Borrelia bissettiae sp. nov. and *Borrelia californiensis* sp. nov. prevail in diverse enzootic transmission cycles

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Two species of the genus *Borrelia*, *Borrelia bissettiae* sp. nov. and *Borrelia californiensis* sp. nov., were first described by Postic and co-workers on the basis of genetic analyses of several loci. Multilocus sequence analysis of eight housekeeping loci confirmed that these two *Borrelia* genomospecies are distinct members of the *Borrelia burgdorferi sensu lato* complex. *B. bissettiae* sp. nov. was initially described in transmission cycles involving *Neotoma fuscipes* wood rats and *Ixodes pacificus* ticks in California, and *Neotoma mexicana* and *Ixodes spinipalpis* in Colorado. The preferred host of *B. californiensis* sp. nov. appears to be the California kangaroo rat, *Dipodomys californicus*; *Ixodes jellisoni*, *I. spinipalipis* and *I. pacificus* ticks are naturally infected with it. Thus, the ecological associations of the two genomospecies and their genetic distance from all other known *Borrelia* genomospecies species justify their description as separate genomospecies: *B. bissettiae* sp. nov. (type strain DN127^T=DSM 17990^T=CIP 109136^T) and *B. californiensis* (type strain CA446^T=DSM 17989^T=ATCC BAA-2689^T).

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*. *B. burgdorferi sensu lato* is a heterogeneous species complex that currently consists of at least 20 recognized or proposed genomospecies. These bacteria are maintained in natural transmission cycles among vertebrate reservoir hosts and ticks of the *Ixodes persulcatus* species complex or other species of the genus *Ixodes*, such as *Ixodes spinipalpis* (Brown & Lane, 1992, 1996; Kurtenbach *et al.*, 2006).

The strain designated the *Borrelia bissettiae* sp. nov. type strain, DN127^T, was isolated from a questing *Ixodes pacificus* tick collected in Del Norte County, California, during the 1980s (Bissett & Hill, 1987). Additional strains of this genomospecies have been isolated from *I. pacificus* or *Ixodes neotomae* (now *I. spinipalpis*) in California and Colorado (Bissett & Hill, 1987; Brown & Lane, 1992; Maupin *et al.*, 1994; Schneider *et al.*, 2000) and from rodents captured in the Chicago area of Illinois (Picken & Picken, 2000). Postic *et al.* (1998) proposed that these strains constitute a distinct genomospecies within the *B. burgdorferi sensu lato* complex and named it *B. bissettii* sp. nov. In the USA, *B. bissettiae* enzootic transmission cycles were also found in some southern states involving *Ixodes affinis* and various rodent-host species (Oliver *et al.*, 2003). In the far west of the USA, *B. bissettiae* was associated with the dusky-footed woodrat, *Neotoma fuscipes* Baird (Brown *et al.*, 2006). In the same geographical region, *B. bissettiae* was detected in a host-seeking avian tick, *Ixodes auritulus*, co-infected with *B. burgdorferi* (Padgett & Bonilla, 2011) and, more recently, in the blood of several bird species and *I. pacificus* immatures infesting birds (Newman *et al.*, 2015). In the latter study, the infection prevalence in *I. pacificus* larvae was much lower for *B. bissettiae* than it was for *B. burgdorferi*; thus, the role of birds as either primary or secondary reservoir hosts for *B. bissettiae* remains to be established.

Abbreviations: IGS, intergenic spacer; LB, Lyme borreliosis; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the sequences obtained in this study are KT709291–KT709458 and KT709517–KT709532. Sequence data are also available at the *Borrelia* MLST website at http://www.pubMLST.org/borrelia, hosted at the University of Oxford, UK (ID numbers 1002–1022, 1277 and 1450).

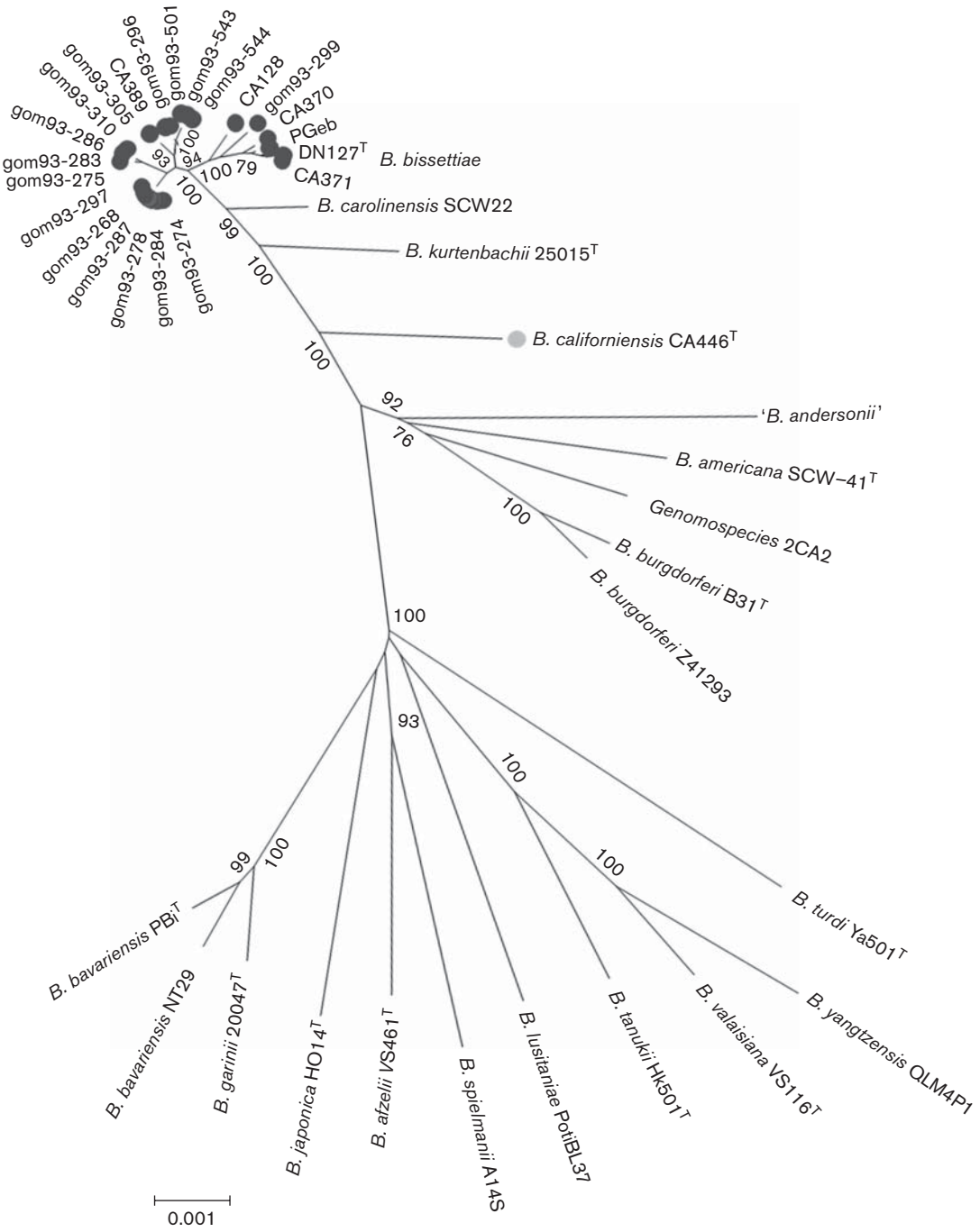

Fig. 1. Molecular phylogenetic analysis of *Borrelia bissettiae* sp. nov. (dark grey dots) and *Borrelia californiensis* sp. nov. (light grey dot) strains. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993). The tree with the highest log-likelihood (−21015.8975) is shown. Bootstrap values (500 replications) are shown next to nodes. Initial tree(s) for the heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter=0.2602)]. All positions containing gaps and missing data were eliminated. There were a total of 4779 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). Bar, 0.001 substitutions per site.

Table 1. *Borrelia bissettiae* sp. nov. and *B. californiensis* sp. nov. isolates from California and Colorado, USA, evaluated in previous studies

ST*	Strain ID	Country of origin	Region	Genomospecies	Biological source of isolate	Year of collection	Typed with:	pubMLST ID/ GenBank accession no.
156	CA128	USA	Mendocino County, CA	*B. bissettiae*	*I. neotomae* (now *I. spinipalpis*) ex *N. fuscipes*	1991	MLSA	1002
282	CA370	USA	Alameda County, CA	*B. bissettiae*	*N. fuscipes* ear biopsy	1992	MLSA	1003
283	CA371	USA	Alameda County, CA	*B. bissettiae*	*N. fuscipes* ear biopsy	1992	MLSA	1004
270	CA389	USA	Alameda County, CA	*B. bissettiae*	*I. pacificus*	1993	MLSA	1005
272	DN127-Cl9-2/p7	USA	Del Norte County, CA	*B. bissettiae*	*I. pacificus*	1985	MLSA	1006
273	gom93-268	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *Neotoma mexicana*	1993	MLSA	1007
273	gom93-274	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1008
273	gom93-275	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1009
273	gom93-278	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1010
160	gom93-283	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1011
273	gom93-284	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1012
274	gom93-286	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1013
273	gom93-287	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1014
271	gom93-296	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1015
273	gom93-297	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1016
158	gom93-299	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1017
275	gom93-305	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *Peromyscus difficilis*	1993	MLSA	1018
276	gom93-310	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1019
277	gom93-501	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1020
277	gom93-543	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1021
277	gom93-544	USA	Larimer County, CO	*B. bissettiae*	*I. spinipalpis* ex *N. mexicana*	1993	MLSA	1022
667	PGeb	Germany	Baden-Württemberg	*B. bissettiae*	*Human*	1996	MLSA	1874
447	CA443	USA	Mendocino County, CA	*B. californiensis*	*D. californicus* ear biopsy	1995	MLSA	1450
447	CA446	USA	Mendocino County, CA	*B. californiensis*	*D. californicus* ear biopsy	1995	MLSA	1277
NA	CA552	USA	Mendocino County, CA	*B. californiensis*	*Ixodes jellisoni* ex *D. californicus*	1998	rrf-rrl IGS	AY182059
NA	CA507	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1997	rrf-rrl IGS	AY182056
NA	CA504	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1997	rrf-rrl IGS	AY182055
NA	CA502	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1997	rrf-rrl IGS	AY182054
NA	CA462	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1996	rrf-rrl IGS	AY182053
NA	CA448	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1995	rrf-rrl IGS	AY182052
NA	CA442	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1995	rrf-rrl IGS	AF073254
NA	CA411	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1994	rrf-rrl IGS	AY182048
NA	CA31	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1990	rrf-rrl IGS	AJ006372
NA	CA22	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1990	rrf-rrl IGS	AY177631
NA	CA134	USA	Mendocino County, CA	*B. californiensis*	*I. pacificus* ex *D. californicus*	1991	rrf-rrl IGS	AY182042
NA	CA468	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1996	rrf-rrl IGS	AY177641
NA	CA404	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1993	rrf-rrl IGS	AJ006371
NA	CA33	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1990	rrf-rrl IGS	AY177632

Table 1. (cont.)

ST*	Strain ID	Country of origin	Region	Genomospecies	Biological source of isolate	Year of collection	Typed with:	pubMLST ID/ GenBank accession no.
NA	CA20	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1990	rrf-rrl IGS	AY180239
NA	CA142	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1991	rrf-rrl IGS	AY182043
NA	CA409	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1993	rrf-rrl IGS	AF073255
NA	CA547	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1998	rrf-rrl IGS	AY177642
NA	CA445	USA	Mendocino County, CA	*B. californiensis*	*D. californicus*	1995	rrf-rrl IGS	AF073256

NA, Not applicable. *ST, sequence type.

Of the tick species known to transmit *B. bissettiae* sp. nov. in the USA, i.e. *I. pacificus* and *I. spinipalpis* in the far west and south-west, and *I. affinis* in the south-east (Bissett & Hill, 1987; Lin *et al.*, 2001, 2003; Maupin *et al.*, 1994), only *I. pacificus* attaches to humans with any frequency. This may partly explain why *B. bissettiae* is not considered to be a human pathogen in the USA (Maupin *et al.*, 1994). On the other hand, *B. bissettiae* occasionally infects humans in northern California as demonstrated by the presence of its DNA in a few serum specimens, but signs or symptoms suggestive of clinical Lyme disease are lacking in this region (Girard *et al.*, 2011).

In Europe, *B. bissettiae* sp. nov. DNA has been detected in human patients (Picken *et al.*, 1996a, b; Rudenko *et al.*, 2008, 2009; Strle *et al.*, 1997), and in questing *Ixodes ricinus* ticks (Hulínská *et al.*, 2007; Tappe *et al.*, 2014). One human isolate of *B. bissettiae* (PGeb) was obtained from a German patient without a history of travel, providing direct evidence that *B. bissettiae* occurs in Europe (Fingerle *et al.*, 2008) (Fig. 1).

In phylogenetic analyses of the *rrf-rrl* intergenic spacer (IGS) region of *B. burgdorferi sensu lato*, strains CA443 and CA446 from northern California fell into a clade well separated from all other known genomospecies, a finding consistent with them representing a distinct genomospecies for which the name *Borrelia californiensis* sp. nov. was proposed (Postic *et al.*, 2007). Twenty-three *Borrelia* strains mainly isolated from the California kangaroo rat (*Dipodomys californicus*) clustered together with strains CA404, CA443 and CA446 (Postic *et al.*, 2007). Those data suggest that all such strains belong to the genomospecies *B. californiensis* sp. nov., and that *D. californicus* is a primary reservoir host of this genomospecies. Strains CA443 and CA446 investigated by multilocus sequence analysis (MLSA) with eight housekeeping genes formed a distinct clade that differed from all other species of the genus *Borrelia* (Margos *et al.*, 2010) (Fig. 1). Genetic-distance analysis confirmed the distinctness of these strains from other described species of the genus *Borrelia* (Margos *et al.*, 2010).

The samples used for studies of the two genomospecies are listed in Table 1.

Description of *Borrelia bissettiae* sp. nov.

Borrelia bissettiae [bis.set′ti.ae. N.L. gen. n. *bissettiae* of Bissett, proposed in honour of Dr Marjorie L. Bissett, who isolated and described this spirochaete along with her co-worker Warren Hill (Bissett & Hill, 1987)].

Cells are helical, approximately 0.2 μm by 20 μm, and stain well with Giemsa stain. Unstained cells can be visualized by dark-field microscopy. Flexible and motile with rotational and forward/backwards movement. Cells can be cultured *in vitro* under microaerophilic conditions (Johnson *et al.*, 1984b) using liquid media such as Barbour–Stoenner–Kelly (BSK) medium. Optimal growth occurs at 33–34 °C.

The type strain, DN127^{T}, was isolated from a questing *I. pacificus* tick in the late 1980s. It has been deposited in

the German Microbial Strain Collection (=DSM 17990^T) and at the Institut Pasteur, Paris, France (=CIP 109136^T). *B. bissettiae* can be distinguished from other genomospecies of the genus *Borrelia* via sequences of the 5S–23S IGS, the *rrs* locus and by MLSA (Margos *et al.*, 2010; Postic *et al.*, 1998). The *B. bissettiae* group is heterogeneous as shown by 5S–23S rRNA IGS (Postic *et al.*, 1998), MLSA analyses and by the size of the 16S–23S rRNA IGS fragment (Bunikis *et al.*, 2004) that approximates 1000 or 1100 bp (unpublished data). This bacterium is maintained in nature in diverse transmission cycles involving various rodent reservoir hosts and certain tick species of the genus *Ixodes*. Strains of this species have been found in the USA and Europe. The mean DNA G+C content of the type strain is 27 mol%.

Description of *Borrelia californiensis* sp. nov.

Borrelia californiensis (ca.li.for.ni.en′sis N.L. fem. adj. *californiensis* belonging to California, from where the type strain was isolated) was proposed by Postic *et al.* (2007).

Cells are helical, approximately 0.2 μm by 20 μm, and stain well with Giemsa stain. Unstained cells can be visualized by dark-field microscopy. Flexible and motile with rotational and forward/backwards movement. Cells can be cultured *in vitro* under microaerophilic conditions (Johnson *et al.*, 1984a) using liquid media such as BSK medium. Optimal growth occurs at 33–34 °C.

The type strain, CA446^T, was isolated from an ear-punch biopsy excised from a male *D. californicus* captured in November 1995 by Kerry A. Padgett at the University of California Hopland Research and Extension Center in Mendocino County, California. It has been deposited in the American Type Culture Collection (=ATCC BAA-2689^T) and the German Microbial strain collection (=DSM 17989^T). Sequence analysis of the *rrf-rrl* intergenic spacer and the *rrs* and flagellin genes differentiates *B. californiensis* from *B. bissettiae* (Postic *et al.*, 1998). *B. californiensis* strains are also distinguishable from all other LB species by using two different MLSA schemes (Margos *et al.*, 2010; Postic *et al.*, 2007). *B. californiensis* seems a rather homogeneous species. So far, it is restricted in distribution to northern California where its primary vertebrate host is the California kangaroo rat, *Dipodomys californicus* (Brown & Lane, 1992, 1996; Lane & Brown, 1991). Known vectors include *Ixodes jellisoni*, *I. pacificus* and *I. spinipalpis*. The mean DNA G+C content of the type strain is 27 mol%.

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