewalska et al., 2012), Minas Gerais (Zeringóta et al., 2017), and Rio de Janeiro (Luz et al., 2017), using destructive DNA extraction techniques which precluded the isolation of the bacterium in cell culture. In the absence of a cultured isolate, the ApPR strain was not included in the recently reported phylogenetic analysis of South American strains of *R. parkeri*-like agents (Nieri-Bastos et al., 2018). Herein, we report the successful isolation of strain ApPR from adults of *A. geayi*. The decision to culture this isolate was motivated by preliminary PCR and sequencing data that revealed the presence of ApPR DNA in nymphs originating from females of *A. geayi*, collected from a sloth in Pará state, in the Brazilian Amazon. This unprecedented association represented only the third report of a SFG

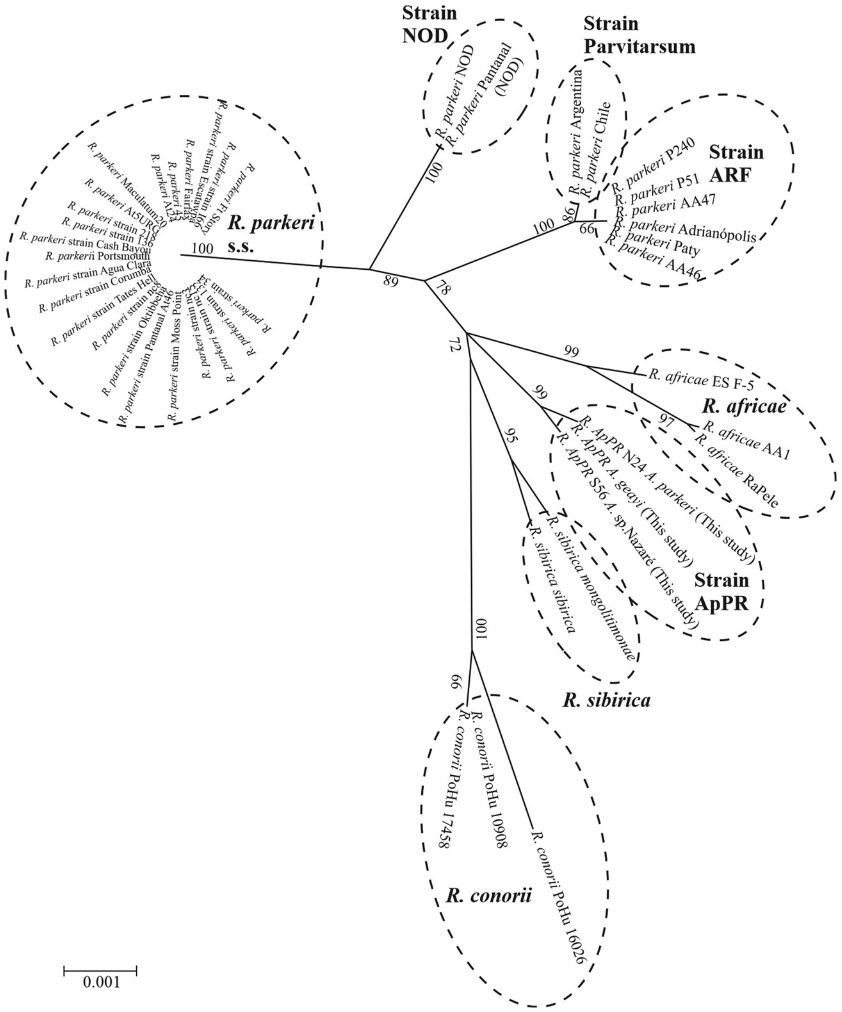

Fig. 2. Phylogenetic analysis of the *Rickettsia* species (strain ApPR), in relation to Old World isolates (*Rickettsia africae, Rickettsia sibirica,* and *Rickettsia conorii*) and New World *Rickettsia parkeri* variants (*Rickettsia parkeri* sensu stricto (s.s), strains Atlantic rainforest, NOD, and Parvitarsum), using the Maximum Likelihood method, based on the Tamura 3-parameter model employing a discrete Gamma distribution (+G) and using concatenated sequences of 3589 aligned nucleotide composed of fragments of the *gltA, ompA, virB4, dnaA, dnaK, mppA-purC, rrl-rrs-ITS* and *rpmE-tRNA* PCR gene fragments. The tree is drawn to scale with branch lengths measured in the number of substitutions per site.

Rickettsia in *A. geayi*, wherein the previous two reports were both of *Rickettsia amblyommatis* in immature specimens of *A. geayi* collected from wild birds in the Amazonian states of Pará and Acre (Ogrzewalska et al., 2010; Lima et al., 2018). The ApPR strain was first reported in a single *A. parkeri* tick collected from a wild bird in the southern state of Paraná (Pacheco et al., 2012), with subsequent records in *A. parkeri* collected from birds in the Southeastern states of São Paulo (Ogrzewalska et al., 2012) and in numerous specimens of *A. parkeri* and *Amblyomma* sp. haplotype Nazaré collected from passerine birds in Minas Gerais (Zeringóta et al., 2017) and Rio de Janeiro (Luz et al., 2017). Importantly, the novel record extends the geographic range of this rickettsial agent to the Amazonian biome in Northern Brazil.

Initial molecular analyses of the F_1 nymphs collected from within the laboratory colony of *A. geayi* demonstrated that 4 of 4 ticks were positive for the presence of the ApPR strain, suggesting that this agent was effectively perpetuated transovarially and transstadially. Although limited to a small number of ticks, the high level of positivity found for *A. geayi* mirrored earlier observations of elevated levels (up to 70%), of infection with ApPR in populations of *A. parkeri* and *Amblyomma* sp. haplotype Nazaré ticks collected from passerine birds (Zeringóta et al., 2017; Luz et al., 2017) suggestive of a symbiotic relationship between the arthropod hosts and the bacterium. Analysis of adults of *A. parvitarsum* collected in Argentina and Chile (Ogrzewalska et al., 2016), also revealed a substantial (62%), level of infection with *R. parkeri* strain parvitarsum. Interestingly, the frequency of intra-population infection in ticks carrying Brazilian strains of *R. parkeri* was generally lower, e.g. (5–10%) for the human pathogens *R. parkeri* s.s. in *A. triste* and *R. parkeri* strain Atlantic rainforest in *A. ovale* (Silveira et al., 2007; Luz et al., 2016; Nieri-Bastos et al., 2016). The characteristic of low-level, intra-population infection rates as reported for *R. rickettsii* and *R. conorii*, is believed to reflect negative impacts of human/animal pathogenic species of *Rickettsia* upon their tick hosts (Socolovschi et al., 2009, 2012). In fact, negative impacts of either *R. parkeri* s.s. or *R. parkeri* strain Atlantic rainforest have been observed upon their natural tick vectors, *A. triste* and *A. ovale,* respectively (Nieri-Bastos et al., 2013; Krawczak et al., 2016b).

The choice of the specimens used in the phylogenetic analysis, reflected our intention to evaluate a broad range of features with potential to impact upon genetic variability namely; host (bird, non-human primate or sloth), geographic location (Minas Gerais, Rio de Janeiro, and Pará), tick species (*A. geayi*, *A. parkeri* and *Amblyomma* sp. halotype Nazaré) and year of collection (2014–2017). The ticks N24 (*A. parkeri*), S56 and S72 (both *Amblyomma* sp. haplotype Nazaré) were collected from birds in an area of primary Atlantic rainforest within the Itatiaia National Park (INP), as part of follow-up survey to the study of Luz et al. (2017), that had revealed an abundant diversity of ticks and rickettsial agents within that habitat. The nymph of *Amblyomma* sp. haplotype Nazaré recovered from the non-human primate in Minas Gerais, was one of eight specimens recovered from that host and represented the first report of that tick parasitizing non-human primates, a feature shared by the related species *A. parkeri* (Martins et al., 2013).

Based on the sequencing of a limited number of genes, the ApPR strain was initially reported as representing a novel genotype of South American *R. parkeri* (Pacheco et al., 2012). The multi-locus analysis performed herein, rejected that assertion and instead showed the strain ApPR to be more closely related to the Old World species of *Rickettsia*.

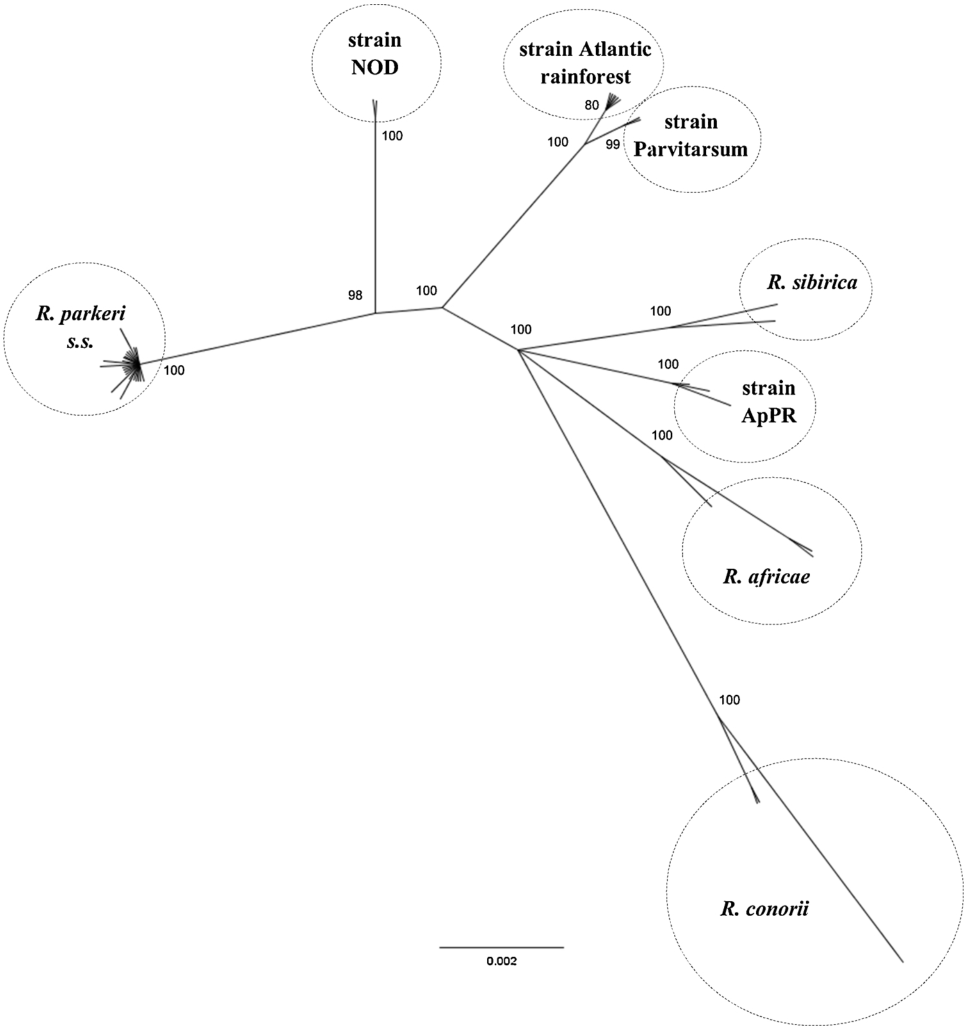

Fig. 3. Phylogenetic analysis of the *Rickettsia* species (strain ApPR), in relation to Old World isolates (*Rickettsia africae, Rickettsia sibirica,* and *Rickettsia conorii*) and New World *Rickettsia parkeri* variants (*Rickettsia parkeri* sensu stricto (s.s), strains Atlantic rainforest, NOD, and Parvitarsum), based on Bayesian inference using a concatenated sequence of 3589 aligned nucleotide sites composed of fragments of the *gltA*, *ompA, virB4, dnaA, dnaK*, genes and the intergenic spacers *mppA-purC, rrl-rrs-ITS* and *rpmE-tRNA*. Numbers at nodes are support values derived from posterior probability. The scale bar is in units of expected substitutions per site.

Thus, phylogenetic analysis using maximum parsimony, maximum likelihood and Bayesian approaches consistently grouped the ApPR isolates as a single cluster positioned between the two Old World species *R. africae* and *R. sibirica*. In addition, the observation that our analyses grouped the other New world rickettsiae (*R. parkeri* strains NOD, Parvitarsum, Atlantic rainforest, and *R. parkeri* s.s from South and North America) into the same clusters as reported by Nieri-Bastos et al. (2018), provided substantial support for the relationship between strain ApPR, *R. africae* and *R. sibirica*. The calculation of genetic divergence, as a compliment to phylogenetic assessments of relatedness between species, is commonplace and was applied in the current investigation as it was in the study of Nieri-Bastos et al. (2018). Direct comparison between ApPR and the New World isolates of *R. parkeri* did not appear to provide support for the tree-based groupings. However, when the divergence values recorded between the different New World *R. parkeri* isolates and *R. africae* and *R. sibirica* were taken into consideration, the approximation of ApPR to the Old World species became more apparent. Given the clear evolutionary distance between the ApPR bacterium and the New World members of the *R. parkeri* complex we proposed the recognition of a new species "*Candidatus* Rickettsia paranaensis", as for Paraná state, where the strain ApPR-infected *A. parkeri* ticks were first reported by Pacheco et al. (2012).

It is evident that the presence of a new species of SGF *Rickettsia,* with an ample distribution within Brazilian territories, associated with three species of *Amblyomma* ticks recovered from three distinct groups of hosts, represents a significant finding. As was noted for each of the other South American strains of *R. parkeri* (Nieri-Bastos et al., 2018), strain ApPR showed tropism for a specific group of related tick species. It is worth considering, that the evolutionary divergence from the *R. parkeri* group may have developed as a consequence of the relationships with specific ticks that appear to be symbiotic rather than detrimental to the arthropod hosts. The maintenance of ApPR as symbionts may have evolved as a means to protect their hosts by reducing susceptibility (i.e., conferring resistance) to other more virulent *Rickettsia* via the phenomenon of rickettsial exclusion, as first described in *Dermacentor andersoni* ticks infected with *R. peacockii*, in the United States of America (Burgdorfer et al., 1981) and subsequently in *Amblyomma dubitatum* ticks infected with *R. bellii* in Brazil (Sakai et al., 2014).

The identification of "*Candidatus* R. paranaensis" as a distinct species may help to resolve some of the confusion that persists in relation to the etiology of *R. parkeri* in South America. In this context, it is recommended that efforts should be made to develop cost effective and accessible methods for the rapid differentiation of these genetic variants, as an aid to improving our understanding of this emerging human disease problem.

Table 2
Divergence values calculated between groups and within groups of *Rickettsia* set in different clades[a], using concatenated sequences of 3572 nucleotides in length[b].

Clades								
	ApPR	A1 + A2	B	C	D	E	F	G + H
ApPR	0.06							
A1 + A2	0.63	0.01						
B	0.49	0.51	0.00					
C	0.56	0.63	0.56	0.00				
D	0.56	0.60	0.56	0.06	0.00			
E	0.54	0.74	0.71	0.66	0.66	0.17		
F	0.51	0.71	0.62	0.62	0.62	0.63	0.22	
G + H	0.90	1.13	1.09	0.96	0.96	1.04	0.92	0.30

[a] Each letter represents a clade as reported by Nieri-Bastos et al. (2018); A1: *Rickettsia parkeri* s.s. isolates from North America; A2: *R. parkeri* s.s. isolates from South America; B: strain NOD isolates; C: strain Parvitarsum isolates; D: strain Atlantic rainforest isolates; E: *R. africae*; F: *R. sibirica sibirica*; G: *R. conorii* Malish isolates; H: *R. conorii* Israeli. A1 and A2, as well as G and H were considered to belong to the same clades as divergence between them was less than the overall distance (0.49%).

[b] Based on an alignment of a concatenated sequence of 3572 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 *mppEpur* (197 nt), *rrl-rrf-ITS* (323 nt), and *rpmE-tRNAfMet* (343 nt).

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Ethical statement

This study was conducted in accordance with the regulations of the Ethics Committee for the Use of Animals (CEUA), of the Federal Rural University of Rio de Janeiro (UFRRJ), Brazil, under protocol number 3379250617.

Declaration of Competing Interest

The authors declare no conflict of interest.

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