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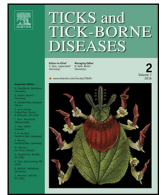

A novel spotted fever group *Rickettsia* infecting *Amblyomma parvitarsum* (Acari: Ixodidae) in highlands of Argentina and Chile

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ABSTRACT

The tick *Amblyomma parvitarsum* (Acari: Ixodidae) has established populations in Andean and Patagonic environments of South America. For the present study, adults of *A. parvitarsum* were collected in highland areas (elevation >3500 m) of Argentina and Chile during 2009–2013, and tested by PCR for rickettsial infection in the laboratory, and isolation of rickettsiae in Vero cell culture by the shell vial technique. Overall, 51 (62.2%) out of 82 *A. parvitarsum* adult ticks were infected by spotted fever group (SFG) rickettsiae, which generated DNA sequences 100% identical to each other, and when submitted to BLAST analysis, they were 99.3% identical to corresponding sequence of the *ompA* gene of *Rickettsia* sp. strain Atlantic rainforest. Rickettsiae were successfully isolated in Vero cell culture from two ticks, one from Argentina and one from Chile. DNA extracted from the third passage of the isolates of Argentina and Chile were processed by PCR, resulting in partial sequences for three rickettsial genes (*gltA*, *ompB*, *ompA*). These sequences were concatenated and aligned with rickettsial corresponding sequences available in GenBank. Phylogenetic analysis revealed that the *A. pavitarsum* rickettsial agent grouped under high bootstrap support in a clade composed by the SFG pathogens *R. sibirica*, *R. africae*, *R. parkeri*, *Rickettsia* sp. strain Atlantic rainforest, and two unnamed SFG agents of unknown pathogenicty, *Rickettsia* sp. strain NOD, and *Rickettsia* sp. strain ApPR. The pathogenic role of this *A. parvitarsum* rickettsia cannot be discarded, since several species of tick-borne rickettsiae that were considered nonpathogenic for decades are now associated with human infections.

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1. Introduction

Bacteria of the genus *Rickettsia* (Rickettsiales: Rickettsiaceae) are obligate intracellular organisms that multiply freely in the cytosol of eukaryotic cells of a variety of invertebrate hosts, including insects and ticks. Many *Rickettsia* species undergo transovarial transmission in their invertebrate hosts, which can sustain rickettsial infection for a number of successive generations. Some *Rickettsia* species infect hematophagous arthropods (e.g., fleas, ticks), which can act as vectors of pathogenic rickettsiae to vertebrate hosts, including humans (Parola et al., 2013). The pathogenic *Rickettsia* species have been divided into two main groups, based on genotypic and phenotypic criteria: the typhus group (TG), typically transmitted by fleas and lice; and the spotted fever group (SFG), mostly transmitted by ticks (Parola et al., 2013). A recent review listed 11 *Rickettsia* species occurring in South America, mostly SFG agents associated with ticks (Labruna et al., 2011). Additionally, a

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number of SFG unnamed agents have been reported infecting South American ticks (Parola et al., 2013).

The tick *Amblyomma parvitarsum* (Acari: Ixodidae) has established populations in Andean and Patagonic environments of Argentina, Bolivia, Chile, and Peru, where the adult tick stage feeds chiefly on native camelids, while immature ticks seem to feed primarily on lizards (Muñoz-Leal et al., 2014). Until the present study, no organism had been reported infecting *A. parvitarsum* ticks. Herein, we report a novel rickettsial agent infecting *A. parvitarsum* ticks in Argentina and Chile.

2. Materials and methods

Adults of *A. parvitarsum* were collected in two localities of Argentina during 2009 and 2013, and one locality of Chile during 2012 (Table 1). Ticks were determined following Estrada-Peña et al. (2005). While the 2009 ticks were preserved in absolute ethanol before been sent to the laboratory, the 2012 and 2013 ticks were sent alive to the laboratory, where they were frozen at −80 °C upon arrival.

All ticks (except for eight ticks from the 2012 and 2013 collections, as stated below) were individually subjected to DNA extraction by the guanidine isothiocyanate phenol technique (Sangioni et al., 2005) and tested for *Rickettsia* by two different PCR protocols. Firstly, all DNA samples were tested with primers CS-78 and CS-323 targeting a relatively conserved fragment of the citrate synthase gene (*gltA*) that occurs in all *Rickettsia* species (Labruna et al., 2004). Samples yielding a visible amplicon of the expected size (compatible with 398-bp) were then tested by a second PCR protocol with primers Rr190.70F and Rr190.602R, which amplify a 532 bp fragment of the 190-kDa outer membrane protein gene (*ompA*) from only some *Rickettsia* species belonging to the SFG (Regnery et al., 1991).

Attempts to isolate rickettsiae in cell culture were performed with eight ticks (four from the 2012 collection and four from the 2013 collection). For this purpose, each tick was thawed and immediately subjected to the shell vial technique for isolation of rickettsiae in Vero cell culture, as previously described (Labruna et al., 2004). Briefly, cultures of Vero cells were inoculated with tick-body homogenates and incubated at 28 °C. The percentage of Vero cells infected with rickettsiae was monitored by the use of Giménez staining of cells scraped from each inoculated monolayer. After the establishment of each isolate in the laboratory (i.e., at least 3 cell passages with >90% infected cells), rickettsial DNA was extracted from the infected cells (Labruna et al., 2004). The extracted DNA was tested in a battery of different PCR protocols, using primer pairs targeting a 632-bp fragment of the *ompA* gene (Roux et al., 1996), a 856-bp fragment of the rickettsial 135-kDa outer membrane protein gene (*ompB*) (Roux and Raoult, 2000), and two overlapping fragments (398-bp and 834-bp) of the *gltA* gene (Labruna et al., 2004). From each of the 8 ticks processed by shell vial, part of the tick homogenate was also tested by PCR targeting fragments of the *gltA* and *ompA* genes, as stated above for ticks. PCR products were purified and sequenced in an automatic sequencer (model ABI 3500 Genetic Analyzer; Applied Biosystems/Thermo Fisher Scientific, Foster City, CA) according to the manufacturer's protocol. The generated sequences were submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) to infer the closest similarities available in GenBank.

Partial DNA sequences obtained from the amplified PCR products (*gltA*, *ompA*, and *ompB*) of the *A. parvitarsum* rickettsial isolate were concatenated and aligned with corresponding sequences of different SFG *Rickettsia* species and yet unnamed rickettsial agents available in GenBank using Clustal X (Thompson et al., 1997) and adjusted manually using GeneDoc (Nicholas et al., 1997). Phylogenetic analyses were performed using PAUP version 4.0b10 (Swofford, 2002) to maximum parsimony (MP); the confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1000 replicates. Bayesian analysis (BA) was performed with MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) software with 1,000,000 generations using the GTR+I+G substitution model. Corresponding sequences of *Rickettsia australis* were used as outgroup.

3. Results

Overall, 51 (62.2%) out of 82 *A. parvitarsum* adult ticks were infected by rickettsiae, including ticks from Argentina and Chile (Table 1). All infected ticks yielded PCR products by both *gltA*- and *ompA*-PCR assays. The overall infection rates per tick gender were 62.1% (18/29) for male ticks and 61.9% (26/42) for female ticks from Argentina (grouping 2009 and 2013 data). In Chile, where no male ticks were collected, 63.6% (7/11) female ticks were infected by rickettsiae. DNA sequences generated from the *ompA*-PCR products were all 100% identical to each other, and when submitted to BLAST analysis, they were 99.3% (444/447-bp) identical to the corresponding sequence of *Rickettsia* sp. strain Atlantic rainforest (JQ906784), 98.9% (471/476-bp) to *Rickettsia africae* (CP001612), 98.7% (469/475-bp) to *Rickettsia* endosymbiont of *Amblyomma tuberculatum* (JF934878), and 98.5% (469/476-bp) to *Rickettsia sibirica* (CP001612).

Rickettsiae were successfully isolated in Vero cell culture from two ticks, one from Argentina and one from Chile. These two isolates were successfully established in the laboratory, with

Table 1
Rickettsial infection in *Amblyomma parvitarsum* adult ticks from Argentina and Chile.

Locality						Ticks		
Name	Province or Region	Country	Coordinates	Elevation	Date	Source	No. collected	No. infected by *Rickettsia* (% infection)
Parque Nacional San Guillermo	San Juan	Argentina	29°28′ S, 69°19′ W	3600	February 2009	Environment[a]	45	29 (64.4)
45 km southwest of provincial route 129 from San Antonio de los Cobres	Salta	Argentina	24°22′ S, 66°42′ W	4500	February 2013	Environment[a]	26	15 (57.7)
Visviri	Arica and Parinacota	Chile	17°35′ S, 69°28′ W	4069	August 2012	Vicuñas [b]	11	7 (63.6)
Total							82	51 (62.2)

[a] Ticks collected through visual inspection of the soil within vicuñas' resting places.
[b] *Vicugna vicugna* (Artiodactyla: Camelidae).

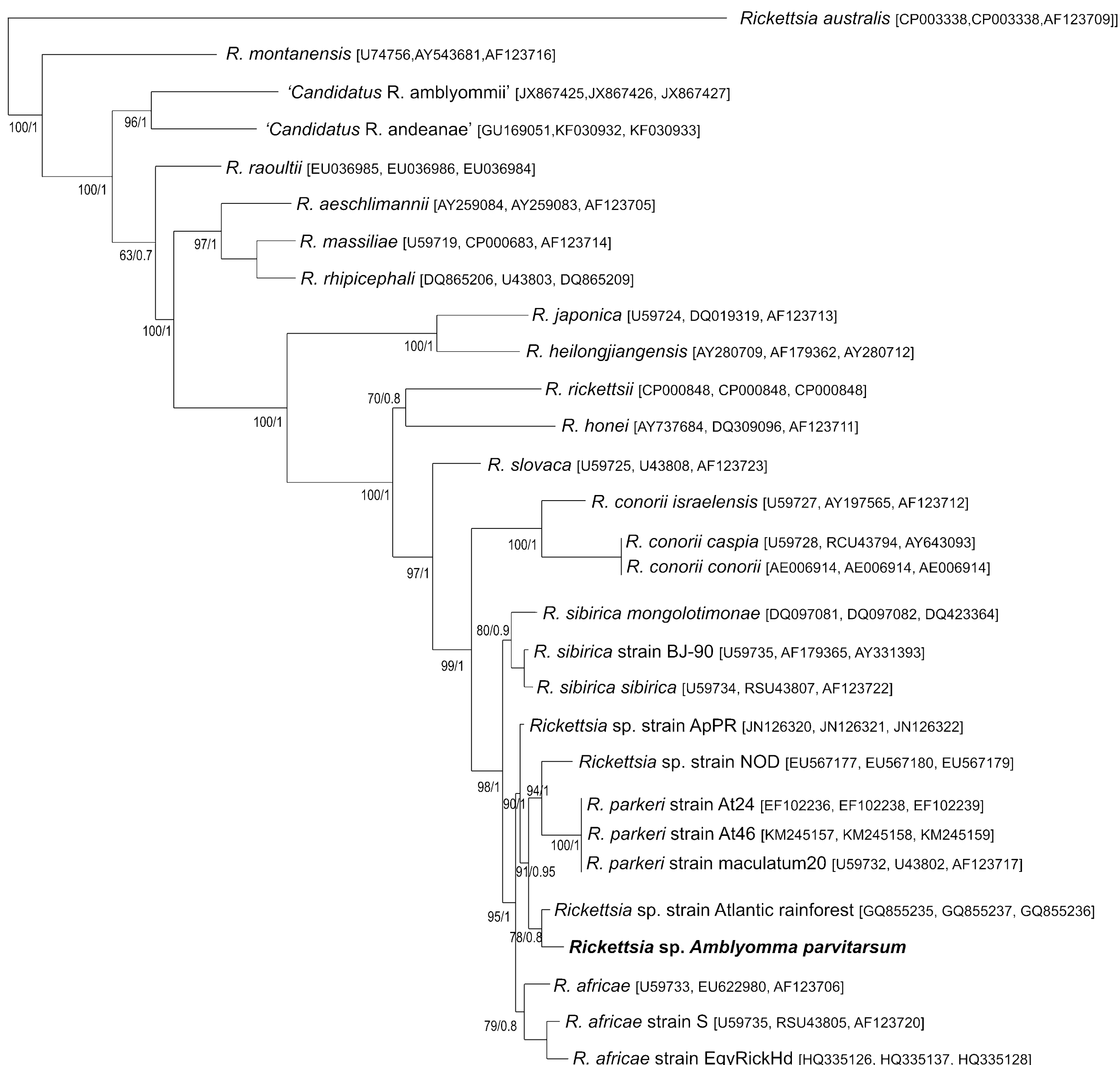

Fig. 1. Molecular phylogenetic analysis of *Rickettsia* sp. isolated from *Amblyomma parvitarsum* from Argentina and Chile. A total of 2137 unambiguously aligned nucleotide sites (268 parsimony informative sites) of three rickettsial genes (*gltA*, *ompA*, *ompB*) were concatenated and subjected to analysis by maximum-parsimony and Bayesian methods. Corresponding sequences of *Rickettsia australis* were used as an outgroup. Numbers at nodes are support values derived from bootstrap and posterior probability for MP and BA analyses (MP/BA). Bootstrap support values ≥70/≥0.7 for phylogenetic groupings are indicated at the nodes. Numbers in brackets are GenBank accession numbers for partial sequences of *gltA*, *ompA* and *ompB* genes, respectively.

several passages, each one infecting >90% of the cells. The isolates have been cryopreserved in our rickettsial collection. DNA extracted from the third passage of the isolates of Argentina and Chile were processed by PCR, resulting in partial sequences identical to each other for each of the three rickettsial genes, *gltA* (1052-bp), *ompB* (789-bp), and *ompA* (590-bp). By BLAST analysis, the *gltA* sequence was 99.9% (1051/1052-bp) identical to the corresponding sequences of *Rickettsia* sp. strain Atlantic rainforest (KJ855083), *Rickettsia* sp. strain ApPR (JN126320), *R. sibirica* (KM288711), *Rickettsia* endosymbiont of *A. tuberculatum* (JF934883), and 99.8% (1050/1052-bp) identical to *Rickettsia parkeri* (KF782319). The *ompB* sequence was 99.6% (751/754-bp) identical to the corresponding sequence of *Rickettsia* sp. strain Atlantic rainforest (KJ855086), 99.3% (748/753) to *R. africae* (CP001612) and *Rickettsia* sp. strain NOD (EU567179). The *ompA* sequence was 99.3% (586/590-bp) identical to the corresponding sequence of *Rickettsia* sp. strain Atlantic rainforest (KJ855085), 99.1% (585/590) to *R. africae* strain S (RSU43805), and 98.8% (583/590-bp) to *Rickettsia* endosymbiont of *A. tuberculatum* (JF934878). The *ompA* sequences of the two isolates were 100% identical to the *ompA* partial sequences generated from the ticks that were processed solely by PCR.

The concatenated phylogenetic analysis included a total of 2137 nucleotides (*gltA*: 1045; *ompA*: 397; *ompB*: 695). The *A. pavitarsum* rickettsial agent grouped under high bootstrap support (98 for MP; 1 for BA) in a clade composed by the SFG pathogens *R. sibirica*, *R. africae*, *R. parkeri*, *Rickettsia* sp. strain Atlantic rainforest, and two unnamed SFG agents of unknown pathogenicty, *Rickettsia* sp. strain

NOD, and *Rickettsia* sp. strain ApPR. Within this clade, the *A. pavitarsum* rickettsial agent grouped with *Rickettsia* sp. strain Atlantic rainforest under moderate bootstrap support (78 for MP; 0.8 for BA).

The *gltA*, *ompA*, and *ompB* partial sequences generated for the rickettsial isolates from *A. parvitarsum* in the present study have been deposited in GenBank under the accession numbers KR296943–KR296945.

4. Discussion

The present study shows that most of the *A. parvitarsum* ticks (≈60%) from three geographically distinct populations were infected by a novel SFG agent, which is phylogenetically closely related to three SFG *Rickettsia* species, *R. africae*, *R. sibirica*, and *R. parkeri.* These three *Rickettsia* species are important human pathogens within their distribution area, where they are etiological agents of spotted fever with very similar clinical profiles, characterized by fever, headache, myalgia, rash, inoculation eschar, and regional lymphadenopathy. The disease is usually mild and is seldom associated with severe complications (Parola et al., 2013). Among the unnamed *Rickettsia* species that also grouped with the *A. parvitarsum* rickettsia in the phylogenetic analysis (Fig. 1), at least *Rickettsia* sp. strain Atlantic rainforest is known to cause human disease, also with the same clinical profile described above (Spolidorio et al., 2010; Silva et al., 2011). The two other unmanned agents, *Rickettsia* sp. strain NOD (Ogrzewalska et al., 2009) and *Rickettsia* sp. strain ApPR (Pacheco et al., 2012), are currently considered of unknown pathogenicity. The *Rickettsia* endosymbiont of *A. tuberculatum*, also considered of unknown pathogenicity (Zemtsova et al., 2012), was not included in the phylogenetic analysis because there was no corresponding *ompB* sequence available in GenBank.

Fournier and Raoult (2009) proposed that a new *Rickettsia* species should not exhibit ≥99.9, 99.2, and 98.8% similarity for the *gltA*, *ompB*, and *ompA* genes, respectively, with the most homologous validated species. If we adopt this procedure to our new isolate from *Amblyomma parvitarsum*, it could not be considered a new species because its *gltA* partial sequence is 99.9% identical to *R. sibirica* (KM288711), its *ompB* partial sequence is 99.3% identical to *R. africae* (CP001612), and its *ompA* sequence is 98.9% identical to *R. africae* (CP001612). Several other papers have discussed that the criteria proposed by Fournier and Raoult (2009) are not suitable to be applied for new rickettsial isolates that are close-related to *R. parkeri, R. africae* and *R. sibirica* (Spolidorio et al., 2010; Pacheco et al., 2012). Therefore, further studies are needed to define the taxonomic status of the *A. parvitarsum* rickettsial agent.

We report a novel SFG agent infecting *A. parvitarsum* ticks, which is phylogenetically related to several tick-borne rickettsial pathogens. Indeed, the pathogenic role of this *A. parvitarsum* rickettsia cannot be discarded, since several species of tick-borne rickettsiae that were considered nonpathogenic for decades are now associated with human infections (Parola et al., 2013). Since human infestation by *A. parvitarsum* has never been reported (Guglielmone et al., 2014), human infection by the *A. parvitarsum* rickettsia is likely to be very rare under natural conditions. It is possible that the current absence of reports of human infestation by *A. parvitarsum* could be just a result of misreporting, since this tick is restricted to remote areas of South America (Andean highlands and Argentinean Patagonia), where very few studies with ticks have been done (Muñoz-Leal et al., 2014). Finally, human densities are generally very low within the distribution area of *A. parvitarsum*, a fact that also contributes to low human exposure to this tick species.

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