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A New *Borrelia* Species Defined by Multilocus Sequence Analysis of Housekeeping Genes▽†

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Analysis of Lyme borreliosis (LB) spirochetes, using a novel multilocus sequence analysis scheme, revealed that OspA serotype 4 strains (a rodent-associated ecotype) of *Borrelia garinii* were sufficiently genetically distinct from bird-associated *B. garinii* strains to deserve species status. We suggest that OspA serotype 4 strains be raised to species status and named *Borrelia bavariensis* sp. nov. The rooted phylogenetic trees provide novel insights into the evolutionary history of LB spirochetes.

Multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) have been shown to be powerful and pragmatic molecular methods for typing large numbers of microbial strains for population genetics studies, delineation of species, and assignment of strains to defined bacterial species (4, 13, 27, 40, 44). To date, MLST/MLSA schemes have been applied only to a few vector-borne microbial populations (1, 6, 30, 37, 40, 41, 47).

Lyme borreliosis (LB) spirochetes comprise a diverse group of zoonotic bacteria which are transmitted among vertebrate hosts by ixodid (hard) ticks. The most common agents of human LB are *Borrelia burgdorferi* (sensu stricto), *Borrelia afzelii*, *Borrelia garinii*, *Borrelia lusitaniae*, and *Borrelia spielmanii* (7, 8, 12, 35). To date, 15 species have been named within the group of LB spirochetes (6, 31, 32, 37, 38, 41). While several of these LB species have been delineated using whole DNA-DNA hybridization (3, 20, 33), most ecological or epidemiological studies have been using single loci (5, 9–11, 29, 34, 36, 38, 42, 51, 53). Although some of these loci have been convenient for species assignment of strains or to address particular epidemiological questions, they may be unsuitable to resolve evolutionary relationships among LB species, because it is not possible to define any outgroup. For example, both the 5S-23S intergenic spacer (5S-23S IGS) and the gene encoding the outer surface protein A (*ospA*) are present only in LB spirochete genomes (36, 43). The advantage of using appropriate housekeeping genes of LB group spirochetes is that phylogenetic trees can be rooted with sequences of relapsing fever spirochetes. This renders the data amenable to detailed evolutionary studies of LB spirochetes.

LB group spirochetes differ remarkably in their patterns and levels of host association, which are likely to affect their population structures (22, 24, 46, 48). Of the three main Eurasian *Borrelia* species, *B. afzelii* is adapted to rodents, whereas *B. valaisiana* and most strains of *B. garinii* are maintained by birds (12, 15, 16, 23, 26, 45). However, *B. garinii* OspA serotype 4 strains in Europe have been shown to be transmitted by rodents (17, 18) and, therefore, constitute a distinct ecotype within *B. garinii*. These strains have also been associated with high pathogenicity in humans, and their finer-scale geographical distribution seems highly focal (10, 34, 52, 53).

In this study, we analyzed the intra- and interspecific phylogenetic relationships of *B. burgdorferi*, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *B. bissettii*, and *B. spielmanii* by means of a novel MLSA scheme based on chromosomal housekeeping genes (30, 48).

***Borrelia* samples analyzed.** *Borrelia* strains used in this study are listed in Table 1.

DNA extraction, primers, and PCR conditions. Genomic DNA was extracted and purified from cultured isolates as described earlier (10, 30, 48). The loci analyzed comprised the "housekeeping" genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*), the 5S-23S IGS, *ospA*, and *ospC*. Primers and PCR conditions have been described in detail previously (14, 25, 30, 34). Two new outer primers (clpA1237F and clpA2218R) and a new inner forward primer (clpA1258F) were designed for *clpA*:

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† Supplemental material for this article may be found at http://aem.asm.org/.

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TABLE 1. LB species and strains used in this study

Strain	*Borrelia* species	Biological source (sample type)[a]	Geographic source[b]	Collector	Culture collection[c]	Source[d]	GenBank accession no.
VS461^T	*B. afzelii*	*Ixodes ricinus*	Switzerland	O. Peter		M. Cornet	
PKo	*B. afzelii*	Human	Germany	B. Wilske			NC_008277
IBS-11	*B. afzelii*	Human	Alsace, France	B. Jaulhac		M. Cornet	
IBS-12	*B. afzelii*	Human	Alsace, France	B. Jaulhac		M. Cornet	
IBS-13	*B. afzelii*	Human	Alsace, France	B. Jaulhac		M. Cornet	
IPT109	*B. afzelii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT110	*B. afzelii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT118	*B. afzelii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT122	*B. afzelii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT138	*B. afzelii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT142	*B. afzelii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT152	*B. afzelii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT154	*B. afzelii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT164	*B. afzelii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT179	*B. afzelii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
20047^T	*B. garinii*	*I. ricinus*	France	J. F. Anderson		M. Cornet	
PBi	*B. garinii*[e]	Human (CSF)	Ingolstadt, Germany	B. Wilske		V. Fingerle	
PFek	*B. garinii*[e]	Human (CSF)	Munich, Germany	B. Wilske		V. Fingerle	
PTrob	*B. garinii*[e]	Human (skin)	Slovenia	B. Wilske		V. Fingerle	
PRab	*B. garinii*[e]	Human (synovia)	Villach, Austria	B. Wilske		V. Fingerle	
POb	*B. garinii*[e]	Human (skin)	Munich, Germany	B. Wilske		V. Fingerle	
PFin	*B. garinii*[e]	Human (CSF)	Munich, Germany	B. Wilske		V. Fingerle	
PBN	*B. garinii*[e]	Human (CSF)	Munich, Germany	B. Wilske		V. Fingerle	
PScf	*B. garinii*[e]	Human (CSF)	Munich, Germany	B. Wilske		V. Fingerle	
PHoe	*B. garinii*[e]	Human (CSF)	Munich, Germany	B. Wilske		V. Fingerle	
PBaeI	*B. garinii*[e]	Human (CSF)	Munich, Germany	B. Wilske		V. Fingerle	
IPT28	*B. garinii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT114	*B. garinii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT126	*B. garinii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT130	*B. garinii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT139	*B. garinii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT140	*B. garinii*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT145	*B. garinii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT155	*B. garinii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT156	*B. garinii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT157	*B. garinii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT158	*B. garinii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT165	*B. garinii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT167	*B. garinii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT168	*B. garinii*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT169	*B. garinii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT171	*B. garinii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT172	*B. garinii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT178	*B. garinii*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT189	*B. garinii*	*I. ricinus*	Normandy, France		CNRB	M. Cornet	
IPT195	*B. garinii*	*I. ricinus*	Normandy, France		CNRB	M. Cornet	
VS116^T	*B. valaisiana*	*I. ricinus*	Switzerland	O. Peter		M. Cornet	
IPT29	*B. valaisiana*	*I. ricinus*	Meuse, France		CNRB	M. Cornet	
IPT31	*B. valaisiana*	*I. ricinus*	Meuse, France		CNRB	M. Cornet	
IPT33	*B. valaisiana*	*I. ricinus*	Meuse, France		CNRB	M. Cornet	
IPT47	*B. valaisiana*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT85	*B. valaisiana*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT102	*B. valaisiana*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT111	*B. valaisiana*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT121	*B. valaisiana*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT144	*B. valaisiana*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT153	*B. valaisiana*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT163	*B. valaisiana*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT174	*B. valaisiana*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT177	*B. valaisiana*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT184	*B. valaisiana*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT186	*B. valaisiana*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT187	*B. valaisiana*	*I. ricinus*	Limousin, France		CNRB	M. Cornet	
IPT188	*B. valaisiana*	*I. ricinus*	Normandy, France		CNRB	M. Cornet	
IPT2	*B. burgdorferi*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT19	*B. burgdorferi*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	

Continued on following page

TABLE 1—*Continued*

Strain	*Borrelia* species	Biological source (sample type)[a]	Geographic source[b]	Collector	Culture collection[c]	Source[d]	GenBank accession no.
IPT23	*B. burgdorferi*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT39	*B. burgdorferi*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT58	*B. burgdorferi*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT69	*B. burgdorferi*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT135	*B. burgdorferi*	*I. ricinus*	Auvergne, France		CNRB	M. Cornet	
IPT137	*B. burgdorferi*	*I. ricinus*	Alsace, France		CNRB	M. Cornet	
IPT190	*B. burgdorferi*	*I. ricinus*	Normandy, France		CNRB	M. Cornet	
IPT191	*B. burgdorferi*	*I. ricinus*	Normandy, France		CNRB	M. Cornet	
IPT193	*B. burgdorferi*	*I. ricinus*	Normandy, France		CNRB	M. Cornet	
IPT198	*B. burgdorferi*	*I. ricinus*	Normandy, France		CNRB	M. Cornet	
NE49	*B. burgdorferi*	*I. ricinus*	Switzerland	L. Gern		M. Cornet	
Z41293	*B. burgdorferi*	*I. ricinus*	Germany	M. Wittenbrink		M. Cornet	
Z41493	*B. burgdorferi*	*I. ricinus*	Germany	M. Wittenbrink		M. Cornet	
B31^T	*B. burgdorferi*	*I. scapularis*	Shelter Island, NY, USA	A. Barbour			NC_001318
PoHL1	*B. lusitaniae*	Human	Lisbon, Portugal	M. Collares-Pereira		M. Collares-Pereira	
PoTiBL37	*B. lusitaniae*	*I. ricinus*	Mafra, Portugal	S. Baptista		M. Collares-Pereira	
PoTiBGr41	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr82	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr130	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr131	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr136	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr143	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr209	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr211	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr213	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr288	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr293	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
PoTiBGr409	*B. lusitaniae*	*I. ricinus*	Grândola, Portugal	A. Quaresma		M. Collares-Pereira	
CA128	*B. bissettii*	*I. spinipalpis*	California, USA	R. S. Lane	CDC	M. Schriefer	
gom93-283	*B. bissettii*	*I. spinipalpis*	Colorado, USA	G. O. Maupin	CDC	M. Schriefer	
gom93-299	*B. bissettii*	*I. spinipalpis*	Colorado, USA	G. O. Maupin	CDC	M. Schriefer	
A14S	*B. spielmanii*	Human	The Netherlands				GPID 28635
DAH	*B. hermsii*	Human	Washington, DC, USA				NC_010673
91E135	*B. turicatae*	*Ornithodoros turicatae*	USA				NC_008710
Ly	*B. duttonii*	Human	Tanzania			M. Drancourt	

[a] CSF, cerebrospinal fluid.
[b] USA, United States of America.
[c] CNRB, Centre National de Référence des *Borrelia*, Institute Pasteur, Paris, France; CDC, Centers for Disease Control and Prevention, Fort Collins, CO.
[d] The strains were provided by M. Cornet, V. Fingerle, M. Collares-Pereira, M. Schriefer, and M. Drancourt.
[e] In this study, we suggest that these strains, assigned previously to *B. garinii*, be made a new species, *Borrelia bavariensis* sp. nov.

clpA1237F, 5′-AAAGATAGATTTCTTCCAGAC-3′; clpA 2218R, 5′-GAATTTCATCTATTAAAAGCTTTC-3′; and clpA 1258F, 5′-AAAGCTTTTGATATTTTAGATG-3′. PCR conditions remained the same as published previously (30).

Sequence analyses. Sequence analyses were performed as described elsewhere (30), and further information can be found in supplemental material. Values of the pairwise genetic distances enabled us to determine the threshold levels of sequence divergence between species using strains B31, Z41293, and NE49 (37, 40).

MLSA and phylogeny. MLSA and phylogenetic analyses were performed as described previously (30). MEGA 4 (21) was used for sequence alignments, and MrBayes 3.1.2 software (19) was used for phylogenetic analyses. Only the phylogenetic trees generated using MLSA were rooted. *B. lusitaniae* sequences were omitted from *ospA* and *ospC* analyses because of insufficient sequence overlap for phylogenetic analyses (48). For detailed information, see supplemental material.

In this study, a novel MLSA scheme has been used to study the phylogenetic relationships within and among the most common species of LB group spirochetes, i.e., *Borrelia burgdorferi*, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *B. bissettii*, and *B. spielmanii* (Table 1). Species defined by other methods formed discrete clusters in phylogenetic trees of concatenated housekeeping genes (Fig. 1). Strains from different species did not share any alleles, suggesting that horizontal gene transfer at these chromosomal loci did not occur between species of LB group spirochetes (see Table S2 in the supplemental material). *B. garinii* represented the most diverse species in this analysis. However, OspA serotype 4 strains (PBi and PBi-like strains) formed a fairly uniform group that was distinct from all other *B. garinii* strains (Fig. 1) (see Tables S2 and S3 in the supplemental material). Our study suggests that these strains, previously assigned to *B. garinii*, are sufficiently divergent genetically and ecologically to raise them to species status.

For LB group spirochetes, new species have been delineated recently using a different MLSA scheme which showed agreement with whole DNA-DNA hybridization (37, 40). To compare the genetic distances obtained by our MLSA scheme with

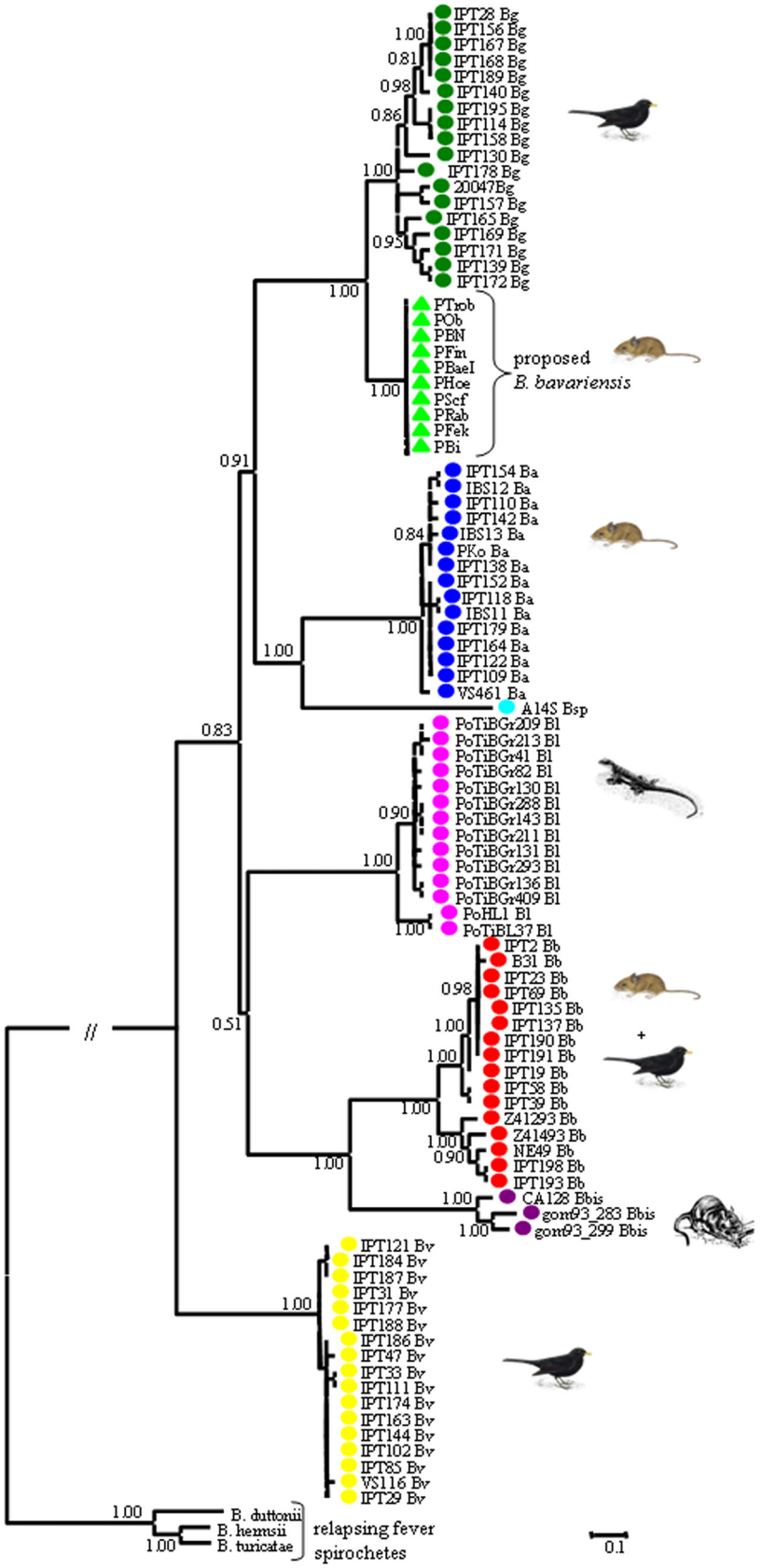

FIG. 1. Rooted Bayesian phylogenetic inference of concatenated housekeeping gene sequences of LB group spirochetes. Posterior probability values of clades are provided. Previously assigned *Borrelia* species are color coded as follows: red, *B. burgdorferi* (Bb) sensu stricto; blue, *B. afzelii* (Ba); green, *B. garinii* (Bg) or *B. bavariensis* sp. nov.; yellow, *B. valaisiana* (Bv); purple, *B. bissettii* (Bbis); sky blue, *B. spielmanii* (Bsp); and pink, *B. lusitaniae* (Bl). The tree was rooted with sequences of the relapsing fever spirochetes *Borrelia duttonii*, *B. hermsii*, and *B. turicatae*. The branch length of the outgroup is not according to scale as indicated by the slashes. The bar labeled 0.1 depicts 10% divergence.

previously reported values, we included type strains of *B. burgdorferi*, *B. afzelii*, *B. garinii*, and *B. valaisiana*. The pairwise genetic distances between the type strains ranged from 0.06 to 0.08 (see Table S4 in the supplemental material), a similar range as described previously (37, 40). *B. burgdorferi* strains Z41293 and NE49 have been used to define species threshold levels (pairwise genetic distance for strains B31/Z41293 = 0.021) for the MLSA scheme developed by Richter and colleagues (37, 40). Using these strains, we determined the species threshold level for our MLSA system to be 0.0170 (see Table S4 in the supplemental material).

The pairwise genetic distance of the concatenated housekeeping genes between *B. garinii* type strain 20047 and PBi-like strains was 0.0200, which is much higher than the genetic distance of strain 20047 to other bird-transmitted *B. garinii* isolates. This genetic distance in combination with the ecological differences (see below) would justify raising OspA serotype 4 strains to species status. We propose the name *Borrelia bavariensis* sp. nov., because it was first found in Bavaria, Germany.

In the Bayesian phylogenetic tree (Fig. 1) based on the concatenated sequences of the eight housekeeping genes, strain PBi and related OspA serotype 4 strains formed a distinct subclade, being basal to the remaining *B. garinii* strains in 100% of the trees. This was found regardless of the tree building method used (Fig. 1) (see Fig. S5 in the supplemental material). In phylogenetic analysis of the 5S-23S IGS, strain PBi and related OspA serotype 4 strains (with the exception of strain PHoe) also formed a distinct cluster within the *B. garinii* clade (see Fig. S2 in the supplemental material).

In our study, the housekeeping genes showed different evolutionary pathways compared with the plasmid-located genes *ospA* and *ospC* (see Fig. S3 and S4 in the supplemental material), which is consistent with reports on plasmid exchange and/or genetic recombination (2, 26, 39, 49, 50). While in the tree generated using *ospC* sequences, strains from all species were dispersed into different clusters, including mixed-species clusters (see Fig. S4 in the supplemental material), in the *ospA* tree both *B. garinii* and *B. valaisiana* strains were split into two separate clusters (see Fig. S3 in the supplemental material). The division of *B. valaisiana* strains into two separate groups using OspA protein sequences has been reported previously, and it has been proposed that their genes evolved from two distinct ancestors (50).

The evolutionary relationships among the LB group spirochetes included in this study support the hypothesis that bird specialization has been acquired at least twice independently (i.e., in *B. valaisiana* and in *B. garinii*). *B. valaisiana* and *B. garinii* were found to form distinct sequence clusters in phylogenetic trees of MLSA sequences (Fig. 1). However, they are, with the exception of OspA serotype 4 strains, very similar ecologically, in that they occur sympatrically and utilize the same spectrum of tick vectors and vertebrate hosts, i.e., birds (16, 22, 24, 45). It is likely that *B. valaisiana* and *B. garinii* evolved allopatrically, which may explain their pronounced genetic distance. Strains assigned to *B. garinii* by other methods, on the other hand, represent at least two ecotypes (rodent-associated ecotype versus bird-associated ecotype), which are congruent with distinct clusters revealed by MLSA. Although a previous study suggested that OspA serotype 4 strains represent a recently emerged clonal lineage within *B. garinii* (28), such strains (PBi and related strains) were found to be at the base of the *B. garinii* clade in the MLSA tree. This suggests that specialization of OspA serotype 4 strains to rodents is a more ancient trait and that genetic elements of *B. garinii* that allow its transmission by birds were acquired more recently. A more recent adaptation of *B. garinii* to birds as reservoir hosts could also explain the present-day sympatric distribution of *B. valaisiana* and *B. garinii*. It also suggests that adaptation to avian hosts evolved at least twice independently in the LB group of spirochetes (i.e., in *B. valaisiana* and *B. garinii*). Phylogenetic studies of other bird-associated LB species, such as *B. turdi*, may provide more information on the evolution of host specialization.

The consistency in species clustering and the fact that trees can be rooted using sequences of relapsing fever spirochetes highlights the suitability of MLSA based on housekeeping genes for evolutionary studies of LB group spirochetes. LB spirochetes comprise distinct ecotypes that are broadly defined by their spectrum of vertebrate hosts (22, 24). Ecotypes of LB group spirochetes can, therefore, be determined more easily than for free-living bacteria. Raising sufficiently divergent sequence clusters of LB group spirochetes, which correspond to ecotypes, to bacterial species status would have the advantage of being ecologically, epidemiologically, and clinically predictive.

Nucleotide sequence accession numbers. The sequences of the housekeeping genes have been submitted to the MLST/MLSA website hosted at Imperial College London (http://www.mlst.net) and can be accessed via the strain name or sequence type. The GenBank (http://www.ncbi.nlm.nih.gov/) accession numbers are as follows: for 5S-23S IGS, FJ546482 to FJ546547 and GQ178225 to GQ178231; for *ospA*, FJ546596 to FJ546657 and GQ178232 to GQ178244; and for *ospC*, FJ546548 to FJ546595, GQ178223, and GQ178224.

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