**Borrelia sinica** sp. nov., a Lyme disease-related Borrelia species isolated in China

Toshiyuki Masuzawa,¹ Nobuhiro Takada,² Midori Kudeken,¹ Takako Fukui,¹ Yasuhiro Yano,² Fubito Ishiguro,³ Yoshiaki Kawamura,⁴ Yasuyuki Imai¹ and Takayuki Ezaki⁴

A survey was performed for Lyme disease borrelia in the southern part of China, in Zhejiang, Sichuan and Anhui provinces, along the Yangtze River valley, in May of 1997 and 1998. Twenty isolates from *Ixodes granulatus*, *Ixodes ovatus*, *Apodemus agrarius* and *Niviventer confucianus* were obtained. These isolates were characterized by RFLP of the 5S–23S rDNA intergenic spacer, sequence analysis of the intergenic spacer, 16S rDNA and flagellin gene, DNA–DNA hybridization analysis, SDS-PAGE and Western blotting with mAbs. Six isolates from *A. agrarius*, five from *I. granulatus* collected in Zhejiang province and one from *N. confucianus* in Sichuan province were highly similar to strains 10MT and 5MT isolated in Korea and classified as *Borrelia valaisiana*. Four isolates from *A. agrarius* and *I. granulatus* collected in Zhejiang province generated unique RFLP patterns and phylogenetic analysis of the 16S rDNA and flagellin gene sequences suggested that the isolates should be classified as *B. valaisiana*. Furthermore, three isolates (CMN1a, CMN2, CMN3⁷) from *N. confucianus* captured in Sichuan province and one (CWO1) from *I. ovatus* in Anhui province showed lower 16S rDNA sequence similarity (less than 99.0%) to sequences of previously described Lyme disease-related *Borrelia* species. DNA–DNA hybridization results revealed that strains CMN3⁷ and CMN1a were clearly distinct from all other known Lyme disease *Borrelia* species. Electron microscope observation showed the spirochaetes to be morphologically similar to those of *Borrelia*, but the cells contained only four periplasmic flagella inserted at each end of the spirochaetes. Based on these results, a new *Borrelia* species, *Borrelia sinica* sp. nov., is proposed. Strain CMN3⁷ is the type strain of this new species.

**Keywords**: *Borrelia sinica, Borrelia valaisiana*, China, *Ixodes ovatus*, Lyme disease

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**INTRODUCTION**

Lyme borreliosis is the most prevalent tick-borne zoonotic disease in Europe, North America and Far-Eastern countries such as Japan, Russia and China (Steere, 1989). The causative agent, *Borrelia burgdorferi* (Johnson et al., 1984), was isolated from the tick *Ixodes dammini* (Burgdorfer et al., 1982). *B. burgdorferi sensu lato* is classified into 10 species: *B. burgdorferi sensu stricto*, isolated in North America and Europe; *Borrelia garinii* (Baranton et al., 1992) and *Borrelia afzelii* (Canica et al., 1993), isolated in Europe and east Asian countries; *Borrelia japonica*, *Borrelia turdi* and *Borrelia bissetti*, respectively isolated from *Ixodes ovatus*, *Ixodes tanuki* and *Ixodes turdi* in Japan (Fukunaga et al., 1996; Kawabata et al., 1993); *Borrelia andersonii*, isolated from *Ixodes dentatus* in North America (Marconi et al., 1995); *Borrelia valaisiana* (Wang et al., 1997) and *Borrelia lusitaniae* (Le Fleche et al., 1997), isolated from *Ixodes ricinus* in Europe; and *Borrelia bissetti* (Postic et al., 1998), isolated in North America. Furthermore, *B. valaisiana*...
has been found in Korea, Taiwan and Japan (Kee et al., 1996; Masuzawa et al., 1999, 2000; Wang et al., 1997).

We previously performed a survey in the north-eastern part of China and isolates from Ixodes persulcatus and from the rodent Apodemus peninsulae were identified as B. afzelii and B. garinii, but no isolates were determined as B. burgdorferi sensu stricto (Li et al., 1998). In order to examine the prevalence of Lyme disease Borrelia and to discover new Borrelia species, we performed a survey in the southern part of China in 1997 and 1998. Consequently, 20 isolates were obtained from Ixodes granulatus, I. ovatus, Apodemus agrarius and Niviventer confucianus. The aim of this study was to characterize these isolates based on their genetic and phenotypic properties. On the basis of the results of the present study, we propose that isolates from N. confucianus captured in Sichuan province and I. ovatus collected in Anhui province should be described as Borrelia sinica sp. nov.

**METHODS**

**Survey sites and isolation of Borrelia.** The Borrelia isolates from China are listed in Table 1. Ticks and wild rodents were collected in the southern part of China, from Zhejiang, Sichuan and Anhui provinces, in May of 1997 and 1998. Ixodid ticks were collected by dragging vegetation at woodland areas. At the same time, wild rodents were also captured in Sherman live traps. Spirochaetes were isolated from earlobe tissues of rodents (A. agrarius and N. confucianus) and the midgut of two species of tick (I. granulatus and I. ovatus) by the method described previously (Takada et al., 1998) and were cultivated at 30°C in Barbour–Stoenner–Kelly II medium (Barbour, 1984).

**PCR and RFLP analysis of 5S–23S rDNA intergenic spacer.** Primers corresponding to the 3' end of the 5S rDNA (5'-CTGCCAGTTCGGGAGA-3') and the 5' end of the 23S rDNA (5'-TCTAGCCATTCCACCATA-3') (Masuzawa et al., 1996a; Postic et al., 1994) were synthesized. PCR was performed by the method described previously (Postic et al., 1994). The amplification products of 5S–23S rDNA amplicons were digested with MseI and Dral and the digested DNA was electrophoresed through 16% polyacrylamide gels. DNA bands were visualized by ethidium bromide staining.

**Sequencing of 5S–23S rDNA intergenic spacer, the flagellin gene and 16S rDNA.** 16S rDNA sequences and flagellin genes were amplified with primer pairs 16SF1 (5'-ATAACGGAAGTGTGTGATCGGC-3') and 16SR (5'-CACCGCAGCTTTCAGTACG-3'), corresponding to the 5' and 3' ends of the 16S rDNA, and flaF (5'-GATTATAATCATATAAGGTATTCGC-3') and flaR2 (5'-CTCCTCATAAAGGCGTGAGTTTGCA-3'), corresponding to the 5' and 3' ends of fla. The amplicons from 16S rDNA (about 1350 bp), the flagellin gene (about 970 bp) and the intergenic spacer sequence (about 250 bp) were cloned into the pGEM-T vector and the recombinant plasmids were transformed into Escherichia coli JM109 using the pGEM-T vector system (Promega) according to the manufacturer’s instructions. The

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolated from:</th>
<th>Location</th>
<th>Size (bp)</th>
<th>Amplicon</th>
<th>Dral fragments</th>
<th>MseI fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. valaisiana</td>
<td>A. agrarius</td>
<td>Hangzhou, Zhejiang</td>
<td>254</td>
<td>173, 81</td>
<td>150, 58, 24, 22</td>
<td></td>
</tr>
<tr>
<td>CKA2a*, CKA3b*, CKA5b</td>
<td>I. granulatus</td>
<td>Hangzhou, Zhejiang</td>
<td>~ 250</td>
<td>CKA2a type</td>
<td>CKA2a type</td>
<td></td>
</tr>
<tr>
<td>CKG1, CKG2, CKG3, CKG5, CKG6</td>
<td>N. confucianus</td>
<td>Nanchuan, Sichuan</td>
<td>~ 250</td>
<td>CKA2a type</td>
<td>CKA2a type</td>
<td></td>
</tr>
<tr>
<td>CNGN1b</td>
<td>A. agrarius</td>
<td>Hangzhou, Zhejiang</td>
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<td>173, 81</td>
<td>107, 58, 43, 24, 22</td>
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</tr>
<tr>
<td>CKA2b*, CKA4a, CKA4b</td>
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<td>107, 58, 43, 24, 22</td>
<td></td>
</tr>
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<td>173, 81</td>
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<tr>
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<td>107, 58, 43, 22</td>
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</tr>
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<td>Borrelia sinica sp. nov.</td>
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<tr>
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<td>Mt Huangshan, Anhui</td>
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<td>144, 50, 42</td>
<td>107, 49, 38, 29, 13</td>
<td></td>
</tr>
<tr>
<td>CW1*</td>
<td>I. ovatus</td>
<td>Mt Huangshan, Anhui</td>
<td>236</td>
<td>144, 50, 42</td>
<td>107, 49, 38, 29, 13</td>
<td></td>
</tr>
<tr>
<td>Reference strains</td>
<td>L. ricius</td>
<td>Switzerland</td>
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<td>203, 52</td>
<td>174, 51, 23, 7</td>
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<tr>
<td>B. valaisiana VS116*</td>
<td>L. ricius</td>
<td>Korea</td>
<td>254</td>
<td>173, 81</td>
<td>150, 58, 24, 22</td>
<td></td>
</tr>
<tr>
<td>B. valaisiana 10MT*</td>
<td>L. nipponensis</td>
<td>Korea</td>
<td>254</td>
<td>173, 81</td>
<td>150, 58, 24, 22</td>
<td></td>
</tr>
</tbody>
</table>

*Exact fragment sizes were determined from the sequences. Sequences of strains CK2a (accession no. AB022124), CK3b (AB022125), CK42b (AB022126), CKA1 (AB022127), CKA3a (AB022128), CNGN1a (AB022129), CW1 (AB022130) and CNGN3* (AB022131) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: VS116* (L30134), 5MT (AB013914) and 10MT (AB013915).
Borrelia sinica sp. nov.

Fig. 1. Phylogenetic tree based on flagellin gene sequences. Sequences of strains CKA1 (accession no. AB022132), CKA2a (AB022133), CMN1b (AB022134), CKA3a (AB022135), CKA4a (AB022136), CMN1a (AB022137), CMN3 T (AB022138) and CKA3b (AB022139) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: VS116 T (D82854), 10MT (AB014678), 5MT (AB014677), Am501 (D82855), B31 T (X15661), 20047 T (D82846), HT17 (D63363), HT19 (D63371), HT37 (D63369), VS461 T (D63365), HT61 (D63366), HT16 (D63366), HO14 T (D82852), 21123 (D83764), Hk501 T (D82847), Ya501 T (D82849), PotiB2 T (D85856), DN127 (D82857) and the relapsing fever borrelia, Borrelia hermsii strain HS1 (M86838). Bar, sequence divergence of 1–20%. Chinese isolates are indicated in bold type.

Nucleotide sequence accession numbers. The intergenic spacer, flagellin gene and 16S rDNA sequences were submitted to DDBJ, EMBL and GenBank with the accession numbers listed in the legends to Figs 1 and 2 and in Table 1.

Determination of DNA–DNA hybridization. DNAs were extracted and purified from strains CMN3 T and CMN1a and 10 strains of B. burgdorferi sensu lato species by the method described previously (Kawabata et al., 1993). Purified reference DNAs were used to determine DNA–DNA hybridization by the microplate hybridization method (Ezaki et al., 1988; Kawabata et al., 1993). Aliquots containing 1 µg heat-denatured DNA solution were incubated for 12 h at 37°C in microdilution plates (Dynatech Laboratories). The DNA solution was discarded and then dried overnight at 60°C. Two-hundred microlitres of pre-hybridization solution [2 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 x Denhardt’s solution, 50% formamide] containing 200 µg denatured salmon sperm DNA ml⁻¹ was added to microdilution wells that had been coated with reference DNAs. The microdilution plates were incubated at 37°C for 1 h and then washed. Photobiotin-labelled DNA was prepared as described previously (Ezaki et al., 1989) and 50 ng labelled DNA was then added. Hybridization was performed with hybridization solution (2 x SSC) containing 50% formamide at 26°C (Blüthmann et al., 1973). After hybridization, the microdilution plates were washed with 2 x SSC buffer. Streptavidin-β-D-galactosidase (Gibco) was added to the wells and incubated at 37°C for 30 min. After incubation, the wells were washed and 4-methylumbelliferyl β-D-galactopyranoside (Wako) was added as substrate. After incubation, the fluorescence intensity was measured with a MicroFluor reader (Dynatech Laboratories) at a wavelength of 360 nm for excitation and 450 nm for emission. The experiment was performed in triplicate.

Recombinant plasmids were extracted from E. coli cultures using a Plasmid Miniprep kit (Bio-Rad) and sequenced by the dye-deoxy chain-termination method using a Taq Dye-Deoxy Terminator cycle sequencing kit with primers M13 (f129) and M13 reverse and a model 373A DNA sequencer (Applied Biosystems) or Sequi Them EXEL II Long-Read DNA sequencing kit (Epicentre Technology) and LI-COR 4000 DNA sequencer. At least three clones were sequenced.

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Fig. 2. Phylogenetic tree based on 16S rDNA sequences. Sequences of strains CNM2\textsuperscript{T} (AB022101), CKA3a (AB022140), CKA1 (AB022141), CKA2a (AB022142), CKA4a (AB022143), CMN1a (AB022144), CMN2 (AB022145) and CWO1 (AB022146) were determined in this study. Previously published sequences, deposited with the following accession numbers, were also utilized: 5MT (U44938), 9MT (L39080), 10MT (AB016974), VS116\textsuperscript{T} (X98232), UK (X98233), Am501 (D67021), B31\textsuperscript{T} (B59293), DK21 (X85191), 20047\textsuperscript{T} (D67018), PBi (X85199) and \textit{B. hermsii} strain HS1 (U42292). Bar, sequence divergence of 0.2\%. Chinese isolates are indicated in bold type.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were carried out according to methods described previously (Masuzawa et al., 1991). After electrophoresis, the gels were stained with Coomassie brilliant blue. Antigens were transferred onto PVDF membranes (Bio-Rad) and specific antigen bands were subsequently detected by immunostaining with mAbs. The mAbs used were: H9724 and O1441b to flagellin; LA2, H5332, P31b, P31c and P31d to outer surface protein A (OspA) (Masuzawa et al., 1996c); G7 to OspC (Masuzawa et al., 1996b); O62a to heat-shock protein 60 (Hsp60) (Masuzawa et al., 1996c); and D6 to 12 kDa antigen (Baranton et al., 1992).

Electron microscopy. To prepare negatively stained specimens, a drop of the cell suspension concentrated by centrifugation was adsorb onto carbon-coated Formvar film supported on copper grids. The cells were stained with 2\% sodium phosphotungstate for 30 s. The specimens were examined with a Hitachi H-7000 electron microscope at an acceleration voltage of 100 kV. To determine the numbers of flagella, cross-sections of \textit{Borrelia} cells were prepared. Aliquots of 5 ml borrelial culture (strains CMN1a and CMN3\textsuperscript{T}) in BSK II medium were fixed by addition of an equal volume of 4\% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 6 h at 4\°C. The cells were harvested by centrifugation, washed gently in buffer and post-fixed with cold 1\% osmium tetroxide for 3.5 h. The pellet was mixed with 0.5 ml 2\% agarose. The agarose blocks were dehydrated in graded acetone and embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead acetate before observation with a Hitachi H-600 electron microscope at an acceleration voltage of 75 kV.

G + C contents of DNAs. DNA base compositions were determined by HPLC (Noguchi et al., 1988) using a DNA-GC kit (Yamasa Shoyu).

RESULTS AND DISCUSSION

RFLP analysis and sequencing of the 5S–23S rDNA intergenic spacer

The results of RFLP analysis of the 5S–23S rDNA intergenic spacer sequence are summarized in Table 1. Exact fragment sizes generated after digestion with restriction enzymes \textit{DraI} and \textit{MseI} were determined by sequencing analysis of the amplicon. Eight strains (CKA2a, CKA3b, CKG1, CKG2, CKG3, CKG5,
Table 2. DNA–DNA relatedness of *B. sinica* sp. nov. and other species of *B. burgdorferi sensu lato*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Level of DNA relatedness with labelled DNA from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. CMN3&lt;sup&gt;T&lt;/sup&gt;</td>
<td>100.0</td>
</tr>
<tr>
<td>2. CMN1a</td>
<td>84.9</td>
</tr>
<tr>
<td>3. <em>B. burgdorferi</em> B31&lt;sup&gt;T&lt;/sup&gt;</td>
<td>58.5</td>
</tr>
<tr>
<td>4. <em>B. garinii</em> 20047&lt;sup&gt;T&lt;/sup&gt;</td>
<td>52.5</td>
</tr>
<tr>
<td>5. <em>B. afzelii</em> Fi81t</td>
<td>50.8</td>
</tr>
<tr>
<td>6. <em>B. japonica</em> HO14&lt;sup&gt;T&lt;/sup&gt;</td>
<td>52.1</td>
</tr>
<tr>
<td>7. <em>B. afzelii</em> VS461&lt;sup&gt;T&lt;/sup&gt;</td>
<td>43.1</td>
</tr>
<tr>
<td>8. <em>B. turdi</em> Ya501&lt;sup&gt;T&lt;/sup&gt;</td>
<td>46.4</td>
</tr>
<tr>
<td>9. <em>B. andersonii</em> 21123</td>
<td>38.8</td>
</tr>
<tr>
<td>10. <em>B. valaisiana</em> VS116&lt;sup&gt;T&lt;/sup&gt;</td>
<td>30.4</td>
</tr>
<tr>
<td>11. <em>B. lactitiae</em> PotiB2&lt;sup&gt;T&lt;/sup&gt;</td>
<td>47.4</td>
</tr>
<tr>
<td>12. <em>B. bissetti</em> DN127</td>
<td>47.0</td>
</tr>
</tbody>
</table>

<FIG. 3. SDS-PAGE profiles of Chinese *Borrelia* strains. Lanes: M, molecular mass markers; 1, CKA1; 2, CKA2a; 3, CKA3a; 4, CKA4a; 5, CKA1; 6, CKA2b; 7, CMN1a; 8, CMN1b; 9, CMN2; 10, CMN3<sup>T</sup>; 11, CWO1; 12, B31<sup>T</sup>. Ten micrograms of protein was applied to each lane of the 12.5% polyacrylamide gel. Arrowheads indicate the position of OspA reactive to mAb P31c.>

Other eight strains listed in Table 1 generated unique RFLP patterns that could be differentiated from those of species reported previously (Masuzawa *et al*., 1999).

**Phylogenetic analysis based on flagellin gene sequences**

A phylogenetic tree based on flagellin gene sequences (Fig. 1) was constructed on the basis of a sequence-similarity matrix of the flagellin gene. The sequences of the six strains CKA2a, CMN1b, CKA3a, CKA4a, CKA1 and CKA3a showed higher similarity values, ranging from 97-1 to 99-7%, to strains 5MT, 10MT, Am501 and VS116<sup>T</sup> and were clustered with these strains. Strains CMN1a and CMN3<sup>T</sup>, isolated from *N. confucianus* captured in Sichuan, diverged from *B. valaisiana* and other previously described species.

**Phylogenetic analysis of 16S rDNA sequences**

A phylogenetic tree based on 16S rDNA sequences (Fig. 2) was constructed on the basis of a sequence-similarity matrix (1367 bp). The sequences of the four strains CKA2a, CMN1b, CKA3a and CKA4a clustered with *B. valaisiana* strains VS116<sup>T</sup>, UK, Am501, 10MT and 5MT and showed the highest similarity to strains of this group, ranging from 98-8 to 99-9%.

Strains CMN1a, CMN2, CWO1 and CMN3<sup>T</sup> showed relatively lower similarity to previously described species, whereas these strains showed high levels of similarity to each other, ranging from 99-8 to 100%. These observations suggested that these isolates were different from previously described species.

**DNA–DNA hybridization**

Chromosomal DNA–DNA hybridization values among strains of 10 *B. burgdorferi sensu lato* species are shown in Table 2. The levels of DNA relatedness of
strains CMN3\(^\text{T}\) and CMN1a were less than 70\% when compared with the type strains of 10 species described previously. This result indicated that strains CMN3\(^\text{T}\) and CMN1a were not members of previously known species.

### Protein profiles and reactivity with mAbs

Strains classified as *B. valaisiana* showed variable molecular masses of OspA and OspB. Likewise, four strains of the novel taxon had variable sizes of proteins that seemed to be OspA and OspB, ranging from 31 to 35 kDa (Fig. 3). All Chinese strains tested reacted with mAbs H9724 to flagellin and 062a to Hsp60, which are specific to *Borrelia* species or *B. burgdorferi sensu lato* (Table 3). Chinese isolates classified as *B. valaisiana* reacted with mAb P31b, specific for OspA of *B. burgdorferi sensu stricto*, D6, specific for *B. garinii*, or P31d, specific for OspA of *B. afzelii* (Table 3). Chinese isolates classified as *B. valaisiana* reacted with mAb P31c, specific for OspA, as did Korean strains 5MT and 10MT, members of *B. valaisiana*. No reactivity of strains CMN3\(^\text{T}\), CMN1a, CMN2 or CWO1 was observed with mAbs reactive to OspA in this study. This result suggested that the four strains differed from known species. However, the group of *Borrelia* could not be identified and distinguished by SDS-PAGE protein profiles alone.

### Morphology

On electron microscope observation of negatively stained specimens, the cells showed the characteristic borrelial shape, i.e. a helical shape with tapered ends. The length of the cells was variable, about 10–25 \(\mu\)m, and the diameter was 0.25–0.3 \(\mu\)m (Fig. 4a). The maximum number of flagella observed at the tapered end of the cells was four. An insertion point was observed in the preparation (Fig. 4b). In order to determine the number of flagella, cross-sections of *Borrelia* cells were prepared; two to four flagella were observed within the ridge (Fig. 4c–f). These results suggested that cells of the novel taxon have two to four flagella at each end. Cells of *B. burgdorferi sensu lato* have approximately seven to fourteen flagella at each end (*Burgdorfer et al.,* 1982; *Hovind-Hougen et al.,* 1986; *Oliver et al.,* 1993; *Yano et al.,* 1997; *Zung et al.,* 1989). *Barbour & Hayes* (1986) clearly demonstrated seven periplasmic flagellar insertion points in the terminus portion of *B. burgdorferi*. The number of flagella in these novel isolates was thus very low in comparison with other borrelial species. Since the flagella are generally responsible for motility, the number of flagella may also affect their movement. In fact, these isolates cannot move actively in medium and readily developed into cell clusters in medium (data not shown); therefore, the exact doubling time could not be determined.

### G + C content of DNA

The G + C content of the DNA of strain CMN3\(^\text{T}\), isolated from *N. confucianus*, was 29.6 mol\%. This value is in agreement with those reported previously for *B. burgdorferi sensu lato* (*Barbour & Hayes, 1986; Johnson et al.,* 1984; *Kawabata et al.,* 1993). From these findings, a new *Borrelia* species, *Borrelia sinica* sp. nov., was designated.
**Description of Borrelia sinica sp. nov.**

*Borrelia sinica* (si’ni.ca. L. fem. adj. *sinica* of China, the country from which the organisms were isolated).

Morphology was as described previously for the genus (Burgdorfer & Hayes, 1986). However, counting of flagella showed a maximum of four at each end. Cultivated in BSK II medium at 30–34 °C, similar to *B. burgdorferi* (Johnson et al., 1984). Differentiated from other *B. burgdorferi sensu lato* species by analysis of DraI and MseI restriction polymorphism of the 5S–23S rDNA (rrf–rrl) spacer. The G+C content of the DNA is 29.6 mol% for the type strain.

The type strain, CMN3T, has been deposited in the Japan Collection of Microorganisms (RIKEN) as strain JCM 10505T. This strain was isolated from *N. confucianus* captured in Sichuan province, China.

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