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Multilocus sequence analysis of *Borrelia bissettii* strains from North America reveals a new *Borrelia* species, *Borrelia kurtenbachii*

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Abstract

Using multilocus sequence analyses (MLSA), we investigated the phylogenetic relationship of spirochaete strains from North America previously assigned to the genospecies *Borrelia bissettii*. We amplified internal fragments of 8 housekeeping genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) located on the main linear chromosome by polymerase chain reaction. Phylogenetic analysis of concatenated sequences of the 8 loci showed that the *B. bissettii* clade consisted of 4 closely related clusters which included strains from California (including the type strain DN127-C19-2/p7) and Colorado that were isolated from *Ixodes pacificus*, *I. spinipalpis*, or infected reservoir hosts. Several strains isolated from *I. scapularis* clustered distantly from *B. bissettii*. Genetic distance analyses confirmed that these strains are more distant to *B. bissettii* than they are to *B. carolinensis*, a recently described *Borrelia* species, which suggests that they

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constitute a new *Borrelia* genospecies. We propose that it be named *Borrelia kurtenbachii* sp. nov. in honour of the late Klaus Kurtenbach. The data suggest that ecological differences between *B. bissettii* and the new *Borrelia* genospecies reflect different transmission cycles. In view of these findings, the distinct vertebrate host-tick vector associations and the distributions of *B. bissettii* and *B. kurtenbachii* require further investigation.

Keywords

Borrelia bissettii; *Borrelia kurtenbachii* sp. nov.; *Ixodes*; Multilocus sequence analysis; Molecular ecology

Introduction

Lyme borreliosis (LB) is caused by several species of bacteria belonging to the LB group of spirochaetes, also referred to as *Borrelia burgdorferi* sensu lato (s.l.) species complex. These bacteria are maintained in nature by numerous vertebrate hosts and are transmitted by ticks in the *Ixodes persulcatus* species complex (reviewed by Kurtenbach et al., 2006). Today, LB is the most frequently reported vector-borne disease in the temperate zones of the Northern Hemisphere. In the United States, human cases exceeding 20,000 per year currently are being reported with 90% of the cases occurring in 2 regional foci, the Northeast and Upper Midwest. Human cases are far less frequently reported in the western and southern regions of the country (Bacon et al., 2008). In North America, *B. burgdorferi* sensu stricto (hereinafter referred to as *B. burgdorferi*) is the sole species associated with human disease (Mathiesen et al., 1997; Wormser et al., 2008). The reasons for the skewed spatial distribution of LB cases in the United States are not entirely clear but may be due to ecological factors, such as the abundance of, and spirochaete infection prevalences in, tick vectors, the presence or absence of disseminating, disease-causing *B. burgdorferi* genotypes, differences in tick host-seeking behaviour (in particular the immature stages), or possibly other factors such as land-use changes and regional differences in human behaviour (Burgdorfer et al., 1985; Oliver, 1996; Lane and Quistad, 1998; Wright et al., 2000; Slowik and Lane, 2001; Lane et al., 2004; Diuk-Wasser et al., 2006; Eisen et al., 2006; Killilea et al., 2008; Gatewood et al., 2009; Hamer et al., 2010; Humphrey et al., 2010).

The aetiological agent of LB was identified in 1981 and named *Borrelia burgdorferi* after its discoverer, Willy Burgdorfer (Burgdorfer et al., 1982; Johnson et al., 1984). Over the following 2 decades, many isolates derived from human patients, ticks, or reservoir host animals were analysed biochemically and genetically, and a high diversity within *B. burgdorferi* was found which led to the delineation of new genospecies in Europe, Asia, and North America (Baranton et al., 1992; Kawabata et al., 1993; Maupin et al., 1994; Postic et al., 1994; Marconi et al., 1995; Fukunaga et al., 1996; Le Fleche et al., 1997; Mathiesen et al., 1997; Wang et al., 1997, ¹⁹⁹⁹; Postic et al., 1998; Masuzawa et al., 1999, 2001; Lin et al., 2001). The LB group of spirochaetes now forms a species complex consisting of 17 named genospecies (Kurtenbach et al., 2010) reflecting the complex ecology of these bacteria (Kurtenbach et al., 2006). Of the named species, 6 occur in North America including *B. andersonii*, *B. bissettii*, *B. californiensis*, *B. carolinensis*, *B. americana*, and *B. burgdorferi*. The true species richness doubtless is higher as several strains phylogenetically distinct from all other species have not yet been named (Postic et al., 1998, 2007).

B. bissettii enzootic transmission cycles in the United States involving tick species such as *I. spinipalpis*, *I. minor*, *I. affinis*, and various vertebrate host species were found in Colorado, California, and some Southern states (Bissett and Hill, 1987; Brown and Lane, 1992; Maupin et al., 1994; Lane and Quistad, 1998; Schneider et al., 2000; Oliver et al., 2003). In

1990, Anderson and coworkers described a non-pathogenic variant of *B. burgdorferi*, strain 25015. This strain was isolated from 4 blood-engorged *I. scapularis* larvae (then termed *I. dammini*) that had been removed from 3 *Peromyscus leucopus* mice collected in Dutchess County, New York, in 1987 (Anderson et al., 1988, 1990). In 1998, the species *B. bissettii* was delineated using restriction patterns of the *rrf-rrl* intergenic spacer as well as sequences of the intergenic spacer and 16S rDNA. Samples included mainly strains isolated from *I. pacificus* or *I. neotomae* (now *I. spinipalpis*) in California (Postic et al., 1998). Strain 25015 was included in the genospecies *B. bissettii*, although differences in *rrf-rrl* restriction patterns between strain 25015 and DN127 were observed (Mathiesen et al., 1997; Postic et al., 1998; Lin et al., 2003). In 1996, 1997, and 1998, *Borrelia* isolates were obtained from the white-footed mouse (*Peromyscus leucopus*), the meadow vole (*Microtus pennsylvanicus*), and the meadow jumping mouse (*Zapus hudsonius*) in forested, suburban areas of Chicago, Illinois (Picken and Picken, 2000). These isolates were compared to B31, DN127, and 25015 by RFLP analysis. They were designated *B. bissettii* because most of them exhibited a restriction pattern similar to that of strain 25015, and 2 strains exhibited a macrorestriction pattern like that of the type strain DN127 (Picken and Picken, 2000).

DNA-DNA hybridization and sequence analyses of the 16S rRNA locus were used as standard procedures to delineate bacterial species for many years, but were not without drawbacks (Gevers et al., 2005; Stackebrandt and Ebers, 2006). The invention of multilocus sequence typing (MLST) during the late 1990s (Maiden et al., 1998; Enright and Spratt, 1999; Spratt, 1999; Feil and Enright, 2004) led to the development of multilocus sequence analysis (MLSA), a tool that has been used since to resolve inter-species relationships of bacteria and to delineate bacterial species (Gevers et al., 2005; Bishop et al., 2009). Several different multiple-loci schemes have been used for the LB group of spirochaetes (Richter et al., 2006; Postic et al., 2007; Margos et al., 2008; Rudenko et al., 2009a, 2009b). One of them employs housekeeping genes (Margos et al., 2008) and resembles typical MLSA schemes reported for other microorganisms (see <http://www.mlst.net>).

Here, we used this MLSA system (Margos et al., 2008, 2009) to investigate the phylogenetic relationships among *B. bissettii* strains isolated from reservoir hosts or ticks removed from them in different regions of North America. The data confirm that *B. bissettii* forms a heterogeneous clade (Postic et al., 1998). We also demonstrate that strain 25015 and several other strains collected in Illinois and Nova Scotia that were assigned previously to *B. bissettii* represent a phylogenetically distinct group, and we propose to name this group *Borrelia kurtenbachii* sp. nov.

Materials and methods

Tick collection and isolation of LB spirochaetes

The samples used in this study are listed in Table 1. They mainly represent cultured isolates principally obtained from ticks that were collected from rodents trapped in California, Colorado, Illinois, and New York. Isolation of LB spirochaetes followed standard procedures (Maupin et al., 1994). One sample, NS07-121, was not a cultured isolate but DNA-extracted from a nymphal *I. scapularis* collected in Nova Scotia from a human in 2007.

Molecular methods and MLST analyses

Borrelia DNA was extracted from cultured isolates or frozen stocks using DNA STAT-60 (Tel-Test, Friendswood, TX, USA). Briefly, cells were lysed in 1 ml of DNA isolation reagent and 0.2 ml chloroform. After centrifugation ($12,000 \times g$ for 15 min at 4°C), the upper layer was isolated and added to an equal volume of isopropanol, followed by

incubation at room temperature for 5 min. The sample was centrifuged at $12,000 \times g$ for 10 min at 4°C, followed by a wash in 75% ethanol. After drying, the sample was resuspended in 50 µl H₂O. For strain NS07-121, no isolation was attempted, but tick and *Borrelia* DNA was purified as described earlier (Ogden et al., 2010).

Amplification of the 8 housekeeping genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) was conducted by nested PCR using primers and conditions principally as described previously (Margos et al., 2009; Ogden et al., 2010; see also <http://borrelia.mlst.net>). Template DNA was diluted to 10 pg/µl, and 2 µl were used in a total reaction volume of 15 µl for the first amplification step except for *clpA* for which 4 µl of template DNA were used instead. The nested step of amplification was slightly modified: HotStarTaq Mastermix (Qiagen, Germany) was used and 1 pmol/µl of primer in a final reaction volume of 30 µl. Three µl from the first amplification step were used as template DNA. A touchdown PCR starting at 58°C with a decrease of 1°C at each cycle until 50°C was used for the second amplification step, followed by 34 cycles of amplification at an annealing temperature of 50°C. All PCR products were sequenced in both forward and reverse directions. Quality control of DNA traces was conducted manually in DNASTAR (Lasergene plc, USA). Sequences with more than one base peak at the same position in forward and reverse sequence were considered mixed isolates and removed from further analysis.

Sequences of individual housekeeping genes were compared to each other and to sequences in the MLST database. Sequences were assigned allelic numbers, and novel sequences received consecutive numbers. New consecutive sequence type numbers were assigned to allelic profiles with novel combinations or containing novel alleles (Table 2) (Margos et al., 2008). Sequences were aligned and concatenated in Mega 4.0 (Kumar et al., 2004), which also was used to determine genetic distances and to generate a neighbour-joining tree using the Maximum Composite Likelihood model. All sequences are available on the MLST website, <http://borrelia.mlst.net>, hosted at Imperial College London, U.K.

Results

A total of 33 strains designated previously as *B. bissettii* (Postic et al., 1998; Picken and Picken, 2000) was analysed by MLST. Of these isolates, 30% (n=10) contained mixed infections probably reflecting isolation from hosts infected with multiple strains. This included the type strain DN127, but further analysis of a cloned isolate (c19-2/p7; 2/18/93) yielded unambiguous results. The remaining 23 samples fell into 2 clades. One clade consisted of 4 closely related clusters from California (i.e., CA128, CA389, CA370, CA371, and DN127-C19-2/p7) and Colorado (all strains isolated in 1993, designated here gom93-267 to gom93-544). The second clade was genetically distant and included strain 25015 (Fig. 1, Table 3). The pair-wise genetic similarities of strains in the first clade were >98.5% (Table 3), which is above the genetic distance used to designate distinct genospecies in the MLST scheme used here (i.e. 98.3%) (Margos et al., 2009). Some isolates of different geographic origin (i.e. Colorado – isolate gom93-299, California – DN127-C19-2/p7) have a high degree of similarity, and therefore occurred in the same cluster (Fig. 1). The number of alleles for the *B. bissettii* strains found in individual genes varied from 4 to 9 (Table 4). The G+C contents of the gene fragments ranged from 26.6 to 36.5% and are close to the average G+C contents of the *B. burgdorferi* main chromosome (Fraser et al., 1997). The nucleotide diversity per site was particularly high for the *pyrG* locus (Table 4) due to high diversity of this gene in DN127-C19-2/p7.

The genetic similarity of the strains that formed the second clade (Fig. 1) (i.e. 25015, 96-255, 97-236u, NS07-121) ranged from 98.7 to 99.9%. The genetic distance of these strains from those of *B. bissettii* and *B. carolinensis* was >3%, and it was even higher for all

the other *Borrelia* species included (Table 3). Therefore, we conclude that the strains belonging to the second clade represent a novel genospecies. In fact, the genetic distances between those strains and the remaining *B. bissettii* strains was higher than it was between *B. bissettii* and *B. carolinensis* (Fig. 1, Table 2), a recently described species from South Carolina (Rudenko et al., 2009a). The second clade included 2 samples from Illinois (96-255, 97-236u), one from New York (25015), and one from Nova Scotia, (NS07-121) (Table 1).

Overall, the phylogenetic tree produced 2 major clades – one that clustered the Eurasian species, the other clustering the North American species. One genospecies, *B. sinica*, was not included in this study, but we consider it highly unlikely that the strains we propose as a new genospecies are similar to *B. sinica*. Firstly, *B. sinica* is only known from *I. ovatus* and murid rodents in southern China (Masuzawa et al. 2001). Secondly, *B. bissettii* strain DN127, a phylogenetic neighbour of 25015, was quite distant from *B. sinica* when analyzed by DNA-DNA hybridization (Masuzawa et al. 2001). Thirdly, published *DraI* and *MseI* restriction fragment length patterns of the 5S-23S *rrf-rrl* intergenic spacer of strain 25015 and *B. sinica* differ considerably (Masuzawa et al., 2001; Chu et al., 2008; Lin et al., 2001).

Discussion

In this study, we have reinvestigated the phylogenetic relatedness of spirochaete strains previously assigned to the genospecies *B. bissettii* (Postic et al., 1998; Picken and Picken, 2000). We found that while strains from California and Colorado clustered with the *B. bissettii* type strain DN127-C19-2/p7, other strains from Illinois and New York fell into a different cluster. A genetic distance analysis indicated that these strains are more distant to *B. bissettii* than they are to *B. carolinensis*, a newly described *Borrelia* species (Rudenko et al., 2009a). Thus, we propose to name this group *Borrelia kurtenbachii* sp. nov., in honour of the late Klaus Kurtenbach who contributed significantly to our understanding of the ecology and evolution of the LB spirochaetes (Kurtenbach et al., 2006).

All isolates from Colorado were obtained either from *I. spinipalpis* removed from the Mexican woodrat *Neotoma mexicana* or from the woodrats themselves, whereas strains from California were either isolated from *I. pacificus* or *I. spinipalpis* that had been removed from the dusky-footed woodrat *Neotoma fuscipes*. These strains clustered together phylogenetically, and the genetic distance analysis indicated that they are true members of the species *B. bissettii*. Natural transmission cycles of *B. bissettii* involve either *I. spinipalpis* or *I. pacificus*; in contrast, strain 25015 and the Canadian strain were both detected in the eastern North American tick vector, *I. scapularis*. Although it has been demonstrated experimentally that *B. bissettii* can be transmitted between hosts by *I. scapularis* (Oliver et al., 2003), our data support the notion that *B. kurtenbachii* strains may be maintained in enzootic transmission cycles that differ ecologically from those of *B. bissettii*. This needs, however, to be further explored since isolation of strain 25015 from blood-fed larvae does not comprise proof of vector competence. Both strains from Illinois were isolated from the rodent *Microtus pennsylvanicus* (Picken and Picken, 2000). In the southeastern United States, strains assigned to *B. bissettii* have been isolated from the eastern woodrat (*Neotoma floridana*), the cotton rat (*Sigmodon hispidus*), the cotton mouse (*Peromyscus gossypinus*), and the vectors *I. affinis* and *I. minor* (Lin et al., 2001, 2005; Oliver et al., 2003). However, the data presented here render the association of *B. bissettii* with *I. minor* uncertain because the strain isolated from this tick showed a *DraI* restriction pattern similar to that of strain 25015, and it also differed in its *MseI* restriction pattern from all other *B. bissettii* isolates (Lin et al., 2001). Taken together, our data suggest that the transmission cycles of *B. bissettii* and *B. kurtenbachii* differ, but the vector(s) for *B. kurtenbachii* needs to be determined throughout its geographic distribution, particularly in Illinois.

In Europe, *B. bissettii* DNA has been detected in, and was isolated from, human patients (Picken et al., 1996a, 1996b; Strle et al., 1997; Fingerle et al., 2008; Rudenko et al., 2008, 2009c), but it rarely has been found in questing *I. ricinus* ticks (Hulinska et al., 2007). It is, therefore, tempting to speculate that the transmission of *B. bissettii* is maintained by an unknown vector in an enzootic cycle with occasional spill over into the human population. Alternatively, if *B. bissettii* is transmitted by *I. ricinus*, the spirochaete distribution may be highly focal and restricted by its host associations since human cases have been recorded quite regionally. However, any association of *B. bissettii* with particular tick vectors and hosts in Europe is currently at best speculative and requires further investigation. This is also true for *B. kurtenbachii*(-related) strains that may occur in Europe (Picken et al., 1996a, 1996b). Of the tick species known to transmit *B. bissettii* in the United States, i.e., *I. pacificus* and *I. spinipalpis* in the Far West and Southwest and *I. affinis* in the Southeast (Bissett and Hill, 1987; Maupin et al., 1994; Lin et al., 2001, 2003), only *I. pacificus* attaches to humans with any frequency. This may partly explain why *B. bissettii* has not been incriminated as a human pathogen in the United States so far (Maupin et al., 1994). In addition, using a murine model for LB, it was demonstrated that *B. bissettii* strains in the United States differed considerably in their potential to induce arthritis in mice (Schneider et al., 2008). Early investigations into the pathogenic potential of strain 25015 revealed that this strain was non-arthritisogenic in mice (Anderson et al., 1990), although subsequent studies demonstrated that it might be mildly pathogenic when passaged repeatedly through laboratory mice (Fikrig et al., 1992). In North America, strain 25015 and related strains have been detected in *I. scapularis*, a tick that frequently attaches to humans. However, the rarity of finding *B. bissettii*-like strains in *I. scapularis* in recent years (Hoen et al., 2009; Hamer et al., 2010) or in patients underpins the need for further studies to clarify whether *I. scapularis* is the primary vector for *B. kurtenbachii* or whether the rare findings of this *Borrelia* species in *I. scapularis* can be attributed to spill-over from another enzootic transmission cycle. Although the number of *B. kurtenbachii* strains evaluated during the present study is restricted, most of the strains found in the previous study in Illinois revealed a macrorestriction pattern similar to that of strain 25015 (Picken and Picken, 2000). Such strains also have been described from the southeastern United States and perhaps Wisconsin indicating that *B. kurtenbachii* may be more widespread in its distribution and common in certain habitats than realized so far (Picken and Picken, 2000; Lin et al., 2001, 2005; Oliver et al., 2003; Caporale et al., 2005). At the time when *B. bissettii* was described, strain 25015 was the only example found in *I. scapularis* in upstate New York (Postic et al., 1998) suggesting that these strains are more common in Illinois and the southern United States. As mentioned above, the discovery of these strains in New York and eastern Canada are unusual because such strains have not been detected in host-seeking *I. scapularis* during recent studies in the northeastern or upper midwestern United States (Hoen et al., 2009; Hamer et al., 2010). In Illinois, the bacteria were isolated from voles and mice in consecutive years, and a rodent-associated transmission cycle might limit the distributional range. More research is needed to clarify the relationships among strains in these locations.

In the United States, the species with the widest distribution is *B. burgdorferi* (s.s.), which occurs in the southern, northeastern, Midwestern, and western states (Fig. 2). *B. andersonii* and *B. kurtenbachii* seem to be restricted to the east of the Rocky Mountains, whereas *B. carolinensis* and *B. californiensis* appear to be limited to South Carolina and California, respectively. Two species, *B. bissettii* and *B. americana*, are found in the southeastern and far-western regions, with *B. bissettii* also being present in Colorado. The occurrence of several genospecies across the continent suggests a dynamic evolutionary history of LB spirochaetes in the United States and is in agreement with recent suggestions that *B. burgdorferi* has been present in the country for several thousand or even millions of years (Hoen et al., 2009). They also imply that demographic events related to glacial and interglacial periods may have shaped the current population structure. Collectively, our own

and previous findings on the diversity of LB spirochaetes in the United States underscore the need for additional research to fully comprehend the ecology and evolutionary history of these spirochaetes and the impact of this spirochaetal diversity, if any, on human health.

Description of *Borrelia kurtenbachii* sp. nov

Borrelia kurtenbachii (kur.ten.ba'chi. L. gen in honour of the late Klaus Kurtenbach and his numerous contributions to research on the Lyme borreliosis group of spirochaetes).

The type strain 25015^T was isolated from an *I. scapularis* larva in upstate New York (Anderson et al., 1990). The morphology matches that of previously described *Borrelia* species in that it displays the typical helical morphology of spirochaetal bacteria (Barbour and Hayes, 1986). Its in vitro culture properties are as described for the genus (Johnson et al., 1984). *B. kurtenbachii* can be distinguished from all other LB species by means of its restriction pattern of the *rrf-rrl* intergenic spacer (Postic et al., 1998) and its housekeeping gene sequences using multilocus sequence analysis.

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Fig. 1.

Phylogenetic analysis of Lyme borreliosis (LB) genospecies. The tree was generated using concatenated sequences of 8 chromosomally located housekeeping gene fragments. For the phylogenetic reconstruction, the maximum composite likelihood model available in MEGA 4 was used with 1000 bootstrap replicates. Scale bar indicates 1% divergence.

Fig. 2.
Present day distribution of LB species in the United States based on published records.

Table 1

Borrelia isolates evaluated during this study.

ST	Strain ID	Country of origin	Region	Species	Biological origin	Year of collection	Collected by
156	CA128	USA	Hopland Field Station, Mendocino County, CA	<i>B. bissettii</i>	<i>Ixodes neotomae</i> (now known as <i>I. spinipalpis</i>) ex <i>Neotoma fuscipes</i>	1991	R.S. Lane
282	CA370	USA	Alameda County, CA	<i>B. bissettii</i>	<i>N. fuscipes</i> ear biopsy	1992	R.S. Lane
283	CA371	USA	Alameda County, CA	<i>B. bissettii</i>	<i>N. fuscipes</i> ear biopsy	1992	R.S. Lane
270	CA389	USA	Tilden Regional Park, Berkeley, CA	<i>B. bissettii</i>	<i>I. pacificus</i>	1993	R.S. Lane
272	DN127-C19-2/p7	USA	Del Norte County, CA	<i>B. bissettii</i>	<i>I. pacificus</i>	1985	J.R. Clover
	gom93-267 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>Neotoma mexicana</i>	1993	Gary O. Maupin
273	gom93-268	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-270 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-271 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-272 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
273	gom93-274	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
273	gom93-275	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-277 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
273	gom93-278	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-280 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
160	gom93-283	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
273	gom93-284	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
274	gom93-286	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
273	gom93-287	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-289 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
271	gom93-296	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
273	gom93-297	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-298 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
158	gom93-299	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
	gom93-300 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>Peromyscus difficilis</i>	1993	Gary O. Maupin
	gom93-304 ^a	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin

ST	Strain ID	Country of origin	Region	Species	Biological origin	Year of collection	Collected by
275	gom93-305	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>P. difficilis</i>	1993	Gary O. Maupin
276	gom93-310	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
277	gom93-501	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
277	gom93-543	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
277	gom93-544	USA	Larimer County, CO	<i>B. bissettii</i>	<i>I. spinipalpis</i> ex <i>N. mexicana</i>	1993	Gary O. Maupin
278	IL 96-255	USA	Poplar Creek, Cook County, IL	<i>B. kurtenbachii^b</i>	<i>Microtus pennsylvanicus</i>	1996	R. Picken
279	IL 97-236u	USA	Poplar Creek, Cook County, IL	<i>B. kurtenbachii^b</i>	<i>M. pennsylvanicus</i>	1996	R. Picken
280	25015	USA	New York State	<i>B. kurtenbachii^b</i>	<i>I. scapularis</i>	1987	J.F. Anderson
281	NS07-121	Canada	Nova Scotia	<i>B. kurtenbachii^b</i>	<i>I. scapularis</i>	2007	N. Ogden

^aThese strains showed mixed infections and could not be included into phylogenetic and multilocus sequence analysis.

^bThese strains form a distinct group that is genetically sufficiently distant from *B. bissettii* to represent a new species. We propose the name *Borrelia kurtenbachii* spec. nov. in honour of Klaus Kurtenbach.

Table 2

Sequence types and allelic profiles of *B. bissetii* and *B. kurtenbachii* isolates.

Strain	ST	<i>clpA</i>	<i>clpX</i>	<i>nifS</i>	<i>pep</i>	<i>pyrG</i>	<i>recG</i>	<i>rplB</i>	<i>urrA</i>
DNI27 ^T , CI9-2p7	272	117	69	68	102	99	99	83	89
CA128	156	91	69	68	81	77	73	69	73
CA389	270	118	70	83	103	100	100	84	90
CA370	282	119	83	88	81	101	101	89	73
CA371	283	120	69	68	104	99	74	83	91
Gom93-268	273	121	84	83	103	102	102	84	92
Gom93-274	273	121	84	83	103	102	102	84	92
Gom93-275	273	121	84	83	103	102	102	84	92
Gom93-278	273	121	84	83	103	102	102	84	92
Gom93-283	160	92	70	69	82	78	74	70	74
Gom93-284	273	121	84	83	103	102	102	84	92
Gom93-286	274	92	70	69	105	78	103	84	93
Gom93-287	273	121	84	83	103	102	102	84	92
Gom93-296	271	118	70	83	103	100	100	84	94
Gom93-297	273	121	84	83	103	102	102	84	92
Gom93-299	158	93	71	70	83	79	74	71	75
Gom93-305	275	122	70	84	106	103	104	84	94
Gom93-310	276	92	70	69	105	78	74	84	93
Gom93-501	277	123	70	85	103	100	100	84	95
Gom93-543	277	123	70	85	103	100	100	84	95
gom93-544	277	123	70	85	103	100	100	84	95
IL 96-255	278	124	85	86	107	104	105	85	96
IL 97-236u	279	125	86	87	108	105	106	86	97
25015	280	126	87	87	109	106	107	87	97
NS07-121	281	127	88	87	107	105	108	88	98

93-544	IL 96-255	IL 97_236u	25015	NS07-121	SCJ1	CA443	QLM4P1	B31	VS461	20047	VS116	B. andersoni	A14S	PBi	CA2	CA8	PoTiBL37	HK501	HO14	Ya501
98.34	96.5	96.55	96.37	96.53	97.32	95.47	90.56	94.13	91.81	92.03	91.33	93.36	90.97	91.91	94.54	94.04	91.82	91.72	91.71	91.53
98.71	96.84	96.88	96.7	96.86	97.69	95.42	90.56	94.1	94.191.56	91.98	91.23	93.36	90.75	91.89	94.24	93.99	91.7	91.72	91.53	91.41
99.81	96.61	96.65	96.55	96.63	97.87	95.26	90.23	93.91	91.45	91.72	90.98	93.14	90.84	91.57	93.75	93.78	91.48	91.59	91.28	91.41
98.48	96.6	96.64	96.46	96.62	97.46	95.51	90.74	94.2	91.92	92.2	91.48	93.31	91.02	92.06	94.56	94.08	91.8	91.82	91.8	91.73
98.41	96.62	96.66	96.48	96.64	97.44	95.54	90.76	94.2	91.94	92.1	91.4	93.43	91.04	91.96	94.6	94.18	91.9	91.82	91.82	91.63
99.26	96.72	96.74	96.64	96.72	97.82	95.44	90.43	94.01	91.52	91.81	91.13	93.29	90.89	91.72	94.01	93.92	91.63	91.69	91.43	91.39
99.26	96.72	96.74	96.64	96.72	97.82	95.44	90.43	94.01	91.52	91.81	91.13	93.29	90.89	91.72	94.01	93.92	91.63	91.69	91.43	91.39
99.26	96.72	96.74	96.64	96.72	97.82	95.44	90.43	94.01	91.52	91.81	91.13	93.29	90.89	91.72	94.01	93.92	91.63	91.69	91.43	91.39
99.26	96.72	96.74	96.64	96.72	97.82	95.44	90.43	94.01	91.52	91.81	91.13	93.29	90.89	91.72	94.01	93.92	91.63	91.69	91.43	91.39
99.01	96.66	96.7	96.59	96.72	97.69	95.47	90.33	93.89	91.43	91.84	91.03	93.1	90.79	91.7	93.91	93.87	91.63	91.57	91.38	91.39
99.26	96.72	96.74	96.64	96.72	97.82	95.44	90.43	94.01	91.52	91.81	91.13	93.29	90.89	91.72	94.01	93.92	91.63	91.69	91.43	91.39
99.01	96.66	96.7	96.59	96.72	97.69	95.47	90.33	93.89	91.43	91.84	91.03	93.1	90.79	91.7	93.91	93.87	91.63	91.57	91.38	91.39
99.26	96.72	96.74	96.64	96.72	97.82	95.44	90.43	94.01	91.52	91.81	91.13	93.29	90.89	91.72	94.01	93.92	91.63	91.69	91.43	91.39
99.83	96.63	96.68	96.57	96.66	97.89	95.28	90.26	93.94	91.47	91.74	91.01	93.17	90.87	91.6	93.77	93.8	91.51	91.62	91.31	91.44
99.26	96.72	96.74	96.64	96.72	97.82	95.44	90.43	94.01	91.52	91.81	91.13	93.29	90.89	91.72	94.01	93.92	91.63	91.69	91.43	91.39
98.71	96.48	96.52	96.35	96.5	97.39	95.4	90.33	94.1	91.52	91.86	91.11	93.21	90.77	91.69	94.44	93.94	91.6	91.52	91.28	91.29
99.52	96.75	96.79	96.68	96.77	97.95	95.37	90.41	94.01	91.57	91.65	91.13	93.19	90.99	91.58	93.87	93.85	91.56	91.6	91.46	91.54
99.03	96.68	96.72	96.61	96.7	97.72	95.44	90.3	93.91	91.4	91.77	91.06	93.07	90.77	91.72	93.89	93.85	91.61	91.59	91.4	91.41
100	96.5	96.54	96.43	96.52	97.85	95.33	90.31	93.89	91.4	91.65	91.11	93.14	90.91	91.53	93.75	93.8	91.39	91.57	91.28	91.36
100	96.5	96.54	96.43	96.52	97.85	95.33	90.31	93.89	91.4	91.65	91.11	93.14	90.91	91.53	93.75	93.8	91.39	91.57	91.28	91.36
0.035		99.66	99.39	99.52	96.95	95.46	90.39	94.01	91.74	91.99	91.21	93.4	90.87	91.99	93.94	93.66	91.56	91.89	91.87	91.51
0.0346	0.0034		99.56	99.56	97.01	95.46	90.36	94.12	91.74	91.91	91.14	93.43	90.82	91.84	93.98	93.66	91.56	91.77	91.89	91.39
0.0357	0.0061	0.0044		99.41	96.9	95.28	90.24	93.92	91.68	91.77	91.02	93.31	90.8	91.73	93.85	93.5	91.59	91.7	91.83	91.35
0.0348	0.0048	0.0044	0.0059		96.97	95.37	90.32	93.98	91.7	91.89	91.04	93.24	90.7	91.75	93.82	93.59	91.61	91.72	91.77	91.27
0.0215	0.0305	0.0299	0.031	0.0303		95.38	90.37	93.97	91.41	91.43	91.05	93.22	90.82	91.53	94.08	93.92	91.62	91.55	91.8	91.42

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	IL 96-255	IL 97_236u	25015	NS07-121	SCJ1	CA443	QLM4P1	B31	VS461	20047	VS116	B. andersoni	A14S	PBi	CA2	CA8	PoTBL37	HK501	HO14	Ya501
0.93-544																				
0.0467	0.0454	0.0454	0.0472	0.0463	0.0462		91.3	94.47	92.35	92.39	91.92	93.9	91.55	92.37	94.89	94.45	92.07	92.01	92.08	91.63
0.0969	0.0961	0.0964	0.0976	0.0968	0.0963	0.087		91.43	91.83	92.05	96.45	90.31	90.94	92.22	91.3	90.8	92.18	94.18	91.71	91.39
0.0611	0.0599	0.0588	0.0608	0.0602	0.0603	0.0553	0.0857		92.18	92.21	91.9	93.97	91.44	92.12	95.43	94.79	92.12	92.07	91.6	91.54
0.086	0.0826	0.0826	0.0832	0.083	0.0859	0.0765	0.0817	0.0782		93.31	92.47	91.9	93.32	93.55	92.61	91.52	93.04	92.61	93.22	92.51
0.0835	0.0801	0.0809	0.0823	0.0811	0.0857	0.0761	0.0795	0.0779	0.0669		92.94	91.71	92.22	98	92.78	91.76	92.99	93.09	93.22	92.7
0.0889	0.0879	0.0886	0.0898	0.0896	0.0895	0.0808	0.0355	0.081	0.0753	0.0706		90.69	91.77	93.11	91.97	91.45	92.47	95.12	92.46	91.88
0.0686	0.066	0.0657	0.0669	0.0676	0.0678	0.061	0.0969	0.0603	0.081	0.0829	0.0931		90.49	91.52	94.2	94.02	91.41	91.27	91.37	90.51
0.0909	0.0913	0.0918	0.092	0.093	0.0918	0.0845	0.0906	0.0856	0.0668	0.0778	0.0823	0.0951		92.41	91.7	90.98	92.08	91.72	91.95	91.52
0.0847	0.0801	0.0816	0.0827	0.0825	0.0847	0.0763	0.0778	0.0788	0.0645	0.02	0.0689	0.0848	0.0759		92.71	91.81	93.28	93.21	93.39	92.83
0.0625	0.0606	0.0602	0.0615	0.0618	0.0592	0.0511	0.087	0.0457	0.0739	0.0722	0.0803	0.058	0.083	0.0729		95.01	92.62	91.97	92.08	91.7
0.062	0.0634	0.0634	0.065	0.0641	0.0608	0.0555	0.092	0.0521	0.0848	0.0824	0.0855	0.0598	0.0902	0.0819	0.0499		91.67	91.78	91.27	91.06
0.0861	0.0844	0.0844	0.0841	0.0839	0.0838	0.0793	0.0782	0.0788	0.0696	0.0701	0.0753	0.0859	0.0792	0.0672	0.0738	0.0833		92.78	92.73	92.18
0.0843	0.0811	0.0823	0.083	0.0828	0.0845	0.0799	0.0582	0.0793	0.0739	0.0691	0.0488	0.0873	0.0828	0.0679	0.0803	0.0824	0.0722		92.75	92.3
0.0872	0.0813	0.0811	0.0817	0.0823	0.082	0.0792	0.0829	0.0804	0.0678	0.0678	0.0754	0.0863	0.0805	0.0661	0.0792	0.0873	0.0727	0.0725		92.03
0.0864	0.0849	0.0861	0.0865	0.0873	0.0858	0.0837	0.0861	0.0846	0.0749	0.073	0.0814	0.0949	0.0848	0.0717	0.083	0.0894	0.0782	0.077	0.0797	

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Table 4

Genetic diversity within *B. bissetii*^a.

Locus	Gene product/Description	Fragment length analysed (bp)	% G/C	π value	No. of alleles
<i>clpA</i>	Clp protease subunit A	579	26.6	0.00929	8
<i>clpX</i>	Clp protease subunit X	624	32.2	0.00349	4
<i>nifS</i>	aminotransferase	564	28.6	0.00427	6
<i>pepX</i>	dipeptidyl aminopeptidase	570	30.6	0.00349	7
<i>pyrG</i>	CTP synthase	603	32.0	0.02009	7
<i>recG</i>	DNA recombinase	651	31.0	0.00631	6
<i>rplB</i>	50S ribosomal protein L2	624	36.5	0.00865	5
<i>uvrA</i>	exonuclease ABC, SU A	570	35.7	0.00882	9
concatenated		4785	31.7	0.00767	

^aOnly samples identified as *B. bissetii* (Table 1) are included.