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Borrelia chilensis, a new member of the *Borrelia burgdorferi* sensu lato complex that extends the range of this genospecies in the Southern Hemisphere

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Summary

Borrelia burgdorferi sensu lato (s.l.), transmitted by *Ixodes* spp. ticks, is the causative agent of Lyme disease. Although *Ixodes* spp. ticks are distributed in both Northern and Southern Hemispheres, evidence for the presence of *B. burgdorferi* s.l. in South America apart from Uruguay is lacking. We now report the presence of culturable spirochetes with flat-wave morphology and borrelial DNA in endemic *Ixodes stilesi* ticks collected in Chile from environmental vegetation and long-tailed rice rats (*Oligoryzomys longicaudatus*). Cultured spirochetes and borrelial DNA in ticks were characterized by multilocus sequence typing and by sequencing five other loci (16S and 23S ribosomal genes, 5S-23S intergenic spacer, *flaB*, *ospC*). Phylogenetic analysis placed this spirochete as a new genospecies within the Lyme borreliosis

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group. Its plasmid profile determined by PCR and pulsed-field gel electrophoresis differed from that of *B. burgdorferi* B31A3. We propose naming this new South American member of the Lyme borreliosis group *Borrelia chilensis* VA1, in honor of its country of origin.

Keywords

housekeeping genes; MLST/MLSA; *Ixodes stilesi*; *Borrelia*

Introduction

The *Borrelia* genus currently contains at least 34 species of spirochetes (http://www.ncbi.nlm.nih.gov/Taxonomy), many of which cause diseases in human beings and domestic animals (Collares-Pereira et al., 2004; Dykhuizen and Brisson, 2010; Bouchard et al., 2011; Stanek and Reiter, 2011). In human beings, *Borrelia* sp. are agents of two major diseases: Lyme borreliosis (LB) (Radolf et al., 2012) and relapsing fever (RF) (Cutler, 2010). *Borrelia* are distributed throughout the world and are maintained in nature in a cycle with various arthropod vectors and mammalian, avian, or reptilian hosts (Piesman and Gern, 2004; Dsouli et al., 2006; Marie-Angele et al., 2006; Tilly et al., 2008; Vitorino et al., 2008; Takano et al., 2010; Bouchard et al., 2011; Margos et al., 2011; Vollmer et al., 2011; Brisson et al., 2012). They fall into three groups, *Borrelia burgdorferi* sensu lato (s.l.) complex (LB group) some of whose member cause LB (Stanek and Reiter, 2011; Margos et al., 2011; Rudenko et al., 2011), the RF group whose members cause RF (Cutler, 2010), and the reptile-associated (REP) group whose members infect reptiles but are not known to cause human disease (Takano et al., 2010).

LB is caused by several members of the *Borrelia burgdorferi* sensu lato (s.l.) complex and is transmitted by hard-bodied *Ixodes* ticks (Piesman and Fikrig, 2010). Of the 19 members of the LB group, only nine have been found in or isolated from human patients (*B. afzelii*, *B. bavariensis*, *B. bissetti*, *B. burgdorferi* sensu stricto (s.s.), *B. garinii*, *B. kurtenbachii*, *B. lusitaniae B. spielmanii*, *B. valaisiana*) while the remaining 10 have not (Stanek and Reiter, 2011; Rudenko et al., 2011). Most have been reported only from the Northern Hemisphere (Margos et al., 2011). *B. garinii* is maintained in pelagic transmission cycles in both hemispheres by seabirds and *Ixodes uriae* (Olsén et al., 1993) and *B. burgdorferi* s.l. DNA has recently been demonstrated in ixodid ticks from Uruguay (Barbieri et al., 2013).

RF is caused by at least 13 borrelial species including *B. turicatae*, *B. hermsii*, *B. hispanica*, *B. miyamotoi*, *B. crocidurae*, *B. duttoni*, *B. recurrentis* and closely related *B. anserina* and *B. coriaceae* (Cutler, 2010; Platonov et al., 2011). It is transmitted by soft-bodied *Ornithodoros* ticks and by the human body louse, *Pediculis humanus* (Barbour and Hayes, 1986; Piesman and Fikrig, 2010) as well as by hard-bodied *Ixodes* ticks (Platonov et al., 2011). In contrast to borreliae of the LB group, RF group borreliae are found in both Hemispheres (Kurtenbach et al., 2006; Ataliba et al., 2007; Piesman and Fikrig, 2010; Dykhuizen and Brisson, 2010).

The distribution of tick-transmitted zoonoses depends on the presence of host animals and arthropod vectors involved in their natural life cycle within a particular biotope (Bouchard et

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al., 2011; Margos et al., 2011; Estrada-Peña et al., 2012; Pepin et al., 2012). Chile has a wide range of climatic conditions and many biotopes including some similar to those in the Northern Hemisphere, as well as ixodid ticks and host animals. *Ixodes stilesi* is a well-characterized tick endemic to Chile (Kohls, 1956; González-Acuña and Guglielmone, 2005). It has been shown to parasitize long-tailed pygmy rice rats (colilargos) (*Oligoryzomys longicaudatus***)** and pudu deer (*Pudu puda*), species indigenous to Southern Chile and Southwestern Argentina (Kohls, 1969; Guglielmone et al., 2006a; Guglielmone et al., 2007). While there is some evidence for transmission of zoonotic pathogenic *Babesia* spp. by *I. stilesi* collected from pudu (Tagle, 1971; Rudolph et al., 1975), *B. burgdorferi* was not identified in *Ixodes* ticks collected previously in Chile (Osorio, 2001) and could not be cultured from Chilean patients with signs and symptoms clinically compatible with LB (Abarca et al., 1996; Neira et al., 1996).

In an effort to explore the question of whether *B. burgdorferi* s.l. is present in South America, *I. stilesi* ticks collected from vegetation and colilargos in a forest reserve in Southern Chile and from captive pudus undergoing rehabilitation in the same region were examined. These ticks harbored a novel LB group borrelial species genetically distinct from other LB borreliae from North America, Europe and Asia. We propose this new borrelial genospecies be named *Borrelia chilensis* VA1 in honor of its country of origin.

Results

Identification of ticks obtained from environmental and animal sources in Chile

Thirty-eight *I. stilesi* ticks (both sexes, various stages, all flat, i.e., unfed) were collected from environmental vegetation in the San Martin Experimental Forest Preserve (39°38′ S, 73°07′ W), Valdivia, Chile, 13 in 2005 and 25 in 2008. Fifty-eight *I. stilesi* ticks (both sexes, various stages, engorged and flat) were removed from at least three captive pudus (*Pudu puda*) undergoing rehabilitation on three occasions: 19 in 2002; 27 in 2003; and 12 in 2005. Both groups of ticks were fixed in 70% ethanol shortly after collection. A third group of 12 *I. stilesi* ticks (flat nymphs) removed from four colilargos trapped in the Preserve in September, 2011, were kept alive until they had been identified and used in these studies. All ticks were identified as *I. stilesi* by standard tick keys (Kohls, 1969; Robbins et al., 2001; Guglielmone et al., 2006a). A single engorged larva removed from one of the pudus was identified as a larva of *I. stilesi* by standard tick keys. Sequencing of 16S rDNA amplicons from four randomly chosen collected ticks confirmed their identification as *I. stilesi* (Guglielmone *et al.*, 2007).

Demonstration of spirochetes in living ticks

Spirochetes with the typical flat-wave morphology of *Borrelia* were visible in midgut tissue from one of 12 *I. stilesi* ticks removed from colilargos (Fig. 1A). Microscopy of midgut tissue cultured at 34°C in Barbour-Stoenner-Kelly (BSK)-H media (Barbour, 1984; Barbour and Hayes, 1986) revealed morphologically similar spirochetes after one week of incubation (Fig. 1B). Unfortunately, the *Borrelia* culture was contaminated with a rod-shaped Gram-negative bacterium identified as *Delftia* sp. by 16S rRNA gene sequence analysis using universal primers for 16S RNA genes (Weisburg *et al.*, 1991). *Delftia* sp. are symbiotic

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Gram-negative betaproteobacteria found in some arthropods including ixodid ticks and insects (Benson et al., 2004; Hail et al., 2011; Zouache et al., 2011). During growth in suspension culture, motile *Borrelia* occupied the upper level of culture medium, *Delftia* the lower. Multiple attempts to free the culture from *Delftia* sp. using serial dilution on solid medium and liquid media, ultrafiltration (0.2 μm and 0.45 μm pore sizes), flow cytometry, and antimicrobials (kanamycin, erythromycin, tetracycline, gentamycin, novobiocin, vancomycin, bacitracin, cycloserine, rifampicin, phosphomycin, amphotericin B) were unsuccessful. *B. chilensis was* subcultured at weekly intervals in BSK-H media; early passages were stored at −80°C. Cultured spirochetes frequently grew in long filamentous forms (Figs. 1C, 1D), similar to those previously described during growth of *B. burgdorferi* sensu stricto in culture (Barbour, 1984). This organism was designated *B. chilensis* VA1.

Borrelial DNA in ticks and comparison with DNA from B. chilensis VA1 cultured from a tick

Standard and nested/seminested PCR amplification was used to detect borrelial DNA in ticks obtained from the environment and from pudus and colilargos. Tick 16S rDNA was used as an internal control to define the quality of isolated DNA. Borrelial 16S rDNA (800 bp) was amplified from one flat male tick collected from vegetation in 2005, two flat female ticks collected from vegetation in 2008 and two flat nymphs removed from colilargos in 2011, including the one in which spirochetes were observed (Table 1). The mean prevalence of infection in ticks (10%) was not significantly different between ticks collected from environmental vegetation in 2005-2008 and from colilargos in 2011 ($P > 0.05$, Fisher's exact test). None of the ticks removed from pudus contained borrelial DNA.

Borrelial DNA in ticks was examined by seminested or nested PCR for four additional loci (23S rRNA genes, 5S-23S intergenic spacer (IGS), *flaB*, *ospC*) and for eight chromosomally-located housekeeping genes (*clpA*, *clpX*, *nifS*, *rplB*, *pyrG*, *recG*, *pepX*, *uvrA*) used in a borrelial multilocus sequence typing (MLST) schema developed by (Margos et al., 2008) (Table 1). It was not always possible to amplify all genes from every infected tick (Table 1). Amplification of IGS and *ospC* sequences suggested that this borrelia was a member of the LB group, since these sequences are only present in *B. burgdorferi* s.l. (Fukunaga et al., 1992; Schwartz et al., 1992; Gazumyan et al., 1994; Margos et al., 2008; Rudenko et al., 2011; Stanek and Reiter, 2011; Margos et al., 2011). With regard to the housekeeping genes used in MLST, sequences obtained from the spirochete cultured from tick 5 (*B. chilensis* VA1), were identical to those obtained from tick 5 itself (Table 1). None of these amplified sequences was identical to sequences from described *Borrelia* species in the GenBank and MLST databases. Genetic identity between *ospC* from *B. chilensis* VA1 and *ospC* from other *B. burgdorferi* s.l. in GenBank varied from <77% for *B. valaisiana* VS116 to 82% for *B. burgdorferi* B31. Comparable values for the eight housekeeping genes from *B. chilensis* VA1 and the corresponding genes in the MLST database ranged from 87% (in the case of *clpA*) to 94% (in the case of *clpX*), indicating that they represented new alleles for each of these genes (data not shown).

Phylogenetic analyses of sequences amplified from Chilean ticks

Phylogenetic analysis of borrelial 16S rRNA genes showed that sequences amplified from infected ticks formed a single well-supported clade within the LB borrelia group distinct

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from RF and REP borreliae (Takano et al., 2010) (Fig. 2). Although insufficient overlap between 16S rRNA sequences of amplicons from Chilean and Uruguayan ticks made phlyogenetic comparison impossible, such comparison was possible with *flaB* sequences (Fig. S1). This confirmed that sequences amplified from Chilean ticks belonged to a single well-supported clade within the LB borrelia group that was distinct from all clades of *flaB* sequences amplified from Uruguayan ticks. Additional phylogenetic analyses of 23S rRNA and IGS sequences from several ticks confirmed that borrelial sequences amplified from Chilean ticks belonged to a single well-supported clade with 98% and 99% support, respectively (data not shown).

A phylogenetic tree was constructed using the MLST schema developed by Margos *et al.* (2008) in order to further clarify the taxonomic and evolutionary relationships between the Chilean borrelia, *B. chilensis* VA1, and other borrelial species (Fig. 3). *B. chilensis* VA1 was closely related to LB borrelia and only distantly related to RF borrelia. Pairwise genetic distances of the concatenated housekeeping genes of *B. chilensis* VA1 from RF borrelia were 0.2859 - 0.3011, while pairwise genetic differences from other LB borreliae were 0.0952 - 0.1138 (Table S1). All these distances are much larger than the species threshold of 0.0170 using this typing scheme (Postic et al., 2007; Margos et al., 2009) and are consistent with designating *B. chilensis* VA1 as a new species within the *B. burgdorferi* sensu lato genospecies..

Plasmid content of B. chilensis VA1

The plasmid content of *B. chilensis* VA1 was characterized using PCR and pulsed-field gel electrophoresis (PFGE) on borrelial samples taken from the upper third of culture tubes. Microscopic examination of two replicate preparations showed they contained <1 and 4 contaminating *Delftia* sp. cells per 100 spirochetes. PCR and PFGE results were similar with both preparations. lp17 was not present in *B. chilensis* VA1 by PCR (Fig. 4A) or by PFGE (Fig. 4B), but was identified in *B. burgdorferi* B31A3 by PCR and corresponded to the smallest band in PFGE. Of the larger borrelial plasmids, lp54 and lp56 were identified by PCR in *B. chilensis* VA1, while only lp54 was present in *B. burgdorferi* B31A3 (Fig. 4A). PFGE confirmed the presence of two clearly separate high molecular weight bands in *B. chilensis* VA1 and only one band in *B. burgdorferi* B31A3 (Fig. 4B). Four mid-range molecular weight plasmids were detectable in *B. chilensis* VA1 by PCR (lp28-2, cp26, cp32-2/7, cp32-3) (Fig. 4B) probably corresponding to the four mid-range bands seen on PFGE. This profile was clearly different from the mid-range profile of *B. burgdorferi* B31A3 (Fig. 4B). These results suggest that plasmid profiles of *B. chilensis* VA1 and *B. burgdorferi* B31A3 are distinct.

Discussion

We have shown the presence of motile culturable spirochetes with flat-wave morphology and borrelial DNA in adult and nymphal *I. stilesi* ticks collected from local vegetation and from a rodent endemic to Chile and Argentina. DNA sequencing analysis of PCR amplicons from 16S and 23S rRNA genes, IGS, *flaB* and *ospC* was consistent with this borrelia being a member of the *B. burgdorferi* s.l. complex. Sequences for each gene from individual ticks

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(when multiple sequences were available) formed well-supported single clades within the LB borrelial group. Furthermore, amplicons for IGS, *flaB*, *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA* from a single infected tick were identical to sequences obtained from amplicons of spirochetes cultured from this tick (Table 1). A standard borrelial MLST phylogenetic analysis of eight housekeeping genes confirmed that this borrelia was a previously undescribed member of the *B. burgdorferi* s.l. complex. Taken in toto, these observations provide strong evidence for these sequences representing a new borrelial species and the cultured organism validly representing the borrelial DNA found in ticks. We propose to name this new species *B. chilensis*, and note that this extends the range of LB group borrelia in South America and the Southern Hemisphere.

It was not possible to amplify all genes from all ticks (Table 1), a situation previously noted by others (Margos et al., 2008). The reason for this is not clear. It might be the result of low spirochete load given that microscopy of tick gut fluid showed 0-1 spirochetes per field. Low spirochete load may be a general condition of Southern Hemisphere borrelia as recently demonstrated by the inability of Barbieri et al. to amplify full sequences of a new borrelial species in ticks from Uruguay (Barbieri et al., 2013). Other causes for this lack of amplification could be DNA degradation as a result of the prolonged fixation of ticks in ethanol, the presence of PCR inhibitors in the DNA extracted from the ticks, sequence heterogeneity at the priming sites or some combination of these factors. For example, *B. chilensis ospC* showed marked sequence divergence from the *ospC* genes of other LB borreliae.

Although *B. chilensis* VA1 was always a distinct member of the LB group in all phylogenetic trees constructed using various loci, its phylogenetic relationships varied considerably (Figs. 2, 3, S1). The different branching orders in these trees do not reflect phylogenetic uncertainty or the evolutionary history of the species but rather reflect the evolutionary history of the particularl loci used to generate the trees. The basal position of *B. chilensis* in the MLST tree (Fig. 3) is thus not indicative of its ancestral status in the absence of further analyses (Crisp and Cook, 2005).

While we were unable to grow *B. chilensis* VA1 free from its contaminating *Delftia* sp. after repeated attempts by different methods, we took advantage of the physical separation of spirochetes and Gram-negative betaproteobacteria during culture to effect a partial separation of the two bacteria. PCR and PFGE showed this new *Borrelia* to contain some plasmids not present in *B. burgdorferi* B31A3 while lacking others present in this strain. Some of these latter negative results may not indicate absence of particular plasmids but may be a result of sequence heterogeneity at the priming sites. *Delftia acidovorans* contains four plasmids ranging in size from 60-77 kb (Sota et al., 2006; Król et al., 2012; Król J.E., Rogers, L.M., Sen, D., and Top, E.M., GenBank accession NC_019283, no attendent publication), but the plasmid content of Delftia sp. is in general poorly characterized (Stolze et al., 2012). We believe the plasmids we observed belong to *B. chilensis* given the lower concentration of *Delftia* in our preparations and the absence of any bands in the 60 – 77 kb region in PFGE (Fig. 4). The absence of lp17 in *B.* by both PFGE and PCR analyses could indicate a modified pathogenicity for rodents, since targeted deletion of genes on this plasmid altered tissue invasion in mice (Casselli et al., 2012). Another plasmid associated

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with borrelial pathogenicity for rodents, lp36 (Purser and Norris, 2000; Jewett et al., 2007), could not be amplified from *B. chilensis* by PCR, but its presence in PFGE analysis could not be definitively determined because of the multiplicity of mid-range sized plasmids visible on the gel. Obtaining a pure culture of this bacterium will be essential to determine its pathogenic potential. In this regard, our inability to grow *B. chilensis* VA1 free from its contaminating *Delftia* sp. may suggest a symbiotic relationship between these two bacteria and frustrate such studies.

In addition to describing this new LB borrelial genospecies, we identified one of its likely vectors, *I. stilesi*, and have confirmed previous observations that colilargos (*O. longicaudatus*) were a host for its nymphs (Guglielmone et al., 2007). Detection of all stages of *I. stilesi* in the environment where the tick samples are obtained also confirmed that these endemic ticks can complete their full life cycle in this biotope (Guglielmone et al., 2006a). Detection of *Borrelia* in two of twelve *I. stilesi* nymphs removed from four colilargos might indicate that this rodent is a reservoir for *B. chilensis* in Chile. A recent study of 900 rodents collected in this region is consistent with these suggestions (C. Hernández and R. Murúa, unpublished). This study found only 37 (4%) of them were infested with ticks. Although colilargos accounted for 32 of these infested rodents, three long-haired grass mice (*Abrothrix longipilis*) and two olive grass mice (*A. olivaceus*) were also infested. More studies of the rodent populations in this biotope will be required to determine whether and which of these rodent species can serve as reservoir hosts for borrelia.The fact that no infected *I. stilesi* were collected from pudus suggests that these animals, like deer of the Northern Hemisphere, are not competent reservoir hosts for LB borreliae (Telford, III et al., 1988; Isogai et al., 1994; Brunner et al., 2008; Kjelland et al., 2011). The recent introduction of red deer (Cervus elaphus) to the Chilean biotope where this study was carried out (Jakcsic et al., 2002) could provide an opportunity for an additional host for *Ixodes* ticks.

Even though vector competence was not established in this study, the presence of *B. chilensis* in two active life stages of *I. stilesi* suggests that the spirochete can be maintained long-term in this tick, a trait that is characteristic of *Ixodes* species that transmit borreliae. The Chilean ixodids unique to the neotropical zoogeographic region and their hosts include several other endemic or established species of ixodid ticks besides *I. stilesi* (*I. auritulus*, *I. chilensis*, *I. cornuae*, *I. neuquenensis I. sigelos*, *I. uriae*, *I. taglei*) and many potential hosts (Kohls, 1969; González-Acuña and Guglielmone, 2005; Marín-Vial et al., 2007).

The mean prevalence of infection of *I. stilesi* by *B. chilensis* (10%) is considerably lower than the 20-40% infection rate of *I. scapularis* with North American LB spirochetes in LB endemic areas (Hanincová et al., 2006; Kurtenbach et al., 2006). The relatively low infection rate of *I. stilesi*, coupled with an apparently homogeneous population of *B. chilensis*, suggests that the tick and spirochete might have evolved under conditions where introductions of borrelial species by migrating animals (including birds) were infrequent, perhaps as a result of the geographical isolation of Southern Chile (Matuschka et al., 1998). Alternatively, *I. stilesi* may be nidicolous with a low frequency of carriage of borrelia as in the case of *I. arboricola* (Heylen et al., 2013). These and other factors make it difficult to evaluate the pathogenic potential of *B. chilensis* for human beings and domestic animals in

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this ecotope at this time (Kurtenbach et al., 1995; Guglielmone et al., 2006b; Bouchard et al., 2011).

The possibility that *I. stilesi* is a vector for *B. chilensis* VA1 in this South American ecotope raises a series of questions on the independent origin and co-evolution of this borrelial genospecies and its vector and how they reached this ecotope. It is generally accepted that the North American and European populations of *B. burgdorferi* s.l. are related but have evolved independently (Margos et al., 2008; Qiu et al., 2008; Hoen et al., 2009; Kurtenbach et al., 2010). In this context, it could be suggested that the ancestors of *B. chilensis* and *I. stilesi* migrated from North America with rodent hosts in the Great American Interchange after the closing of the Isthmus of Panama 3 million years ago (Knowles and Richards, 2005; Kirby et al., 2008). Alternatively, the ancestors of *Ixodes* already present in Pangaea passed to Gondwanaland and then to South America where they evolved independently, only coming in contact with rodents infected with *Borrelia* after the Great American Interchange (Balashov, 1994; Duarte et al., 2008). *B. chilensis* could also have been introduced by migratory birds or be a relict population generated by the northern migration of hosts and vectors following retreat of the glaciers (Margos et al., 2011). The unique sequence of the *ospC* gene in *B. chilensis* VA1 suggests that selective forces present in the mammalian population of this ecotope might result in an important degree of host specialization or immune-escape frequency-dependent selection (Brisson and Dykhuizen, 2004; Barbour and Travinsky, 2010; Haven et al., 2011).

In summary, this study provides solid evidence for extending the range of LB borreliae to the western coast of South America. It also serves as a basis for further studies of relationships of *B. chilensis* with *Ixodes* ticks and rodents, and the pathogenic relevance of this genospecies.

Experimental procedures

Collection and identification of ticks

Ticks for this study were collected in the San Martin Experimental Forest Preserve (39°38′ S, 73°07′ W), Universidad Austral de Chile, Valdivia, Chile and from captive pudu deer undergoing rehabilitation at the Wildlife Rescue Center in the Veterinary Hospital at the Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia. The preserve is located in the Valdivian temperate rain forest ecoregion in the Eastern foothills of the Pacific coastal range in Region de los Rios (Region XIV) at an average elevation of 3 m above sea level. Its climate is temperate and wet (average temperature, 12.1°C, range 0° to 30°C; average annual rainfall, 2,500 mm) (Miller, 1976; Veblen and Schlegel, 1982).

Ticks were collected in 2002, 2003, 2005, 2008 and 2011. A group of 38 ticks was collected from the environment during the Austral springs of 2005 and 2008 by dragging 1 m^2 panels of white corduroy cloth over the ground and vegetation (Moreno et al., 2006). A second group of 58 ticks was collected from from at least three captive pudus (*Pudu puda*) undergoing rehabilitation at the Wildlife Rescue Center in the Veterinary Hospital, Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia (one in 2002, one in 2003, and at least one in 2005). All ticks clinging to the drag cloth or attached to the deer were

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removed with forceps, fixed with 70% ethanol and stored in vials at 4°C for later identification and DNA extraction. A third group of 12 ticks was removed with forceps from four colilargos (*O. longicaudatus*) trapped in the Reserve in September, 2011. These ticks were stored alive in sterile glass vials at ambient temperature for later identification, borrelial culture, and DNA extraction. Fixed and living ticks were identified by standard tick keys (Kohls, 1969; Robbins et al., 2001; Guglielmone et al., 2006a) and by sequencing 16S rDNA amplicons generated with primers IXOst1 and IXOst2 (Table S2). These primers were constructed based on the *I. stilesi* rDNA sequence (GenBank accession no. EF362757).

Tick processing and borrelial culture

Individual live ticks were sequentially washed in 3% hydrogen peroxide, 95% ethanol, 0.1% sodium hypochlorite and phosphate-buffered saline, pH 7.2 (PBS) (Varela *et al.*, 2004) and split in two. One part was used for DNA extraction; the other was crushed with a sterile needle in 100 μl of PBS. A small aliquot of this mixture was taken for microscopy. The remainder was inoculated into 3 ml of BSK-H media (Sigma Chemical, St. Louis, MO) supplemented with 6% rabbit serum (Sigma) and *Borrelia* antibiotic mix (Sigma), and cultured at 34°C for 10 weeks (Barbour, 1984; Barbour and Hayes, 1986). Cultures were checked weekly for bacterial growth by fluorescence microscopy after staining with acridine orange (Fisher Scientific, Fair Lawn, NJ), and subcultured when cells reached early stationary phase of growth. Photomicrographs of aliquots of culture were taken with a DVC-1412AC digital camera (Digital Video Camera Company, Inc., Austin, TX), attached to Olympus Bx60 microscope (Olympus Optical, Tokyo, Japan) equipped with a Plan phase-contrast objective (magnification, ×100).

DNA extraction, PCR and sequencing

Ticks were processed individually. Total DNA from a whole or partial tick or from fourth passage *Borrelia* culture was extracted using DNeasy Blood and Tissue kit (QIAGEN Sciences, Germantown, MD) according the manufacturer's instructions, and was immediately used for PCR analysis or was stored at −20°C for later analysis. Several tubes from which tick tissue had been omitted were included in each group of assays as negative controls to detect cross-contamination. DNA extraction was done in a room physically separate from those where PCR analysis and gel electrophoresis were performed to avoid contamination. All tick DNA samples were subjected to PCR amplification using the primers shown in Table S2. DNA from ticks positive for borrelial 16S rRNA and genomic DNA from fourth passage cultures of *B. chilensis* VA1 was assayed by PCR for 23S rDNA,; IGS; *flaB*; *ospC*; and eight housekeeping genes (borrelial MLST) using published primers and protocols (Postic, *et al.*, 1994, de Silva, *et al.*, 1998, Wang, *et al.*, 1999; Margos et al., 2008). Genomic DNA of *B. burgdorferi* B31A3 was used as a positive control for all PCR. All PCR amplicons were isolated and purified from agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol, and were sequenced in both forward and reverse directions in GENEWIZ (South Plainfield, NJ). Sequences of each gene and their traces were carefully reviewed, tested individually, and searched against sequences available in the GenBank using BLAST and in the MLST (http://borrelia.mlst.net) databases using implemented facilities.

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Phylogenetic analysis

Sequences for analysis were obtained from GenBank (using BLAST on complete genomes as necessary) and from *B. burgdorferi* MLST database (http://borrelia.mlst.net/) (Table S3). Phylogenetic trees were constructed and pairwise distances between sequences computed from multiple sequence alignment by log expectation (MUSCLE)-aligned loci sequences and from concatenated sequences of eight housekeeping genes using the Maximum Likelihood method in MEGA5 with bootstrap test (n = 1000 replicates) (Tamura et al., 2011). Best-fit models chosen in MEGA included: Kimura 2-parameter model (Kimura, 1980) with a discrete Gamma distribution (16S, IGS); Kimura 2-parameter model (Kimura, 1980) (23S); Tamura 3-parameter model (Tamura, 1992) with a discrete Gamma distribution (*flaB*, *ospC*); and General Time Reversible model (Nei and Kumar, 2000) with a discrete Gamma distribution, some sites to be evolutionarily invariable (MLST). Trees were rooted using RF group borreliae as the outgroup (Margos et al., 2009)

Analysis of plasmid content

Cells from 10 ml (10^7 cells/ml) fourth passage cultures of *B. chilensis* VA1 and second passage of *B. burgdorferi* B31A3 after isolation from infected mice were centrifuged at 7500 × g for 10 min to pellet the bacteria. Cells were washed twice in PBS, resuspended in 300 μl of 50 mM Tris, pH 7.6, 50 mM NaCl, mixed with 300 μl of 1.8 % agarose (Bio-Rad Laboratories, Hercules, CA), and placed in the PFGE insert mold (Bio-Rad). Cells in these agarose blocks were lysed with a buffer containing 50 mM Tris-HCl, pH 8, 50 mM ethylendiaminetetraacetic acid (EDTA), 1% sodium dodecylsulphate and proteinase K (1 mg/ml) at 50°C for 24 h, then washed extensively in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and used for PFGE (Walker et al., 1995) (CHEF-DR II pulsed-field electrophoresis system, Bio-Rad) in 0.5X TBE with pulse time increasing from 0.7 to 2.2 sec at 14°C for 19 h at 6 V/cm using MidRange PFG marker I (New England Biolabs, Ipswitch, MA, USA) as molecular reference marker. Plasmid patterns were visualized after ethidium bromide staining. Analysis of plasmid content in *B. chilensis* VA1 was done by PCR using primers and conditions developed for the plasmids of *B. burgdorferi* B31 (Iyer et al., 2003).

Nucleotide sequence accession numbers

GenBank accession numbers of sequences reported in the present study: *nifS*, JX564989; *rplB*, JX564990; *pyrG*, JX564991; *recG*, JX564992; *pepX*, JX564993; *clpX*, JX564994; *clpA*, JX564995; *uvrA*, JX564996; 23S rRNA gene, JX669128; 16S rRNA gene, JX669129; *ospC*, JX669130; *flaB*, JX669131; 5S-23S IGS, JX669132. MLST allele numbers of sequences reported in the present study: *clpA*, 170; *clpX*, 133; *nifS*, 123; *pepX*, 148; *pyrG*, 148; *recG*, 148; *rplB*, 130; *uvrA*, 140. The *B. chilensis* VA1 allelic profile is designated MLST serotype ST430.

Supplementary Material

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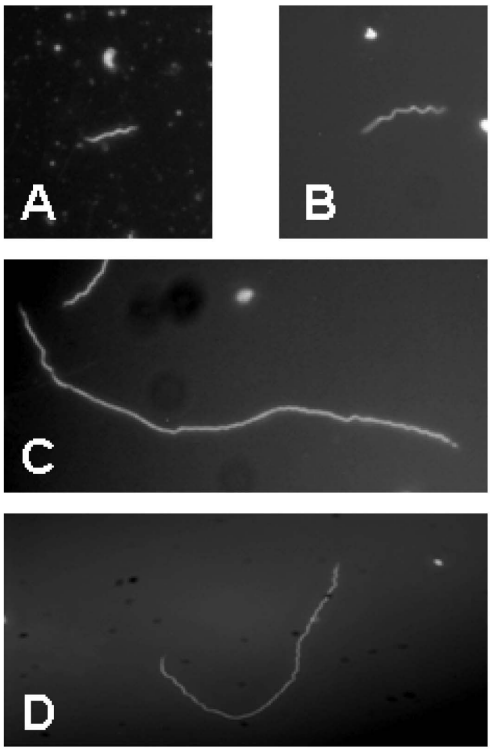

Fig. 1.

Morphology of spirochetes from *I. stilesi* midgut (A), or after *in vitro* culture for (B) 5 or (C) 12 days or after (D) 9 passages in BSK-H media (magnification, 100×).

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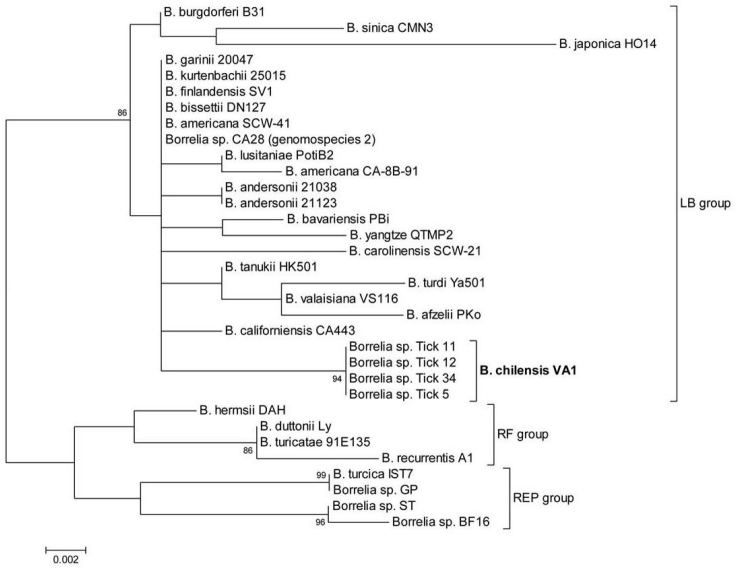

Fig. 2.

Phylogenetic bootstrap analysis of 16S rDNA of *B. chilensis* VA1 and other borrelial species using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura , 1980). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; +*G*, parameter = 0.1000). The tree with the highest log likelihood (−785.9) is drawn to scale with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is indicated next to the branches; values <75% have been omitted. The analysis involved 33 nucleotide sequences with 355 positions in the final data set after all positions containing gaps and missing data had been eliminated. See Experimental Procedures for details.

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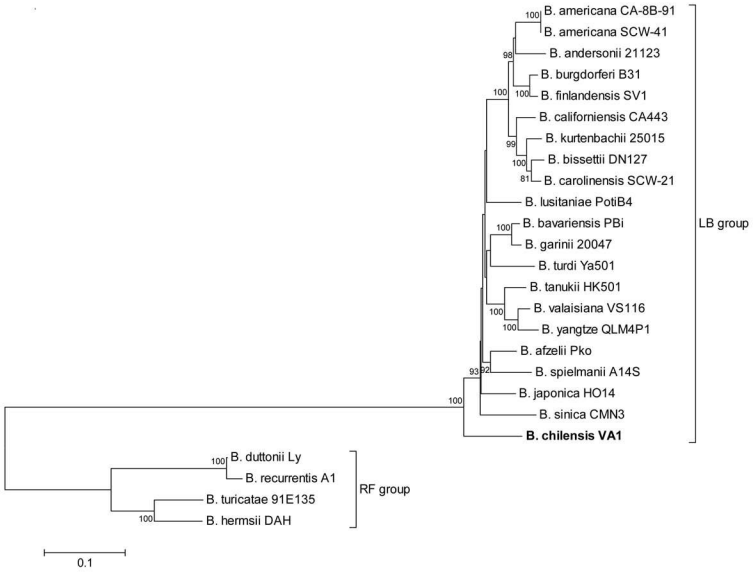

Fig. 3.

Phylogenetic bootstrap analysis (n = 1,000) of concatenated sequences of eight housekeeping genes of *B. chilensis* VA1 and other borrelial species using the Maximum Likelihood method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; +*G*, parameter = 0.3517). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.0000% sites). The tree with the highest log likelihood (−28,311) is drawn to scale with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is indicated next to the branches; values <75% have been omitted. The analysis involved 24 nucleotide sequences with 4767 positions in the final dataset after all positions containing gaps and missing data were eliminated. See Experimental Procedures for details.

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A.

Plasmid	*B. burgdorferi* B31A3	*B. chilensis* VA1
lp17	+[a]	-
lp25	+	-
lp28-1	-	-
lp28-2	+	+
lp28-3	-	-
lp28-4	+	-
lp36	+	-
lp38	+	-
lp54	+	+
lp56	-	+
cp26	+	+
cp32-1	+	-
cp32-2/7	-	+
cp32-3	+	+
cp32-4	+	-
cp32-5	+	-
cp32-6	+	-
cp32-8	+	-
cp32-9	-	-
cp32-11	-	-

a. +, amplicon obtained; -, no amplicon obtained.

B.

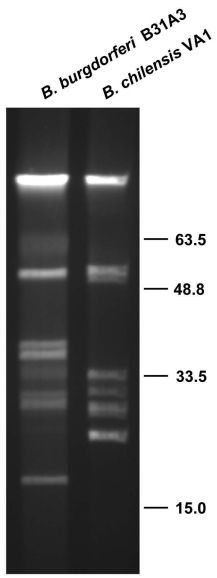

Fig. 4.

Plasmid profiles of *B. burgdorferi* B31A3 and *B. chilensis* VA1 analyzed by (A) PCR using primers designed for *B. burgdorferi* B31 and (B) PFGE of whole-cell DNA prepared in agarose plugs. See Experimental Procedures for details.

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Table 1

B. chilensis VA1 sequences in *I. stilesi* ticks and in *B. chilensis* VA1 cultured from *I. stilesi*

		Source		Stage		State	rRNA			Housekeeping genes									
Date Collected	**Description**	**Rice rats**	**Environmental Vegetation**	**Nymph**	**Adult**	**Flat**	**16S**	**23S**	**IGS**	***flaB***	***ospC***	***clpA***	***clpX***	***nifS***	***pepX***	***pyrG***	***recG***	***rplB***	***uvrA***
2005[a]	Tick 34		+		Male	+	+		+	+			+		+	+		+	+
2008[b]	Tick 11		+		Female	+	+	+		+			+	+	+	+	+	+	
2008[b]	Tick 12		+		Female	+	+	+		+				+		+			
2011[c]	Tick 3	+		+		+							+	+	+	+		+	+
2011[c]	Tick 5	+		+		+	+	+	+	+		+	+	+	+	+	+	+	+
	Bch VA1[d]								+	+	+	+	+	+	+	+	+	+	+

[a]2005: 13 ticks from environmental vegetation, fixed in 70% ethanol at 4°C: 1 positive for *B. chilensis* VA1 (7.7 % prevalence).

[b]2008: 25 ticks from environmental vegetation, fixed in 70% ethanol at 4°C: 2 positive for *B. chilensis* VA1 (8.0 % prevalence).

[c]2011: 12 live ticks from four long-tailed pygmy rice rats (colilargos) (*Oligoryzomys longicaudatus*), 2 ticks positive for *B. chilensis* VA1 (17 % prevalence). Difference in prevalence of infection between ticks obtained from environmental vegetation and ticks removed from rice rats is not significant (P > 0.5, Fisher's exact test).

[d]*B. chilensis* (Bch) VA1 cultured from tick 5 (fourth passage). Sequences for *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB* and *uvrA* were identical to those obtained directly from tick 5.