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Isolation and Characterization of a *Rickettsia* from the Ovary of a Western Black-legged Tick, *Ixodes pacificus*

Maryam Alowaysi[1,§], **Junyan Chen**[1,§], **Sierra Stark**[1], **Kristine Teague**[1], **Monique LaCourse**[1], **Joanna Proctor**[1], **Katie Vigil**[1], **Jeremy Corrigan**[1], **Aja Harding**[1], **Jinze Li**[1], **Timothy Kurtti**[2], and **Jianmin Zhong**[1,*]

[1]Department of Biological Sciences, Humboldt State University, Arcata, CA 95521, USA.

[2]Department of Entomology, University of Minnesota, St. Paul, MN 55108, USA

Abstract

A rickettsial isolate was obtained from a partially engorged *Ixodes pacificus* female, which was collected from Humboldt County, California. The isolate was provisionally named *Rickettsia* endosymbiont *Ixodes pacificus* (REIP). The REIP isolate displayed the highest nucleotide sequence identity to *Rickettsia* species phylotype G021 in *I. pacificus* (99%, 99%, and 100% for *ompA*, 16S rRNA, and *gltA*, respectively), a bacterium that was previously identified in *I. pacifiucs* by PCR. Analysis of sequences from complete opening frames of five genes, 16S rRNA, *gltA*, *ompA, ompB*, and *sca4*, provided inference to the bacteria's classification among other *Rickettsia* species. The REIP isolate displayed 99.8%, 99.4%, 99.2%, 99.5%, and 99.6% nucleotide sequence identity for 16S rRNA, *gltA*, *ompA, ompB*, and *sca4* gene, respectively, with genes of '*R. monacensis*' str. IrR/Munich, indicating the REIP isolate is closely related to '*R. monacensis'*. Our suggestion was further supported by phylogenetic analysis using concatenated sequences of 16S rRNA, *gltA*, *ompA, ompB*, and *sca4* genes, concatenated sequences of *dksA*-*xerC*, *mppA*-*purC*, and *rpmE*-tRNA^{fMet} intergenic spacer regions. Both phylogenetic trees implied that the REIP isolate is most closely related to '*R. monacensis'* str. IrR/Munich. We propose the bacterium be considered as '*Rickettsia monacensis*' str. Humboldt for its closest phylogenetic relative (=DSM 103975T =ATCC TSD-94T).

Keywords

'*Rickettsia monacensis*' str. Humboldt; *Ixodes pacificus*; phylogenetic analysis; multilocus sequence typing; multi-spacer sequence typing

[*]Corresponding author: Jianmin Zhong. jz15@humboldt.edu. Telephone: 707-826-5554.
[§]These authors contributed equally to this work.

Declaration of interest: None

Introduction

Ixodes pacificus, the Western black-legged tick, is a predominant vector of *Borrelia burgdorferi* sensu stricto and *Anaplasma phagocytophilum*, the etiologic agents of Lyme borreliosis and anaplasmosis, respectively, in the Pacific West Coast region of the United States (Burgdorfer et al., 1985; Foley et al., 2008; Piesman et al., 1999). Ixodid ticks, as well as other arthropods, often harbor rickettsiae that are known as obligate intracellular bacteria and arthropod-borne pathogens of vertebrate hosts (Hardstone Yoshimizu and Billeter, 2018). Presently, 32 *Rickettsia* species within the spotted fever, typhus, *Rickettsia bellii*, and *Rickettsia canadensis* groups have been validated (Abdad et al., 2017; http://www.bacterio.net/rickettsia.html). Utilization of molecular techniques has facilitated the continual detection and isolation of rickettsiae from ticks around the world (Parola et al., 2013; Cazorla et al., 2008; Richards, 2012). Several serological studies have noted that *I. pacificus* harbors a spotted fever group *Rickettsia* that is antigenically related to pathogenic species (Hughes et al., 1976; Philip et al., 1978; Philip et al., 1981). We have previously identified *Rickettsia* species phylotype G021 and *Rickettsia* species phylotype G022 via PCR amplification using genomic DNA extracted from *I. pacificus* ticks; of which, the bacterial prevalence was found to be 100% and 2%, respectively (Phan et al., 2011; Cheng et al., 2013b). Transmission electron microscope and Fluorescence in situ hybridization assays detected the presence of *Rickettsia* species in midgut and ovaries of *I. pacificus* (Phan et al., 2011; Bagheri et al., 2017). It was later noted that *Rickettsia* species phylotype G021 was found to possess 100% transovarial and transstadial efficiency as well as the genetic capacity to synthesize tetrahydrofolate, indicating a possible nutritional relationship between the host and endosymbiont (Bodnar et al., 2018; Cheng et al., 2013a; Kurlovs et al., 2014; Hunter et al., 2015).

Material and methods

Source of ticks and tick cell culture

To isolate *Rickettsia* phylotype G021 from a mixed population of microorganisms in *I. pacificus*, we collected a partially engorged *I. pacificus* female that was donated from McKinleyville Animal Care Center, McKinleyville, CA, in March 2013. The standard Universal Transverse Mercator Grid System coordinate for the tick collection site is 10N 429550 4530570. The isolation of rickettsiae from the engorged tick followed methods reported previously (Kurtti et al., 1996; Kurtti et al. 2015). In brief, ovaries were extirpated from a surface disinfected, partially engorged, female. Ovarian fragments were cultured with tick cell line IRE11 and incubated in a candle jar at 25°C. Once established, the isolate was maintained in tick cell line ISE6 at 34°C (Simser et al., 2002; Munderloh et al., 1994). In the sixth serial transfer, genomic DNA was prepared and submitted to BioProject PRJNA232537 for genome sequencing (SRX476329; SRX476328 and SRX476327).

The isolate was initially named *Rickettsia* endosymbiont *Ixodes pacificus* (REIP). Infection of IRE11 and ISE6 cell lines was conducted by mixing 10^6 cells with 0.3–0.5 mL of REIP-infected IRE11 lysate, resulting in 20–100 rickettsiae per tick cell, in 25 cm^2 flasks (Fisher Scientific, USA). Cells of rickettsiae and IRE11 were counted using a Petroff Hauser counting chamber under a Leica DM750 phase-contrast microscope with Leica

highresolution camera module (Leica Microsystems, USA). Infected cells were cultured for three weeks at 25°C in L-15B300 complete medium supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, 0.1% bovine lipoprotein cholesterol concentrate, and 10 mM HEPES (Kurtti et al., 2005; Munderloh and Kurtti, 1989). Giemsa stain (KaryoMAX®, Thermo Fisher Scientific, USA) was performed on a weekly basis to monitor infection status. Giemsa stained REIP-infected ISE6 cells (or IRE11) were observed under the Leica DM750 microscope.

DNA extraction and PCR amplification

To classify REIP into genus and species, genomic DNA was extracted from 5×10^5 REIP-infected IRE11 cells using DNeasy Blood & Tissue Kit for both MLST and MST (Qiagen, Germany). Amplification of 16S rRNA, *gltA*, *ompA*, *dksA-xerC*, *mppA-purC*, and *rpmE-*tRNA^{fMet} was conducted using PCR using primers specified in Table 1 (Simser et al., 2002; Fournier and Raoult, 2007; Cheng et al., 2013b). Specifically, 2 μL genomic DNA (50 ng/μL), 1 μL 5 mM forward and reverse primers, 10 μL GoTaq® Green Master Mix (Promega, USA), and 6 μL PCR grade water were added in PCR reactions. Negative controls contained all components with the exception of REIP genomic DNA. The β-actin-coding gene was amplified simultaneously to serve as positive control using β-actin specific primers and field-collected *I. pacificus* (Cheng et al., 2013b). PCR reactions were performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, USA) with an initial denaturation at 95°C for 5 minutes; this was followed by 30–45 cycles of denaturation at 95°C for 30 seconds, annealing at 50–60°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension was completed at 72°C for ten minutes. Following amplification, PCR products were run on 2% agarose gel with 1× TAE buffer and stained in 10 μg/mL ethidium bromide. AlphaImager® HP was used to visualize and photograph gels (ProteinSimple, USA).

Cloning and DNA sequencing

PCR amplicons were ligated into StrataClone's pSC-A-amp/kan cloning vector (Agilent Technologies, USA). Transformants were plated on LB agar containing 100 μg/mL ampicillin and 40 μl of 2% X-gal. White colonies were then restreaked on LB/AMP/X-gal plates. Following restreaking, white colonies were grown in LB broth containing 100 mg/mL ampicillin overnight at 37°C. Plasmids were purified by E.Z.N.A.® Plasmid Mini Kit (Omega, USA) and constructs were confirmed by running EcoRI restriction enzyme digests (Promega, USA) on 1% agarose gel with 1× TAE buffer and stained in ethidium bromide. Sequencing of the PCR amplicons was conducted by Elim Biopharmaceuticals, Inc. using M13 reverse primer (5'-GGAAACAGCTATGACCATG-3'). The GenBank accession numbers for the 16S rRNA, *gltA*, and *ompA* genes, *dksA-xerC*, *mppA-purC*, and *rpmE-* tRNA^{fMet} regions for determining the numbers of species and genotypes of the REIP isolate are KX505845, KX505846, KX505847, KX505844, KX505842, KX505843, respectively.

Phylogenetic analysis

We utilized the completed genome sequence of REIP, which is available in NCBI (accession number NZ_LAOP01000001). Homologous sequences of the whole open reading frame of

each of the five genes of REIP, 16S rRNA (NZ_LAOP01000001), *gltA* (KJW02430), *ompA* (KJW02278), *ompB* (KJW03404), and *sca4* (KJW03052), were obtained by BLAST analysis. For phylogenetic reconstructions, homologous sequences for each gene (Supplemental Table) were aligned using Clustal Omega at EMBL-EBI. The resulting aligned sequences of 16S rRNA (1,440 bp), *gltA* (1,233 bp), *ompA* (1,330 bp), *ompB* (2,847 bp) and *sca4* (2,691 bp) genes of REIP and 21 validated *Rickettsia* species were concatenated to generate a sequence of 9,829 characters for each species in Mesquite (version 3.04). Sequences from other validated *Rickettsia* species were not included in this study since their *ompA* gene is either truncated or lacking. The concatenated nucleotide sequences were translated to amino acid sequences, with a purpose of aligning the nucleotide sequences to match the amino acid alignments in Mesquite. Phylogeny of the concatenated sequence was constructed by SeaView (version 4.4.3) (http://pbil.univlyon1.fr/software/seaview.html). Gouy and Guindon S. & Gascuels' method was used to determine evolutionary distance values, which were then used to construct phylograms by neighbor-joining method and maximal parsimony method (Gouy et al., 2010). The pairwise nucleotide sequence identity of each gene between REIP and validated *Rickettsia* species was calculated in SIAS (http://imed.med.ucm.es/Tools/sias.html) using aligned sequences from Clustal Omega.

Results and discussion

We successfully propagated the REIP isolate using embryonic tick cell lines IRE11 and ISE6 (Simser et al., 2002; Munderloh and Kurtti, 1989), in L15B300 media formulated for tick cell culture (Munderloh and Kurtti, 1989; Oliver et al., 2014). Giemsa stained REIP-infected ISE6 cells were observed under light microscope. Coccobacillus-shaped bacteria were viewed residing in the cytoplasm or outside lysed cells; uninfected controls had no visible signs of bacterial inhabitance (Fig. 1).

To confirm that there is only one bacterial species and/or genotype in the REIP isolate, we utilized multilocus sequence typing (MLST) using three genes—16S rRNA, *gltA*, and *ompA*—allowing for the detection of single nucleotide polymorphisms that distinguish *Rickettsia* species (Fournier et al., 2003; Zhu et al., 2005a). However, because coding DNA may not provide enough nucleotide sequence variation to differentiate within some rickettsial species, we also used multi-spacer sequence typing (MST)—utilizing intergenic spacers such as *dksA*-*xerC*, *mppA*-*purC*, and *rpmE*-tRNA^{fMet}—as this method is capable of differentiating at the intraspecies level (Fournier et al., 2004; Zhu et al., 2005b). The three genes and three intergenic spacers were amplified via PCR and amplicons were ligated into StrataClone's pSC-A-amp/kan cloning vector. Sequencing data determined the insert size of clones for *gltA*, *ompA*, 16S rRNA genes of REIP to be 382, 530 and 436 base pair (bp)-long, respectively. PCR amplification and cloning of intergenic spacers determined the insert size of *dksA*-*xerC*, *mppA*-*purC*, and *rpmE*-tRNA^{fMet} regions were 165, 121, 134 and 368 bp, respectively (data not shown). Additional, sequences from 30 clones of 16S rRNA, *gltA*, *ompA*, *dksA*-*xerC*, *mppA*-*purC*, and *rpmE*-tRNA^{fMet} genes of REIP were aligned in ClustalX version 2.1. Nucleotide sequence alignments of thirty clones for each of the genes and intergenic spacers determined that the nucleotide sequences were identical to one another in the amplified regions. Additionally, no difference was noted when comparing

amplified 16S rRNA, *gltA*, and *ompA* sequences to sequences obtained from the genome sequencing project of REIP (Supplemental Table). Considering REIP's nucleotide homology among all thirty clones, we assumed the REIP isolate possessed one genotype.

We utilized complete open reading frames of 16S rRNA, *gltA*, *ompA*, *ompB*, and *sca4* nucleotide sequences of REIP obtained from the genome sequencing project in NCBI (accession number NZ_LAOP01000001) to classify the REIP isolate. The pairwise nucleotide sequence identity comparison was conducted between 1,440 bp 16S rRNA, 1,233 bp *gltA*, 1,330 bp *ompA*, 2,847 bp *ompB*, and 2,691 bp *sca4* genes of REIP and homologous sequences of 21 validated *Rickettsia* species in GenBank. The nucleotide sequence identities of 16S rRNA, *gltA*, *ompA, ompB*, and *sca4* genes of REIP with homologous sequences of *R. buchneri* str. ISO7 (Kurtti et al., 2015) was 99.7%, 99.5%, 91.7%, 76.4%, and 47.3%, respectively (Table 2). Based on Fournier *et al.*'s classification scheme (Fournier et al., 2003), these values confirmed the isolate is a member of the *Rickettsia* genus. For all genes, REIP shared the highest nucleotide sequence identity with '*R. monacensis'* str. IrR/Munich (99.8, 99.4, 99.2, 99.5, and 99.6% for 16S rRNA, *gltA*, *ompA*, *ompB*, and *sca4*, respectively). The identities between the two species for all genes indicate that the REIP isolate is closely related to '*R. monacensis*'. Moreover, the isolate also displayed similarity to *Rickettsia* species phylotype G021. For instance, the isolate shared 99%, 99%, and 100% nucleotide identity to *Rickettsia* species phylotype G021's *ompA*, 16S rRNA, and *gltA* sequences, respectively (Phan et al., 2011). Considering the nucleotide sequences of 30 clones for each gene were identical, we ruled out the possibility that the discrepancies observed between nucleotide sequences for REIP and phylotype G021's genes could have resulted from random errors during PCR. The incongruity is more likely due to bacterial genetic diversity within species or an error when sequencing phylotype G021. Therefore, we are in favor of the conclusion that REIP is the same species as phylotype G021, however possesses a different genotype.

Sequence similarities present between REIP, *R. buchneri*, '*R. monacensis'*, and *Rickettsia* species phylotype G021 were also reflected via phylogenetic analysis. Phylogenetic analysis was conducted by comparing the REIP isolate's concatenated *gltA*, *ompA*, *ompB*, *sca4*, and 16S rRNA nucleotide sequences to homologous sequences of rickettsiae available in GenBank. Trees generated from the concatenated sequences reflected those previously generated by our lab for *Rickettsia* species phylotype G021 (Phan et al., 2011). The tree topology indicated that REIP was clustered with '*R. monacensis'* str. IrR/Munich and that the two bacteria were placed in the same clade as *R. buchneri* str. ISO7. This monophyletic group forms a sister group to the rest of a clade that contains 16 spotted fever group rickettsiae. The concatenated sequences of the five genes of the spotted fever group rickettsiae are distant from the sequences of *R. akari*, *R. australis*, and *R. felis* (Fig. 2). The neighbor joining tree indicated that the monophyletic groups, in which REIP resides, are distinct from the rest of the spotted fever group rickettsiae. We inferred that the REIP isolate is closely related to '*R. monacensis'* and shares similar evolutionary history with *R. buchneri* and '*R. monacensis'* str. IrR/Munich. As such, based on our nucleotide sequence identities and phylogenetic analyses, we propose that the REIP isolate constitutes a new strand of '*R. monacensis'* different from '*R. monacensis'* strain IrR/Munich. Therefore, we propose the name '*Rickettsia monacensis*' str. Humboldt, for the bacterium.

Previous studies have demonstrated the ability of MST to differentiate between genotypes of some *Rickettsia* species by at least one nucleotide mutation (Fournier and Raoult, 2007; Zhu et al., 2005b; Znazen et al., 2013). As such, MST was used to further understand the evolutionary relationship between '*R. monacensis*' str. Humboldt and other *Rickettsia* species. A neighbor joining tree was generated based on the concatenated nucleotide sequence of *dksA-xerC*, *mppA-purC*, and *rpmE*-tRNA^{fMet} regions and their homologous sequences from 22 validated *Rickettsia* species in GenBank (Supplemental Table). There was a limit to what rickettsial strains could be used to generate the tree as some strains don't have all three spacer sequences and these regions for other strains have yet to be sequenced and made available in GenBank. As shown in Figure 3, the phylogram indicated that '*R. monacensis*' str. Humboldt and '*R. monacensis'* str. IrR/Munich form a unique clade. The phylogram also confirmed that '*R. monacensis*' str. Humboldt is closely related to '*R. monacensis'* str. IrR/Munich and that '*R. monacensis*' str. Humboldt, '*R. monacensis'* str. IrR/Munich, *R. buchneri* str. ISO7, and *R. massiliae* str. Mtut1T form a monophyletic group that is distinct from other spotted fever group rickettsiae (Fig. 3).

Based on MLST, MST, and phylogenetic tree constructions of this study, we have drawn a conclusion that the REIP isolate from *I. pacificus* is '*R. monacensis'*. Our conclusion is supported by a recent genome-wide comparison among genomes of *Rickettsia* species using core genome alignment sequence identity (CGASI), a method using both length and sequence identity of genome for species classification. Using a CGASI cutoff of equal or greater than 96.8%, '*R. monacensis*' str. Humboldt from *I. pacificus* and '*R. monacensis*' str. IrR/Munich are classified as the same species. There are only two *Rickettsia* species in the spotted fever group based on CGASI reclassification. In addition to '*R. monacensis*' species, all other *Rickettsia* species in the spotted fever group are classified as the second *Rickettsia* species (Chung et al., 2018). Our conclusion that '*R. monacensis*' str. Humboldt and '*R. monacensis'* str. IrR/Munich are two different strains within the same species is also supported by CGASI since the core genome alignment sequence identity between '*R. monacensis*' str. Humboldt and '*R. monacensis'* str. IrR/Munich is 99.6% (Chung et al., 2018). In addition, the different genome content between the two strains is reflected by unique plasmids that exist in their genomes. Pulse-field gel electrophoresis and Southern blot identified pRM plasmid of '*R. monacensis'* str. IrR/Munich (Baldridge et al., 2007) (GenBank Accession Number EF564599), whereas genome sequencing of '*R. monacensis*' str. Humboldt revealed the presence of pREIP plasmid (KR611317). Although the size of pREIP (102 kb) differs dramatically with that of pRM (23.5 kb), parts of the pREIP share 99% nucleotide sequence identity with pRM (T. Kurtti, personal communication). However, the pREIP and pRM plasmids have low nucleotide homology with other rickettsial plasmids from other spotted fever group rickettsia, including *R. amblyommii*, *R. buchneri*, *R. helvetica*, *R. hoogstraalii*, *R. massiliae*, and *R. peacockii* (Baldridge et al., 2008; Gillespie et al., 2012; T. Kurtti, personal communication).

'R. monacensis' was originally isolated in an *I. ricinus* tick in Munich, Germany (Simser et al., 2002). Later, '*R. monacensis'* infection in *I. ricinus* was identified all along Europe, including Hungary (Sreter-Lancz et al., 2006), Portugal (de Carvalho et al., 2008), Switzerland (Boretti et al., 2009), Sweden (Elfving et al., 2010), Italy (Corrain et al., 2012), the Netherlands (Koetsveld et al., 2016), Turkey (Gargili et al., 2012), Spain (Palomar et al.,

2012), Poland (Rymaszewska and Piotrowski, 2013), Romania (Ionita et al., 2013), Croatia (Tijsse-Klasen et al., 2013), Slovakia (Spitalska et al., 2014), and Estonia (Katargina et al., 2015). In addition to Europe, '*R. monacensis'* has been reported in Africa, Asia, and South and Central America. In Africa, '*R. monacensis'* was isolated from *I. ricinus* in Tunisia (Sfar et al., 2008) and Algeria (Dib et al., 2009). Also, the bacterium infection was documented in *I. nipponensis* ticks in Korea (Lee et al., 2013) and in *I. sinensis* ticks in China (Ye et al., 2014). In South and Central America, *I. boliviensis* and *Rhipicephalus sanguineus* sensu lato were tested positive for '*R. monacensis'* in Nicaragua and Costa Rica (Springer et al., 2018; Troyo et al., 2014). The global distribution of '*R. monacensis'* is, at least partially, attributed to migrating birds, which serve as possible dispersers of the bacterium (Elfving et al., 2010; Biernat et al., 2016). Many not yet isolated rickettsia in *Ixodes* ticks have been reported. Some of them are genetically close to '*R. monacensis*' str. Humboldt and '*R. monacensis'*. For example, the *Rickettsia* species detected in *Amblyomma ovale* (Springer et al., 2018). Future isolation and characterization of the bacteria, especially genomic sequencing and CGASI analysis, are required to confirm that they belong to '*R. monacensis'*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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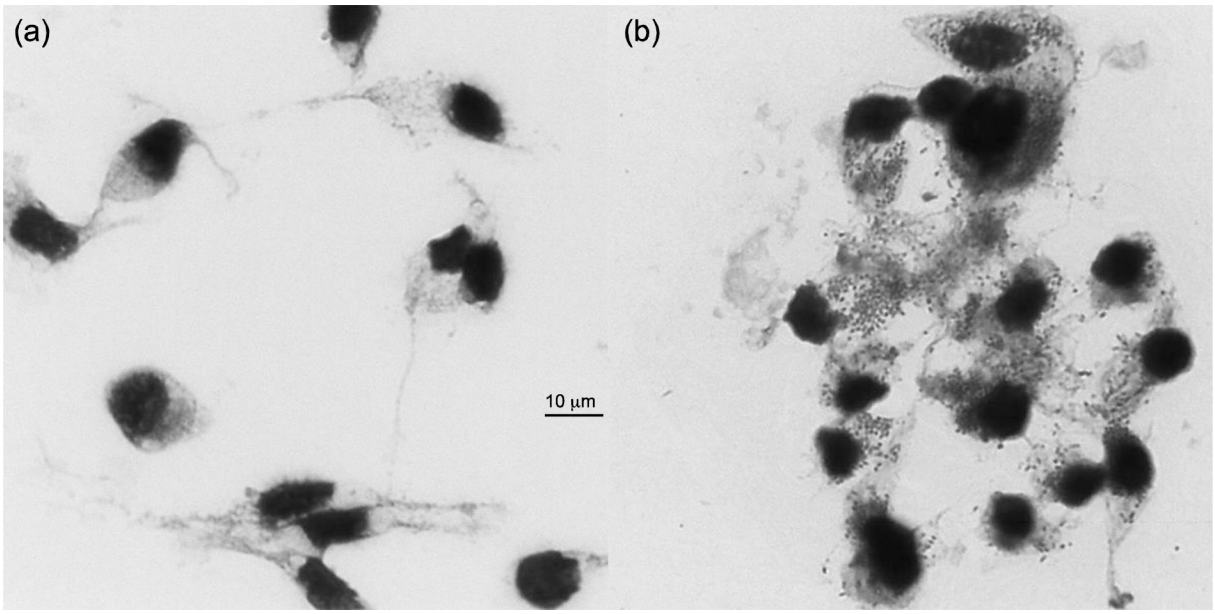

Fig. 1. Giemsa stain of *Rickettsia* endosymbiont *Ixodes pacificus* (REIP)-infected ISE6 cells propagated for three weeks at 26°C.

(a) Uninfected ISE6 control without bacteria residing in the cytoplasm. (b) Heavily infected ISE6 cells three weeks post infection with REIP. Original magnification: x1000. Bar, the scale bar in µm

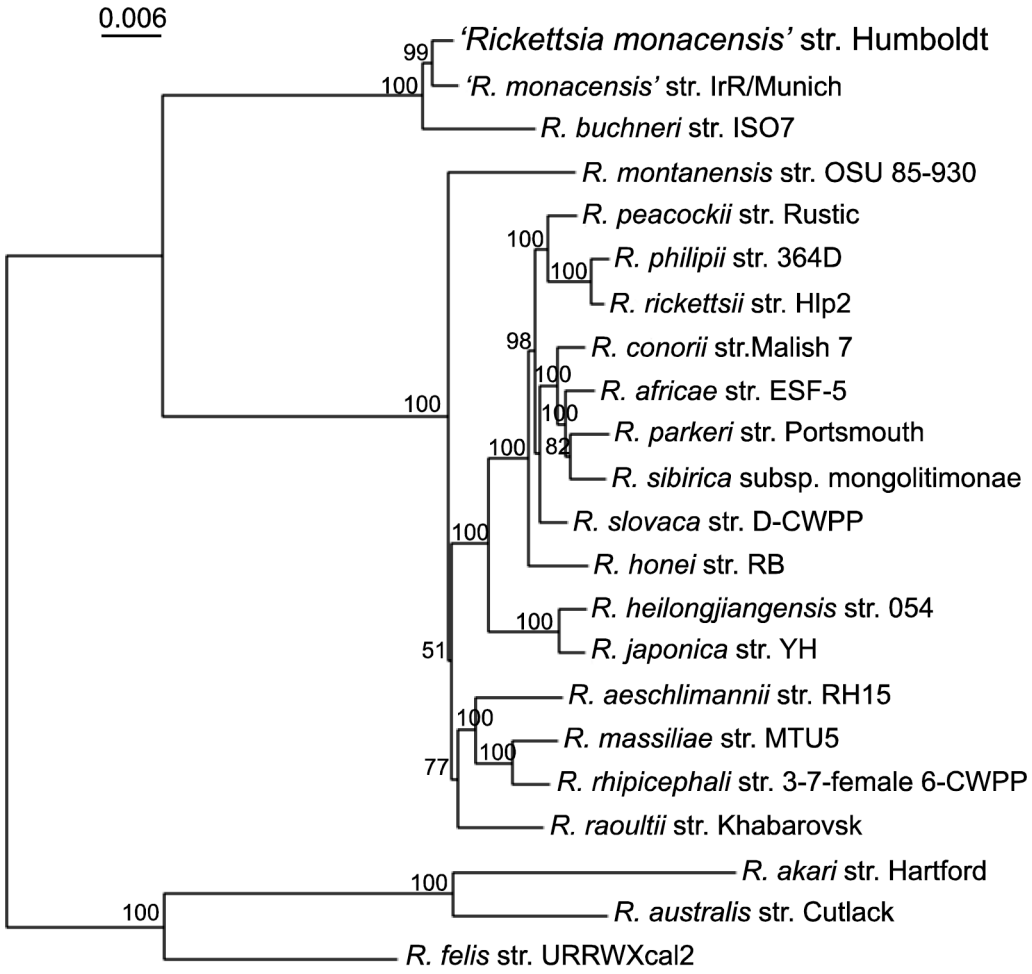

Fig. 2. Phylogenetic tree of concatenated *gltA, ompA, ompB, sca4,* and 16S rRNA gene sequences of '*Rickettsia monacensis*' str. Humboldt.

The tree was generated using neighbor-joining distance method and 1000 bootstrap replicates. Bootstrap values >50% are shown at the nodes. Bar, nucleotide distance. '*R. monacensis*' str. Humboldt (or *Rickettsia* endosymbiont *Ixodes pacificus*) is represented at the top of the tree. Other selected sequences on the tree include: *R. aeschlimannii*, *R. africae*, *R. akari*, *R. australis*, *R. buchneri*, *R. conorii*, *R. felis*, *R. heilongjiangensis*, *R. honei*, *R. japonica*, *R. massiliae*, *'R. monacensis'* str. IrR/Munich, *R. montanensis*, *R. parkeri*, *R. peacockii*, *R. philipii*, *R. raoultii*, *R. rhipicephali*, *R. rickettsii*, *R. sibirica*, and *R. slovaca*. The accession numbers of genes of all *Rickettsia* species are listed in Supplemental Table.

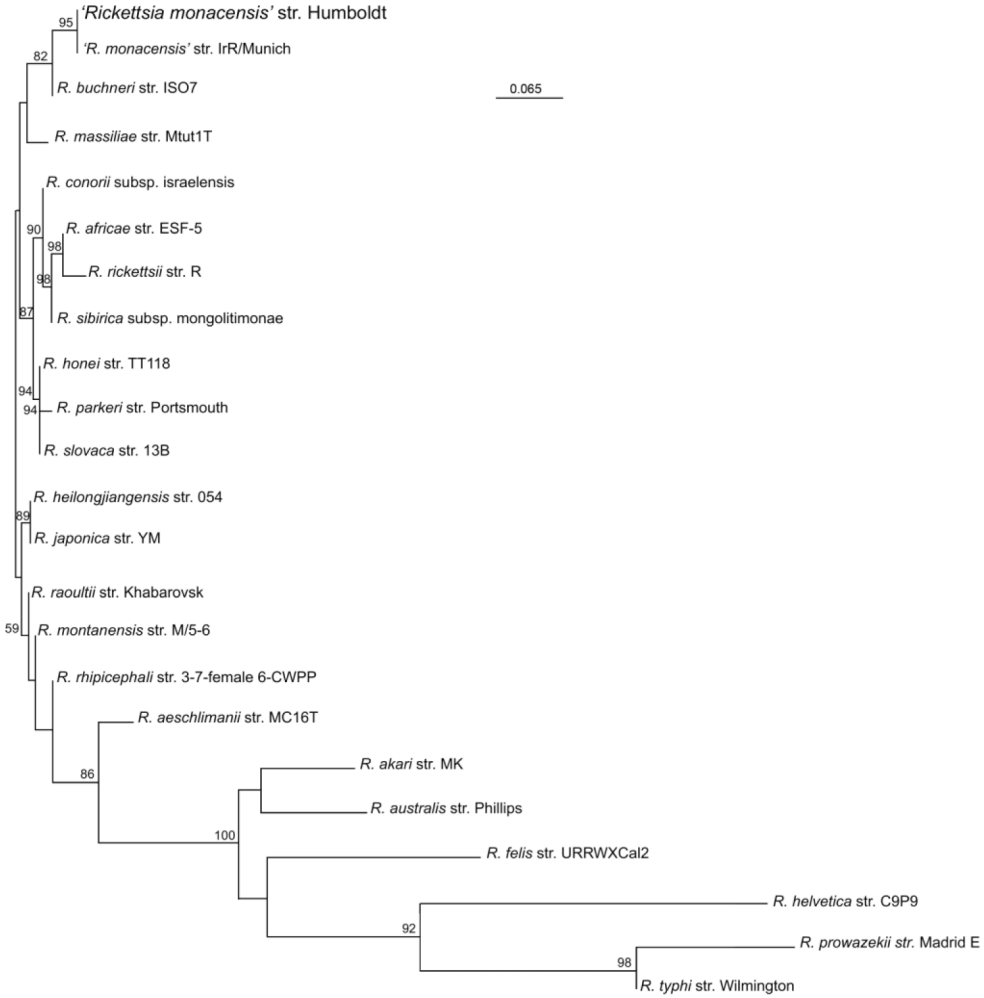

Fig. 3. Phylogenetic tree of concatenated nucleotide sequences of *dksA-xerC*, *mppA-purC*, and *rpmE*-tRNA^{fMet} intergenic spacers of '*Rickettsia monacensis*' str. Humboldt.

The tree was generated using neighbor-joining distance method and 1000 bootstrap replicates. Bootstrap values >50% are shown at the nodes. Bar, nucleotide distance. '*R. monacensis*' str. Humboldt (or *Rickettsia* endosymbiont *Ixodes pacificus)* is represented at the top of the tree. Other selected sequences on the tree include: *R. aeschlimannii*, *R. africae*, *R. akari*, *R. australis*, *R. buchneri*, *R. conorii*, *R. felis*, *R. heilongjiangensis*, *R. helvetica*, *R. japonica*, *R. massiliae*, '*R. monacensis'* str. IrR/Munich, *R. montanensis*, *R. parkeri*, *R. prowazekii*, *R. raoultii*, *R. rhipicephali*, *R. rickettsii*, *R. sibirica*, *R. slovaca*, and *R. typhi*. The accession numbers of genes of all *Rickettsia* species are listed in Supplemental Table.

Table 1.

PCR reaction primers, annealing temperatures, and amplicon sizes.

Gene name	Primer (5'−3')	Annealing temperature	Amplicon size (bp)	Reference
16S rRNA	F-AGAGTTTGATCCTGGCTCAG	58°C	436	Simser et al., 2002
	R-AACGTCATTATCTTCCTTGC			
gltA	F-GGGGGCCTGCTCACGGCGG	50°C	382	Simser et al., 2002
	R-ATTGCAAAAAGTACAGTGAACA			
ompA	F-ATGGCGAATATTTCTCCAAAAA	60°C	530	Simser et al., 2002
	R-AGTGCAGCATTCGCTCCCCCT			
dksA-xerC	F-TTTTCATGACGCTCTTGAGC	58°C	165	Fournier and Raoult, 2007
	R-GTAAAGAAAGAATAATTCCGTGGTT			
mppA-purC	F-GCATATGCRGTRGGTAGTTAT	59°C	1000	Fournier and Raoult, 2007
	R-CACACGCCCAAATTCTAATT			
rpmE-$_{tRNA}^{fMet}$	F-TTCCGGAAATGTAGTAAATCAATC	57°C	368	Fournier and Raoult, 2007
	R-TCAGGTTATGAG CCTGACGA			
β-actin	F-TTGTCCGCGACATCAAGGA	57°C	110	Cheng et al., 2013b
	R-CGGGAAGCTCGTAGGACTTCT			

Table 2.

The nucleotide sequence identity of the *gltA, ompA, ompB, sca4*, and *16S rRNA* genes between '*Rickettsia monacensis*' str. Humboldt and *Rickettsia buchneri* str. ISO7 and *R. monacensis* str. IrR/Munich.

Strains	***'R. monacensis' str. Humboldt***				
	16S rRNA	***gltA***	***ompA***	***ompB***	***sca4***
R. buchneri str. IS07	99.65	99.51	91.71	76.41	47.32
R. monacensis str. IrR/Munich	99.79	99.35	99.17	99.50	99.55