'*Candidatus* *Borrelia texasensis*', from the American dog tick *Dermacentor variabilis*

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TXW-1, a *Borrelia* strain isolated in March 1998 from an adult male *Dermacentor variabilis* tick feeding on a coyote from Webb county, Texas, USA, was characterized by using randomly amplified polymorphic DNA (RAPD) analysis, RFLP and sequence analysis of *flaB* and *rrs* (16S rRNA gene), DNA–DNA hybridization analysis, SDS-PAGE and Western blotting with mAbs. It shows different banding patterns in RFLP analysis of *flaB* and forms distinct branches in phylogenetic analysis derived from *flaB* and *rrs* genes. It differs from other borreliæ based on the banding patterns obtained by RAPD analysis. This strain contains a small, 38-kDa endoflagellar protein. DNA–DNA hybridization experiments revealed that the levels of DNA reassociation between TXW-1 and previously described relapsing fever borreliæ were 38–64 % (*Borrelia turicatae*), 38–40 % (*Borrelia parkeri*), 7–39 % (*Borrelia hermsii*) and 18–30 % (*Borrelia coriaceae*). However, the level of DNA relatedness between *B. parkeri* and *B. turicatae* was 78–78 %. Sequence analyses of *flaB* and *rrs* genes indicate that the similarities of nucleotide sequences among TXW-1 and *B. turicatae* or *B. parkeri* are less than that between *B. turicatae* and *B. parkeri*, and that the genetic distances among TXW-1 and *B. turicatae* or *B. parkeri* are greater than that between *B. turicatae* and *B. parkeri*. TXW-1 lacks an *ospC* gene. Electron microscope observations showed that this spirochaete had different morphological structures compared to previously described relapsing fever borreliæ. All the results obtained from the above-mentioned analyses indicate that TXW-1 is different from other described *Borrelia* species and that it represents a novel species of *Borrelia*. We have been unable to revive frozen cultures and so can not meet the requirements of the Bacteriological Code to deposit viable type material at two different culture collections. Therefore we use the *Candidatus* designation; based on these results, the species '*Candidatus* *Borrelia texasensis*' is proposed.

Relapsing fever is a medically important borreliosis caused by different species of relapsing fever borreliæ. It is found in North America, Europe and Africa and is transmitted by various species of *Ornithodoros* and *Argas* ticks or lice (Barbour & Hayes, 1986). Two major groups of *Borrelia* are the *Borrelia burgdorferi sensu lato* species complex and the relapsing fever borreliæ. To date, 11 genospecies in the *B. burgdorferi* complex and more than 20 species of relapsing fever borreliæ have been reported. Only three of the 11 genospecies of *B. burgdorferi sensu lato* are proven agents

of Lyme disease in humans. *Borrelia recurrentis*, the agent of louse-borne relapsing fever, and several species of tick-borne spirochaetes are included among the relapsing fever borreliæ.

Specific relationships are often found among borreliæ and vector species. Although several different genospecies of *B. burgdorferi sensu lato* can be transmitted by several species of ticks, the ticks are closely related and most are in the *Ixodes ricinus* species complex. The relapsing fever borreliæ and their tick vectors have an even stricter relationship. Some borreliæ are reported to be transmitted by a single tick species (Kelly, 1976; Schwan & Piesman, 2002); however, this tick–spirochaete specificity theory needs reinvestigation using molecular analysis and experimental transmission experiments.

Most relapsing fever borreliæ are transmitted by soft-bodied ticks (*Ornithodoros* or *Argas* species), but there are

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *flaB* gene sequences of TXW-1 are AF467976 and AF264901.

A scanning electron micrograph of a cell of TXW-1 is available as supplementary material in IJSEM Online.

exceptions. For example, *B. recurrentis* is transmitted by the human biting louse, *Pediculus humanus*, and *Borrelia theileri* is vectored by the hard-bodied ticks *Rhipicephalus* (several species) and *Boophilus microplus* (Anderson & Magnarelli, 1993). Moreover, '*Borrelia lonestari*' has been detected and cultured in the hard tick *Amblyomma americanum* from the US states of Texas, New Jersey, New York, Missouri and Georgia (Barbour *et al.*, 1996; Varela *et al.*, 2004), and a *Borrelia* species genetically close to *Borrelia miyamotoi* has been detected in *Ixodes scapularis* from Rhode Island, Connecticut, New York and New Jersey (Scoles *et al.*, 2001). Also, a *B. miyamotoi*-like *Borrelia* species appears to be present in *Ixodes ricinus* ticks in Europe (Fraenkel *et al.*, 2002). In Japan, *B. miyamotoi* is vectored by the closely related *Ixodes persulcatus*, which also is a vector of the Lyme borreliosis spirochaetes *Borrelia garinii* and *Borrelia afzelii* (Fukunaga & Koreki, 1995).

The American tick-borne relapsing fever spirochaetes *Borrelia hermsii*, *Borrelia parkeri* and *Borrelia turicatae* were successfully cultured 30 years ago (Kelly, 1971, 1976, 1984). Although investigation of Lyme borreliosis stimulated improvements in the cultivation of some of the most notable borreliae, such as *B. recurrentis* and *Borrelia duttonii* (Cutler *et al.*, 2000), some species remain uncultivated, such as the borreliae from New England transmitted by *Ixodes scapularis* (Scoles *et al.*, 2001).

The aim of this study was to characterize the genetic and phenotypic features of isolate TXW-1. By using different genotyping and phenotyping methods, we were able to classify this isolate and clarify its taxonomic status. Our results indicate that TXW-1 represents an undescribed species in the relapsing fever borreliae complex. Although we were able to culture this spirochaete, we are currently unable to revive the frozen cultures and thus can not meet the requirements of the Bacteriological Code to deposit viable type material at two different culture collections. Therefore we propose that TXW-1 should be described as '*Candidatus Borrelia texasensis*'.

TXW-1 was isolated in BSK-H medium (Sigma-Aldrich) in March 1998 from an adult male *Dermacentor variabilis* feeding on a coyote from Webb county, Texas. Cultures were incubated at 34 °C for 1–2 weeks until the cell density

reached about 2×10^6 cells ml⁻¹. Spirochaete genomic DNA was extracted by methods described previously (Zingg *et al.*, 1993).

A 348 bp fragment of the flagellin B gene (*flaB*) (for RFLP analysis) was amplified by using a pair of primers, FlaLS (5'-AACAGCTGAAGAGCTTGAATG-3'; positions 438–459) and FlaRS (5'-CTTTGATCACTTATCATTCTAATAGC-3'; 791–766) (Barbour *et al.*, 1996). Another fragment of *flaB* (for sequence analysis) was amplified by using primers FlaLL (5'-ACATATTCAGATGCAGACAGAGGT-3'; 301–324) and FlaRL (5'-GCAATCATAGCCATTGCA-GATTGT-3'; 942–965) (Barbour *et al.*, 1996). A 1532 bp segment of the 16S rRNA gene (*rrs*) was amplified by using a pair of primers fd3 (5'-AGAGTTTGATCCTGGCTTAG-3'; 8–27) and UniB (5'-TACAAGGAGGTGATCCAGC-3'; 1539–1522) (Le Flèche *et al.*, 1997). The outer surface protein C gene (*ospC*) was amplified by using primers *ospC3* (5'-AAGTGCAGATATTAATGACTTTA-3') and *ospC4* (5'-TTTTTTGGACTTTCTGCCACA-3') (Marti Ras *et al.*, 1997).

The 348 bp fragment of *flaB* was digested with *AluI* (Gibco-BRL, Life Technologies) as recommended by the manufacturer. Ten microlitres of the PCR product was digested with 4 U *CelII* (Amersham Pharmacia Biotech) and *DdeI* (Gibco-BRL) for 1 h at 37 °C. The *AluI*, *CelII* and *DdeI* digests were respectively separated in 3.8, 3.0 and 3.8 % (w/v) agarose gels (NuSieve GTG; FMC) containing 0.5 µg ethidium bromide ml⁻¹ in 1 × TBE for 3, 2.5 and 3.5 h at a constant voltage of 100 V. The molecular marker pBR322/*HaeIII* (Sigma-Aldrich) was used. Gels were photographed using the Eagle Eye II System (Stratagene) and a Polaroid GelCam. The RFLP patterns were measured and analysed by using EagleSight software (version 3.2) in the Eagle Eye II System as recommended by the manufacturer.

RFLP of *rrs* was carried out with restriction enzyme *BfaI* (Le Flèche *et al.*, 1997).

The PCR products were purified by QIAquick gel extraction kit (Qiagen). The DNA sequences of *flaB* and *rrs* genes were determined by using an ABI Prism model 377 sequencer. Sequences were aligned manually and by using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were

Fig. 1. *AluI* (a), *CelII* (b) and *DdeI* (c) restriction profiles of amplified fragments of *flaB* from TXW-1 and reference strains. Lanes: 1, B31^T; 2, 21038; 3, DN127; 4, 25015; 5, TXW-1; M, molecular size markers.

Table 1. *AluI*, *CeII* and *DdeI* restriction fragments of amplified partial *flaB* genes

Exact fragment sizes were determined from sequences. The sequence for TXW-1 was determined in the present study. NA, *flaB* sequences were not available in GenBank; restriction patterns were estimated based on RFLP patterns in gels.

Strain	Amplicon size (bp)	<i>AluI</i>		<i>CeII</i>		<i>DdeI</i>		Accession no.
		Pattern	Fragment sizes (bp)	Pattern	Fragment sizes (bp)	Pattern	Fragment sizes (bp)	
<i>B. burgdorferi sensu stricto</i> B31 ^T	354	A1	133, 100, 51, 33, 24, 8, 5	A2	No fragment	A3	180, 174	X16833
<i>B. burgdorferi sensu stricto</i> SH-2-82	354	A1	133, 100, 51, 33, 24, 8, 5	A2	No fragment	A3	180, 174	NA
' <i>B. bissetii</i> ' DN127	354	A1	133, 100, 51, 33, 24, 8, 5	A2	No fragment	C3	117, 102, 72, 63	D82857
' <i>B. bissetii</i> ' 25015	354	A1	133, 100, 51, 33, 24, 8, 5	A2	No fragment	B3	117, 102, 63, 45, 27	NA
' <i>B. andersonii</i> ' 21038	354	D1	133, 68, 51, 45, 33, 24	A2	No fragment	D3	174, 100, 80	D83763
<i>Borrelia</i> sp. TXW-1	348	E1	103, 69, 54, 48, 43, 12, 8, 6, 5	A2	No fragment	F3	171, 162, 15	AF264901
<i>B. garinii</i> 20047 ^T	354	F1	133, 102, 63, 36, 15, 5	A2	No fragment	G3	171, 102, 72, 9	D82846
<i>B. afzelii</i> VS461 ^T	354	G1	133, 69, 55, 45, 33, 8, 6, 5	A2	No fragment	H3	180, 132, 42	D63365
<i>B. valaisiana</i> VS116 ^T	354	H1	133, 124, 51, 33, 8, 5	A2	No fragment	I3	135, 117, 63, 39	D82854
<i>B. lusitaniae</i> PotiB2 ^T	354	I1	133, 55, 51, 45, 33, 24, 8, 5	A2	No fragment	A3	180, 174	D82856
<i>B. japonica</i> HO14 ^T	354	J1	133, 69, 63, 51, 33, 5	A2	No fragment	A3	180, 174	D82852
<i>B. tanukii</i> Hk501 ^T	354	K1	133, 69, 55, 51, 33, 8, 5	A2	No fragment	A3	180, 174	D82847
<i>B. turdi</i> Ya501 ^T	354	L1	133, 69, 55, 51, 33, 8, 5	A2	No fragment	A3	180, 174	D82849
<i>B. turicatae</i> M2007	348	M1	103, 69, 48, 43, 33, 21, 12, 8, 6, 5	A2	No fragment	J3	186, 162	D86862
<i>B. parkeri</i> 6232	348	N1	117, 103, 43, 33, 21, 12, 8, 6, 5	A2	No fragment	J3	186, 162	D82863
<i>B. coriaceae</i> Co53 ^T	345	O1	117, 103, 43, 30, 21, 12, 8, 6, 5	D2	171, 174	K3	174,171	D82864
<i>B. hermsii</i> HS1	348	P1	153, 103, 43, 12, 12, 8, 6, 6, 5	A2	No fragment	K3	177, 171	M86838
<i>B. anserina</i> ES-1	348	Q1	103, 69, 55, 48, 42, 9, 9, 8, 5	A2	No fragment	K3	177, 171	X75201
<i>B. miyamotoi</i> FR64b	348	R1	121, 69, 55, 42, 42, 13, 6	A2	No fragment	L3	No fragment	D82860
' <i>B. lonestari</i> ' Texas	330	S1	117, 87, 55, 36, 16, 13, 6	A2	No fragment	L3	No fragment	U26704
<i>B. crocidurae</i> ORI	351	T1	93, 90, 45, 42, 36, 12, 10, 6, 6, 6, 5	A2	No fragment	L3	No fragment	U28496
<i>B. duttonii</i> 406K	351	U1	103, 69, 55, 45, 36, 12, 8, 6, 6, 6, 5	A2	No fragment	L3	No fragment	D82859
<i>Borrelia</i> sp. Spain	348	V1	124, 109, 51, 45, 8, 6, 5	A2	No fragment	L3	No fragment	U28499
<i>B. hispanica</i> UESV/246	351	W1	99, 69, 55, 45, 36, 18, 10, 8, 6, 5	A2	No fragment	L3	No fragment	U28498

Fig. 2. *Bfal* restriction profiles of amplified fragments of *rrs* genes from TXW-1 and reference strains. Lanes: 1, *B. burgdorferi sensu stricto* B31^T; 2, '*B. bissettii*' Al-1; 3, '*B. bissettii*' FD-1; 4, '*B. andersonii*' MOS-1b; 5, TXW-1; M, molecular size markers.

constructed by neighbour-joining (Saitou & Nei, 1987) using PAUP (Swofford, 2001).

RAPD-PCRs were performed by using previously published primers 1254, 1283 and AP13 (Wang *et al.*, 1998). Reactions were performed as described previously (Wang *et al.*, 1998) in a GeneAmpPCR System 9700 (PE Biosystems). The amplified DNA fragments were separated in 1% agarose (w/v) gels in $0.5 \times$ TBE containing $0.5 \mu\text{g}$ ethidium bromide ml^{-1} . Gels were photographed using the Eagle Eye II System (Stratagene) and PCR-RAPD patterns were analysed as described above for RFLP.

Phenotypic characters of TXW-1 and reference strains were analysed by SDS-PAGE and Western blot. Briefly, reference strains and TXW-1 were incubated in BSK-H medium (Sigma-Aldrich) with 6% rabbit serum at 34°C for 1–2 weeks until the cell density reached about 2×10^6 cells ml^{-1} . Ten millilitres of culture was washed with three cycles of washing with 0.01 M PBS (pH 7.2) with 5 mM MgCl_2 and centrifugation at $10\,000 \text{ r.p.m.}$ ($12\,096 \text{ g}$)

Table 2. *Bfal* restriction fragments of amplified partial *rrs* genes

Exact fragment sizes were determined from sequences. The sequence for TXW-1 was determined in the present study.

Strain	Amplicon size (bp)	<i>Bfal</i> RFLP pattern	Fragment sizes (bp)	Accession no.
<i>B. burgdorferi sensu stricto</i> B31 ^T	1531	A	202, 236, 404, 689	U03396
' <i>B. bissettii</i> ' DN127	1531	A	202, 236, 404, 689	L40596
' <i>B. bissettii</i> ' 25015	1531	A	202, 236, 404, 689	AJ224138
' <i>B. andersonii</i> ' 21038	1531	B	99, 136, 202, 404, 690	L46701
<i>B. garinii</i> 20047 ^T	1531	C	78, 158, 202, 404, 689	D67018
<i>B. afzelii</i> DK1	1531	D	136, 202, 504, 689	X85190
<i>B. japonica</i> HO14 ^T	1524	G	136, 199, 500, 686	L40597
<i>B. valaisiana</i> VS116 ^T	1531	E	58, 78, 202, 504, 689	X98232
<i>B. lusitanae</i> PotiB2 ^T	1531	F	58, 78, 100, 202, 404, 689	X98228
<i>B. tanukii</i> Hk501 ^T	1531	F	58, 78, 100, 202, 404, 689	D67023
<i>B. turdi</i> Ya501 ^T	1530	H	199, 639, 689	D67022
<i>B. sinica</i> CMN2	1537	H	202, 647, 688	AB022145
<i>B. turicatae</i> M2007	1531	I	88, 202, 236, 403, 602	U42299
<i>B. parkeri</i> 6232	1532	I	88, 202, 236, 404, 602	AF307100
<i>Borrelia</i> sp. TXW-1	1532	I	88, 202, 236, 404, 602	AF467976
<i>B. hermsii</i> HS1	1531	I	88, 202, 236, 403, 602	U42292
<i>B. coriaceae</i> Co53 ^T	1531	I	88, 202, 236, 403, 602	U42286
<i>B. miyamotoi</i> HT31 ^T	1532	I	88, 202, 236, 404, 602	D45192
<i>Borrelia</i> sp. FCB-1	1532	I	88, 202, 236, 404, 602	L37837
<i>B. persica</i> UESV/340	1532	I	88, 202, 236, 403, 603	U42297
<i>Borrelia</i> sp. LB-2001	1534	I	88, 202, 238, 404, 602	AY024345
<i>B. anserina</i> ES-1	1530	J	15, 88, 202, 236, 388, 601	U42284
' <i>B. lonestari</i> ' Texas	1532	J	15, 88, 202, 236, 389, 602	U23211
<i>B. crocidurae</i> UESV/1096TEN	1531	J	15, 88, 202, 236, 388, 602	U42302
<i>B. duttonii</i> Ma	1532	J	15, 88, 202, 236, 389, 602	AF107366
<i>B. hispanica</i> UESV/246	1531	J	15, 88, 202, 236, 388, 602	U42294
<i>Borrelia</i> sp. Spain	1531	K	14, 51, 88, 185, 201, 389, 603	U28502

for 10 min. Washed pellets of the spirochaetes were then lysed by adding loading buffer. Twenty microlitres of cell lysate was heated at 100 °C for 10 min and then separated by SDS-16.5 % PAGE. A low-molecular-mass marker (Sigma-Aldrich) was used to determine molecular masses. The gel was then stained with Coomassie brilliant blue R250. Western blotting was carried out by electrotransferring the proteins from the SDS-PAGE gel to a nitrocellulose membrane (Bio-Rad). The membrane was blocked by immersing in 5 % dry milk for 1 h at room temperature. The whole membrane was reacted with mAb H9724 (1:100) for 1 h at room temperature and washed three times in Tris-buffered saline (TBS) with 0.1 % Tween 20 for 5 min each at room temperature. The membrane was then incubated in horseradish peroxidase-labelled anti-mouse second antibody (Kirkegaard & Perry Laboratories, Inc.) at 1:1000 dilution for 1 h at room temperature and subsequently washed three times in TBS with 0.1 % Tween 20 and once in distilled water, 5 min for each washing. The membrane was then incubated in TMB substrate solution at room temperature. The reaction was stopped by immersing the membrane in distilled water for 10–20 s when a suitable colour intensity was observed.

Measurement of DNA–DNA hybridization was performed by dot-blot analysis of samples with ³²P-labelled probes. DNAs were extracted and purified from TXW-1 and

reference strains by the method described previously (Lin *et al.*, 2002). Aliquots of 2.5 µl total genomic DNA purified from the various bacterial strains were spotted at four different concentrations (25, 5, 1.25 and 0.25 ng DNA per dot) onto Hybond-XL nylon membrane (Amersham) and cross-linked with a Stratalinker 1800 UV cross-linker (Stratagene). Subsequently, membranes were prehybridized for 1 h at 60 °C in ExpressHyb solution (Clontech) followed by 1 h hybridization at 60 °C in ExpressHyb solution with [³²P]dCTP-labelled probe made from 100 ng TXW-1 or *B. parkeri* genomic DNA by using an oligolabelling kit (Amersham). After hybridization, membranes were washed with 2 × SSC, 0.05 % SDS at room temperature for 40 min with five changes of wash solution. This was followed by two high-stringency washes at 50 °C for 20 min each with 0.1 × SSC, 0.1 % SDS, according to the manufacturer's instructions (Clontech). Hybridization signal was detected by phosphorimager analysis using a Storm840 Phosphor-Imager system (Molecular Dynamics). Quantification of hybridization signal was performed by the NIH Image 1.62 software (<http://rsb.info.nih.gov/nihi-image/>) and data are given as percentages relative to the homologous probe for four independent hybridizations (mean ± SD).

B. burgdorferi sensu lato complex strains and relapsing fever borreliae consisted of 21 *AluI* RFLP types, two *CellI* RFLP types and 11 *DdeI* RFLP types. TXW-1 formed the

Table 3. Nucleotide similarity (%) between *flaB* sequences from different genospecies of *B. burgdorferi sensu lato* complex and species of relapsing fever borreliae

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. <i>B. burgdorferi</i> s. s. B31 ^T	100																						
2. 'B. bissetii' DN127	97	100																					
3. 'B. andersonii' 21123	96	95	100																				
4. <i>B. garinii</i> 20047 ^T	94	94	93	100																			
5. <i>B. afzelii</i> VS461 ^T	94	94	93	94	100																		
6. <i>B. valaisiana</i> VS116 ^T	94	95	94	94	95	100																	
7. <i>B. lusitaniae</i> PotiB2 ^T	94	94	93	94	93	94	100																
8. <i>B. japonica</i> HO14 ^T	94	94	94	94	94	94	93	100															
9. <i>B. tanukii</i> Hk501 ^T	95	96	95	95	95	97	94	95	100														
10. <i>B. turdi</i> Ya501 ^T	94	95	93	94	93	94	94	93	95	100													
11. <i>Borrelia</i> sp. Am501	94	95	94	94	95	98	94	94	94	94	100												
12. <i>Borrelia</i> sp. TXW-1	84	85	84	85	85	85	85	84	86	85	84	100											
13. <i>B. turicatae</i> M2007	84	84	84	84	85	85	85	83	86	84	84	97	100										
14. <i>B. parkeri</i> 6232	84	85	84	84	85	85	85	83	86	84	84	97	98	100									
15. <i>B. coriaceae</i> Co53 ^T	83	83	82	83	84	84	84	82	84	83	83	91	91	91	100								
16. <i>B. hermsii</i> HS1	84	85	84	84	85	85	84	84	86	83	84	92	92	92	90	100							
17. <i>B. anserina</i> ES-1	85	85	84	85	86	85	85	84	86	85	84	92	93	92	91	92	100						
18. <i>B. miyamotoi</i> HT31 ^T	82	82	82	82	83	83	83	82	83	81	82	90	91	90	88	90	91	100					
19. 'B. lonestari' Texas	84	83	83	83	84	84	84	82	84	83	83	88	89	88	86	87	88	90	100				
20. <i>B. crocidurae</i> ORI	84	84	84	83	84	84	84	83	84	85	83	90	90	91	88	88	90	89	88	100			
21. <i>Borrelia</i> sp. Spain	84	84	83	83	84	84	85	82	84	84	83	89	90	90	88	88	90	88	87	96	100		
22. <i>B. hispanica</i> UESV/246	82	81	81	80	82	81	82	80	82	82	80	87	87	87	85	85	87	85	85	94	94	100	
23. <i>B. duttonii</i> 406K	84	84	84	83	84	84	84	83	85	85	83	90	90	91	89	88	90	89	90	99	96	94	100

s. s., *Sensu stricto*.

Table 4. Nucleotide similarity (%) between 16S rRNA gene sequences from different genospecies of *B. burgdorferi sensu lato* complex and species of relapsing fever borreliae

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. <i>B. burgdorferi</i> s. s. B31 ^T	100																											
2. 'B. bissetii' DN127	99	100																										
3. 'B. bissetii' 25015	99	99	100																									
4. 'B. andersonii' 21038	98	98	98	100																								
5. <i>B. garinii</i> 20047 ^T	99	99	99	98	100																							
6. <i>B. afzelii</i> DK1	98	98	98	98	98	100																						
7. <i>B. valaisiana</i> VS116 ^T	98	98	98	98	98	99	99	100																				
8. <i>B. lusitaniae</i> PotiB2 ^T	99	99	99	98	99	98	99	100																				
9. <i>B. japonica</i> HO14 ^T	98	98	98	97	98	98	97	98	100																			
10. <i>B. tanukii</i> Hk501 ^T	99	99	99	98	99	99	99	99	98	100																		
11. <i>B. turdi</i> Ya501 ^T	98	99	99	98	98	98	99	98	98	99	100																	
12. <i>B. sinica</i> CMN2	99	99	99	98	99	98	98	99	98	98	98	100																
13. <i>Borrelia</i> sp. TXW-1	96	96	96	96	96	96	96	96	95	96	96	96	100															
14. <i>B. turicatae</i> M2007	96	96	96	96	96	96	96	96	95	96	96	96	99	100														
15. <i>B. parkeri</i> 6232	96	96	96	96	96	96	96	96	95	96	96	96	99	99	100													
16. <i>B. coriaceae</i> Co53 ^T	96	96	96	95	96	95	96	96	95	96	96	96	98	98	98	100												
17. <i>B. hermsii</i> HS1	96	96	96	96	96	96	96	96	95	96	96	96	98	99	99	98	100											
18. <i>B. recurrentis</i> A5	96	96	96	96	96	96	96	96	95	96	96	96	98	98	98	98	98	100										
19. <i>B. anserina</i> ES-1	96	96	96	96	96	96	96	96	95	96	96	96	98	98	98	98	98	98	100									
20. <i>B. miyamotoi</i> HT31 ^T	95	96	96	95	95	95	96	95	94	96	95	95	97	98	98	97	97	97	97	100								
21. 'B. lonestari' Texas 20	95	96	96	95	96	95	96	95	95	96	96	95	97	98	98	98	97	97	97	98	100							
22. <i>B. crocidurae</i> UESV/1096TEN	96	96	96	96	96	96	96	96	95	96	96	96	98	99	99	98	98	99	98	97	98	100						
23. <i>Borrelia</i> sp. Spain	95	95	95	95	95	95	95	95	94	95	95	95	97	97	97	97	97	98	97	96	96	98	100					
24. <i>B. hispanica</i> UESV/246	96	96	96	96	96	96	96	96	95	96	96	96	98	99	99	98	98	99	98	97	98	99	98	100				
25. <i>B. duttonii</i> Ma	96	96	96	96	96	96	96	96	95	96	96	96	98	99	99	98	98	99	98	97	98	99	98	99	100			
26. <i>Borrelia</i> sp. FCB-1	96	96	96	96	96	96	96	96	95	96	96	96	99	99	99	98	99	98	98	97	98	99	99	99	99	100		
27. <i>B. persica</i> UESV/340	96	96	96	96	96	96	96	96	95	96	96	96	98	98	98	98	98	98	98	97	97	99	97	99	98	98	100	
28. <i>Borrelia</i> sp. LB-2001	94	95	95	94	95	95	95	94	94	95	94	94	97	97	97	97	97	96	96	98	97	97	96	97	97	97	96	100

unique *AluI* RFLP type E1 and *DdeI* RFLP type F3 (Fig. 1; Table 1). The same strains contained 11 *BfaI* RFLP types; TXW-1 formed pattern I (Fig. 2; Table 2).

Nucleotide identities of *flaB* sequences from TXW-1 and strains of relapsing fever borreliae ranged from 87 to 97%. The similarities of *flaB* nucleotide sequences among TXW-1 and *B. turicatae* (97%) and *B. parkeri* (97%) were less than that between *B. turicatae* and *B. parkeri* (98%) (Table 3). Similar results were found for *rrs* nucleotide sequences. The *rrs* nucleotide sequence similarities among

TXW-1 and *B. turicatae* and *B. parkeri* were 99%. Nucleotide identities among many species were 99% (Table 4).

Repeated RAPD analyses were performed with reference strains B31^T, SH-2-82, DN127, 25015 and 21038 in order to assess the reproducibility of our RAPD fingerprinting procedure. We obtained similar DNA fingerprints for these strains in repeated RAPD analysis. No differences were seen between the RAPD patterns for B31^T at different passages (3 and 8) (data not shown). Based on the RAPD profiles in three individual amplifications with different primers, a

Fig. 3. RAPD fingerprints of TXW-1 and reference strains from different genospecies obtained by using primers 1283 (a), AP13 (b) and 1254 (c). Lanes: 1, 25015; 2, SH-2-82; 3, DN127; 4, 20047^T; 5, 21038; 6, B31^T; 7, TXW-1; M, lambda DNA/*Bst*EII. Molecular sizes (in kbp) are indicated on the left.

Fig. 4. Phylogenetic tree derived from *flaB* nucleotide sequences of TXW-1 and reference strains of *B. burgdorferi sensu lato* and relapsing fever borreliae. The neighbour-joining tree was constructed with PAUP software and based on a comparison of 585–608 bp partial *flaB* sequences. The scale bar represents a calculated distance of 0.01 substitutions per site. Bootstrap confidence levels above 50% are indicated to left of each relevant cluster.

total of seven RAPD types were identified from the seven *Borrelia* strains that were compared (Fig. 3).

Based on sequence analysis of *flaB*, TXW-1 forms a separate branch close to, but separate from, *B. turicatae* and *B. parkeri*. The genetic distance (indicated by branch length) between TXW-1 and *B. turicatae* (0.007) is greater than that between *B. parkeri* and *B. turicatae* (0.005) (Fig. 4). Similarly, the *rrs* phylogenetic tree revealed a separate

branch for TXW-1 within the relapsing fever cluster. The genetic distance (indicated by branch length) between TXW-1 and *B. turicatae* (0.002) is greater than that between *B. parkeri* and *B. turicatae* (0.001) (Fig. 5).

The protein profiles of strains B31^T (*B. burgdorferi sensu stricto*), 20047^T (*B. garinii*), DN127 (*Borrelia bissetii*), 25015 (*B. bissetii*), 21038 (*Borrelia andersonii*), Co53^T (*Borrelia coriaceae*), HS1 (*B. hermsii*), RML (*B. parkeri*),

Fig. 5. Phylogenetic tree derived from *rrs* (16S rRNA gene) nucleotide sequences of TXW-1 and reference strains of *B. burgdorferi sensu lato* and relapsing fever borreliae. The neighbour-joining tree was based on a comparison of 1537 bp of nearly complete *rrs* genes. The scale bar represents 0.005 substitutions per site. See Fig. 4 legend for further details.

Fig. 6. (a) Coomassie brilliant blue-stained protein profiles of whole-cell lysates of TXW-1 and reference strains. Lanes: M, molecular mass markers; 1, *B. burgdorferi sensu stricto* B31^T; 2, *B. garinii* 20047; 3, '*B. bissettii*' DN127; 4, '*B. bissettii*' 25015; 5, '*B. andersonii*' 21038; 6, *B. coriaceae* Co53; 7, *B. hermsii* HS1; 8, *B. parkeri* RML; 9, *B. turicatae* OZ-1; 10, '*Candidatus B. texasensis*' TXW-1. Arrows indicate positions of endoflagellar proteins reactive to mAb H9724. (b) Western blot with mAb H9724 against the flagellar protein. See (a) for further details.

OZ-1 (*B. turicatae*) and TXW-1 ('*Candidatus B. texasensis*') were different from one another (Fig. 6a). Major proteins including 31 kDa OspA, 34 kDa OspB and 22 kDa OspC were clearly resolved for strain B31^T on a Coomassie brilliant blue-stained 16.5% polyacrylamide gel (Fig. 6a). mAb H9724, which is specific for *Borrelia* flagellar protein, was used as a probe for Western blotting. Flagellar proteins of 41 kDa were found in *B. burgdorferi sensu lato* strains B31^T, 20047^T, DN127, 25015 and 21038; however, a 38-kDa flagellar protein was found in TXW-1, similar to those found in relapsing fever borreliae HS1, RML and OZ-1 based on protein profiles and reactivity with mAb 9724 (Fig. 6a, b).

DNA–DNA hybridization is a superior method for examining relationships between closely related taxa and for bacterial taxonomy (Stackebrandt & Goebel, 1994). The ad hoc committee on reconciliation of approaches to bacterial systematics recommended that the level of DNA relatedness for separate species should be less than 70% (Wayne *et al.*, 1987). DNA–DNA hybridization experiments revealed levels of DNA reassociation between TXW-1 and previously described relapsing fever borreliae of 38.64% (*B. turicatae*), 38.40% (*B. parkeri*), 7.39% (*B. hermsii*) and 18.30% (*B. coriaceae*). However, the level of DNA relatedness between *B. parkeri* and *B. turicatae* was 78.78%. Strain TXW-1 exhibited lower levels of DNA relatedness with *B. parkeri* and *B. turicatae* than that between *B. parkeri* and *B. turicatae*. Based on these results, TXW-1 is different from other described borreliae and therefore represents an undescribed species. Its closest relationship is to *B. parkeri* and *B. turicatae*.

It is not known whether the coyote is a natural host for TXW-1 and whether *D. variabilis* can maintain and transmit the spirochaete. The tick may have become infected during the larval or nymphal stages when feeding on unknown hosts. Thus, only limited data are available regarding the vector, vertebrate reservoir host and complete geographical distribution of TXW-1; nothing is known about its infectivity or pathogenicity. Nevertheless, our study provides the description of a novel spirochaete species among the relapsing fever borreliae. The results also enhance understanding of the diversity among borreliae and may be useful for future studies of the evolution of relapsing fever borreliae.

Description of '*Candidatus Borrelia texasensis*'

'*Candidatus Borrelia texasensis*' (te.xas.en'sis. N.L. fem. adj. *texasensis* of Texas, USA, where the organism was isolated).

Cells are actively motile with reversal, rotational and translational movements. Structurally, they consist of an outer membrane that surrounds a protoplasmic cylinder complex and flagella. They have six to ten flexible helical coils and are 0.21–0.24 µm wide and 9.41–11.23 µm long. The wavelengths are 1.12–1.53 µm (a scanning electron micrograph of TXW-1 is available as supplementary material in IJSEM Online). Cells have tapered ends. Microaerophilic spirochaete that can be cultured in BSK-H medium at 34 °C. Gram-negative and stains well with Giemsa's stain. Stained cells are visible by bright-field microscopy but, to observe unstained cells, dark-field microscopy is required. Pathogenicity and infectivity remain to be determined.

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