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Borrelia burgdorferi sensu stricto *ospC* alleles associated with human Lyme borreliosis worldwide were detected in non-human biting tick *Ixodes affinis* and rodent hosts in southeastern U.S.A

Running title: Analysis of *ospC* alleles from southeastern U.S.A.

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Abstract

Comparative analysis of *ospC* genes from 127 *Borrelia burgdorferi* sensu stricto strains collected in Lyme disease endemic and non-endemic European and North American regions revealed close relatedness of geographically distinct populations. *OspC* alleles A, B and L were detected on both continents in vectors and hosts including humans. Six *ospC* alleles, A, B, L, Q, R and V, were prevalent in Europe; 4 of them were detected in samples of human origin. Ten *ospC* alleles, A, B, D, E3, F, G, H, H3, I3 and M, were identified in the far-western U.S.A. Four *ospC* alleles, B, G, H and L, were abundant in the southeastern U.S.A. Here we present the first expanded analysis of *ospC* alleles of *B. burgdorferi* strains from the southeastern U.S.A with respect to their relatedness to strains from other North American and European localities. We demonstrate that *ospC* genotypes commonly associated with human Lyme disease in endemic European and North American regions were detected in *B. burgdorferi* strains isolated from non-human biting tick *Ixodes affinis* and rodent hosts in southeastern U.S.A. We discovered that some *ospC* alleles previously known only from Europe are widely distributed in the southeastern U.S.A., a finding that confirms the hypothesis of trans-oceanic migration of *Borrelia* species.

Introduction

Establishment of *Borrelia* spp. populations in different geographic regions is determined by natural factors (31). The maintenance of spirochete species in nature depends upon the relative abundances of their reservoir hosts and vector ticks, and the intensity of host-vector interactions (48). The worldwide distribution of spirochetes from *Borrelia burgdorferi* sensu lato (s.l.) complex, some of which cause Lyme disease (LD), is facilitated by the long-distance dispersal of infected ticks by migrating hosts (16, 49,

53). A hypothesis for the migration route of *Borrelia* spp. between the continents was proposed and the first evidence of trans-oceanic dispersal of *B. burgdorferi* sensu stricto (s.s.) was presented almost 15 years ago (19, 41, 52, 55).

B. burgdorferi s.s. is the primary, but not the only, species that causes LD around the world (23, 60, 62, 63). Different strains of *B. burgdorferi* s.s. exhibit considerable genetic heterogeneity locally as well as globally. Also, molecular analyses revealed a close relationship and an overlapping of genotypes between European and North American spirochete populations, which confirms the trans-oceanic migration hypothesis and the existence of recombinant genotypes (19, 55). Multiple genotypes of *B. burgdorferi* s.s. have been identified based on the analysis of a spirochete gene (*ospC*) that encodes a highly polymorphic outer surface protein C (38, 39, 74). *Borrelia* OspC antigen is heavily targeted by the host immune system. It establishes the secondary immune response, or immune memory in the hosts (11). Associations between *ospC* genotypes and invasiveness in patients (1, 10, 18, 30, 65) and experimentally infected animals (71, 72) have been reported. The *ospC* gene is more diverse than any other *Borrelia* gene studied to date (11). *B. burgdorferi* s.s. has the ability to infect a wide range of phylogenetically diverse vertebrate hosts, which facilitates the further expansion of the spirochete into new geographical areas (4, 5, 22, 34). Selection pressure from the vertebrate immune system is likely responsible for the high polymorphism of the *ospC* gene (11, 54, 70, 73).

Furthermore, because *B. burgdorferi* s.s. is a host generalist that occurs in birds, rodents and other mammals, its dispersal potential is considerable. More than 240 animal species have been reported as hosts for tick vectors and potential reservoir hosts of *Borrelia* in Europe (22). Such a diverse host spectrum may lead to the establishment of

new enzootic LD foci in Europe. We believe that the current distribution of *B. burgdorferi* s.s. in Europe is much wider than has been reported and is enhanced by involvement of multiple phylogenetically diverse migratory animal species. Such expansion could affect LD risk and helps to explain the observed increased incidence of LD in humans worldwide.

Earlier studies carried out in LD endemic areas demonstrated that the vast majority of known *ospC* alleles are geographically distinct (14, 41, 56, 74). The presence of *Borrelia* spp. in nature is known to be affected by recent urbanization, an increasing overlap between human and *Borrelia* habitats, and a climate change (7, 42, 51, 55, 59, 61, 66). Thus, it is not unexpected that the distributions of *Borrelia* genotypes may have been shifting in recent decades, and may continue to shift. The number of LD cases worldwide has increased recently (28, 67) which may be attributable to *Borrelia* expansion, or to gene transfer that resulted in recombinant genotypes (73).

The objectives of this study were to compare *ospC* alleles from a southeastern U.S.A. population of *B. burgdorferi* s.s. with other *Borrelia* s.s. strains from non-endemic and endemic North American and European localities. The search for evidences that support hypothesis of trans-oceanic migration of *Borrelia* species was another aim of our project. Our study was not meant to be a statistical analysis with emphasis on ranking of *Borrelia ospC* alleles, but rather the invitation to the open discussion to advance the natural history and understanding of enzootiology of *B. burgdorferi* s.s. in southeastern United States, previously considered to be “low-or-no” Lyme disease region.

Materials and Methods

Control sequences

As a control group, 100 of *B. burgdorferi* s.s. strains with different *ospC* types were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) (details in Table 1).

Experimental samples

The experiment group includes 127 samples of *B. burgdorferi* s.s. The sampling in presented study was not exhaustive or random for a variety of reasons as statistical analysis was not a goal of this project. Of these, 58 samples were derived from the vector ticks *Ixodes ricinus, I. affinis, I. pacificus*, and *I. scapularis*. For this purpose each tick was rinsed in 10% bleach for 50 minutes with following 5 minutes wash in 70% ethyl alcohol. Cleaned tick was air dried on sterile filter paper, placed in 100 microliters of BSK-H media and homogenized. All instruments and breakers were autoclaved prior to use. Homogenized mixture was transferred into 5 ml with BSK-H and tubes were maintained at 34^{o}C for 8 weeks. All work was conducted in sterile biohazard hood.

Another 35 samples originated from the rodents *Peromyscus gossypinus*, *Neotoma floridana, Sigmodon hispidus, Tamias senex*, *N. fuscipies* and *Sciurus griseus*. Samples from ear clips were prepared as follows: triangle cut from ear was washed in 70% ethyl alcohol for 4-5 minutes. After that ear clip was soaked for 4-5 minutes in freshly diluted 10% bleach, followed by 1 minute rinse in 95% ethanol. After 2 minutes of air drying under the sterile conditions, tissue was cut into 3-4 pieces and placed into 5 ml of BSK-H media. Tubes were kept at 34^{o}C for 6 weeks. Spirochete cultures from internal organs were initiated as follows: organs were removed from euthanized animals, placed directly in 200 microliters of BSK-H media, chopped in it and left at room temperature for 2-3 minutes. After that 100 microliters of mixture were transferred into 5 ml of BSK-H media and kept at 34^{o}C for 6 weeks. The remaining 34 samples were of human origin. Fifty three samples were collected in Georgia, South Carolina, and Florida in the

120 southeastern U.S.A.; 25 samples were collected in California in the far-western U.S.A;
121 and 49 samples came from a subset of LD endemic European countries, i.e. the Czech
122 Republic, Germany, Hungary, Slovakia, Slovenia, and Switzerland (Table 2).
123 *DNA purification, PCR amplification, and sequencing*
124 Total *Borrelia* DNA was purified using the DNeasy Blood and Tissue kit (Qiagen,
125 U.S.A.). Partial *ospC* genes were amplified using previously described *ospC* primers
126 (74) and protocols (24, 58, 61). PCR products from European and the southeastern
127 U.S.A. samples were sequenced at a University of Washington, while *ospC* products
128 from California strains were sequenced at a University of California, Berkeley
129 sequencing facility. All 127 samples were sequenced at the *ospC* locus directly in both
130 directions, then assembled and edited using DNAStar (DNASTAR, United Kingdom).
131 The BlastN algorithm was used to confirm identity against GenBank.
132 *Nucleotide sequence accession numbers*
133 All sequences obtained in this study have been submitted to the GenBank (Table 2).
134 *Data Analysis*
135 Sequences were aligned using Clustal X (36). Because a high level of recombination
136 was confirmed for *Borrelia ospC* gene, a cladistic analysis was deemed inappropriate (12).
137 Clustering analysis was performed using the Neighbor Joining method with uncorrected
138 (raw) pairwise sequence distances, as modified in BioNJ (20). One thousand bootstrap
139 replicates were performed under a neighbor-joining search to obtain support values for
140 clusters. A 50% majority rule consensus tree was formed, with ties broken randomly if
141 encountered.
142 ***Results***

The comparative analysis presented here includes partial sequences of *ospC* genes from 227 *Borrelia* strains (100 control and 127 experimental) originating from recognized LD endemic areas in Europe, northeastern, Midwestern and far-western U.S.A. and from the southeastern U.S.A that for a long time is considered to be LD non-endemic region.

The southeastern U.S.A. samples of *Borrelia* contained 22 strains isolated from *I. affinis,* 1 from *I. scapularis*, 25 from *P.gossypinus*, 1 from *N. floridana* and 4 from *S. hispidus*. California samples included 20 isolates from *I. pacificus*, 3 from *Sciurus griseus*, 1 from *Tamias senex* and 1 from *Neotoma fuscipies*. Among 49 European *Borrelia* samples, 15 originated from *I. ricinus* and 34 from humans. Most *ospC* amplicons were 525-610 bp long (depending upon what PCR primers were used) and were truncated to 498 bp to achieve a perfect alignment. Four samples had to be removed from the Neighbor-joining analysis because they were too short (<200 bp). These samples were placed in clusters with samples whose sequences were identical over the length of the sequence fragment we were able to obtain for them on the assumption that this represents the best clustering position for the sequence available.

OspC sequences from the experimental samples were identified as one of the known *ospC* alleles by their strong clustering with control sequences in the analysis. In total, 14 *ospC* alleles were identified among the 127 experimental samples (Fig. 1).

Clades B, D, E3, F, G, H, H3, I3, L, M, Q, R and V contained experimental samples, and were all well-supported (bootstrap values of 100%) with apparent monophyly of the clade type samples (those taken from GenBank and previously designated). Clade A received 81% bootstrap support when all samples were included; but ignoring one control sample (X84738), the clade received 100% support. In one anomaly, the *ospC*

allele E3 clade, which included samples from California and one control *ospC* allele E3, clustered tightly with one control type O strain from the northeastern U.S.A. The other control *ospC* allele O strain was placed on a separate branch, alone. This is evidence that what has been classified as *B. burgdorferi* s.s. genotype O is not monophyletic. Overall, clades containing experimental samples were scattered throughout the tree and represented a broad variety of *ospC* alleles.

North American

OspC allele G, the third most abundant type among experimental samples, was detected only in North American samples. It was present equally in vector- and host-derived strains from the southeastern U.S.A. that clustered with strains from the northeastern U.S.A. *OspC* allele G also was detected in 2 *I. pacificus* nymphs.

OspC allele H was the fourth most abundant allele detected among southeastern *B. burgdorferi* s.s. strains cultured from ear clips, bladders, spleen, kidney and hearts of multiple rodent hosts and a single *I. affinis* nymph. *OspC* allele H strains from California were cultured from an *I. pacificus* nymph and a western gray squirrel (*S. griseus*). This *ospC* allele was not detected in ticks or in hosts collected from any European locality sampled in this studies (Table 2).

California-specific *ospC* alleles E3 and H3 were both detected in hosts and *I. pacificus* ticks, the main vector of spirochetes from *B. burgdorferi* s.l. complex from the far-western U.S.A. *OspC* alleles D, I3 and M were detected in *I. pacificus* only. The Neighbor Joining analysis revealed that the only experimental samples that fell into the H3 and I3 clades were from California. Californian *ospC* alleles D and M clustered with northeastern *B. burgdorferi* s.s. D and M control sequences. *OspC* alleles D, E3, H3, I3 and M were not detected among the European or southeastern U.S.A. samples.

The southeastern U.S.A. strains of *B. burgdorferi* s.s. contained *ospC* alleles B, G, H and L, with almost equal representation of alleles B, G and L (30% of each). California samples showed the largest number of *ospC* alleles (A, B, D, E3, F, G, H, H3, I3 and M) found at any one location. *OspC* allele L was not found in California, despite its being widely distributed in ticks and hosts in the southeastern U.S.A. and connected to human LD in Europe.

European

OspC allele Q, R and V were found among the European *B. burgdorferi* s.s. samples only (Table 2). Clades Q and R contained only vector-originated spirochete samples from control and experimental groups; clade V contained both tick and human-originated strains (Fig. 1).

The highest diversity of *B. burgdorferi* s.s. *ospC* alleles (type per number of samples) was detected in Neuchâtel, Switzerland. Five out of 6 (83%) *ospC* alleles detected among all European samples were found in seven spirochete cultures isolated from *I. ricinus* nymphs collected from this single location in Switzerland: *ospC* allele B (one), L (two), Q (one), R (two) and V(one) (Table 2).

Transcontinental

Three *ospC* alleles (A, B and L) were detected in European and North American *B. burgdorferi* s.s. samples.

OspC allele A, associated with the most pathogenic strains of *B. burgdorferi* s.s., was detected in the serum sample of a patient (N12/JQ219683) with a LD diagnosis from southern Czech Republic. Four other *ospC* allele A strains were identified among the Californian isolates (Table 2). All five *ospC* sequences were 100% identical and clustered clearly (100% bootstrap support) with control sequences from LD endemic

European countries, as well as from the northeastern and Midwestern U.S.A. *OspC* allele A was not detected in the samples from the southeastern U.S.A. (Table 2).

OspC allele B was the most abundant among the European samples of human origin (skin, blood, serum, cerebrospinal fluid or joint fluid of the patients) and was the most represented among the host and vector originated southeastern U.S.A. strains (Table 2). One *ospC* allele B strain was cultivated from *N. senex* (Allen's chipmunk) captured in California. The *ospC* allele B clade consisted of two subclades, one containing a preponderance of southeastern samples (SCW/SCCH/BUL) clustered with *ospC* B allele strains from New York and Michigan, another consisting of European control and experimental samples.

Eighty percent of *ospC* allele L (the second most abundant) were detected among southeastern U.S.A. *B. burgdorferi* s.s. strains cultured from non-human biting ticks *I. affinis* or from ear clips, bladders and hearts of local rodent hosts. The remaining 20% of *ospC* allele L strains were isolated from *I. ricinus* nymphs and from the skin of a Slovenian patient (SLV1/JQ236853) diagnosed with acrodermatitis chronica atrophicans (Table 2).

Discussion

Since recognition of Lyme disease in the 1970s discussions of it etiology attracted attention of wide scientific community and the general public. Our analysis of population structure of *B. burgdorferi* s.s. in southeastern United States represents the logical extension of similar studies conducted in highly endemic northeastern and moderate mid-western United States. The presented results are not meant to be a statistical analysis with emphasis of ranking of *Borrelia ospC* alleles, but rather the invitation to the open discussion to advance the natural history and understanding of enzootiology of *B.*

burgdorferi s.s. in southeastern United States, previously considered to be "low-or-no" Lyme disease region.

Even though several different spirochete species cause LD, *B. burgdorferi* s.s. is still considered as the major species that causes clinical illness in the U.S.A. It also causes LD in Europe, though at a lower rate. Molecular analysis reveals overlap of *B. burgdorferi* s.s. genotypes between European and North American spirochete populations (55). While most of *B. burgdorferi* sensu lato species or subtypes in Europe are specialized to infect specific taxa of vertebrate hosts ('specialists'), *B. burgdorferi* s.s., as a 'generalist', has the ability to infect a wide range of phylogenetically diverse vertebrates. In fact, *B. burgdorferi* s.l. spirochetes are one of the few groups of zoonotic pathogens for which a molecular mechanism of host 'specialism' or 'generalism' has been proposed (74).

B. burgdorferi s. s. is transmitted from one vertebrate host to another by *Ixodes* spp. ticks belonging to the *Ixodes ricinus* complex. All parasitic stages of these ticks are able to transmit the pathogen, but the nymphal stage appears to be the most important one (3, 17, 32, 64). A notable exception is the Asian species, *I. persulcatus*, in which the female tick, not the nymph, is a primary vector of *B. burgdorferi* s.l. In Europe, *B. burgdorferi* is transmitted by *I. ricinus* ticks (22). In the U.S.A., *I. scapularis* is the primary vector of *B. burgdorferi* s.s. in the eastern, northeastern and north central regions (48), whereas *I. pacificus* is the primary vector in the far-western part of the U.S.A. (13, 33). The majority of LD cases come from the Northeast (> 80%) (50) where the population of human biting tick vector *I. scapularis* is well established. Lyme disease in a mid-west has received little attention, most probably because the distribution of *I. scapularis* and establishment of local population in that region was recognized only recently (25) and

only around 12% of human LD cases were reported from that region (56). LD in the southeastern United States received even less attention due to the presumable low abundance of *I. scapularis* and recognition of *Amblyomma americanum* that does not transmit *B. burgdorferi*, as a major human-biting tick in this region (69). What is needed to be taken into consideration is, as correctly noticed by Stromdahl and Hickling, that "the lack of detection of a tick species is not proof of that species' absence from the survey area" (69). Collection methods are biased for specific tick species, development stages, collection season and sampling region (Oliver J.H., Jr. unpublished data). Unfortunately, past tick surveys in southeastern United States were affected by the amount of efforts in sampling of different habitats and hosts and the lack of experience with region-specific methodology. *I. scapularis* distribution map from 1945 indicated that this tick species was widely distributed in southeastern U.S.A. (9). Even though southeastern tick population has undergone dramatic changes due to the increasing of wild hosts' population, climate changes, urbanization or geographical spread, it still does not mean that southeastern *I. scapularis* population was decreased so significantly. Rapid expansion of *I. scapularis* ticks in northeastern United States and recent invasion in mid-western U.S.A. originated from a very few migrants from the southeastern region after the recession of Pleistocene ice sheets (29). The mid-western tick populations are much younger than northeastern one, and, both are an order of magnitude younger than southeastern population of *I. scapularis*. This fact led to hypothesis that ticks were introduced or re-introduced to new areas by long-distance migration maintained by birds (25, 29, 43, 44, 49). As distribution pattern of *B. burgdorferi* and re-colonization of new regions by this pathogen is tightly linked to it tick vectors or vertebrate hosts, we can presume, from one side, that northeastern and mid-western strains of *B. burgdorferi* has

the same origin as their main tick vector *I. scapularis*, which is the southeastern United States. From the other side, the strict connection of pathogen distribution pattern to the pattern of distribution of its principal vector is naive, as we still do not know how much the pathogen and vector share common evolutionary or biogeographic history (25, 29).

Several other tick species in the *I. ricinus* complex and those not included in it have been found to maintain *B. burgdorferi* enzootically (2, 13, 21, 45, 48, 49). Recent publication by Hamer and colleagues (2011) showed the strong evidences that confirmed the presence of multiple strains of *B. burgdorferi* in areas with apparent absence of *I. scapularis*, which means the absence of classical spirochete maintenance cycle *I. scapularis* - *P. leucopus* (25). This study supports the previously presented hypotheses of uncoordinated phylogeography of *B. burgdorferi* and its tick vector *I. scapularis* (29) and the impact of the migratory hosts in pathogen expansion (43, 44). Despite the strong association of LD spirochete with *I. scapularis*, the population structure, evolutionary history and biogeography of the pathogen are distinct from its arthropod vector (29).

Except the abundant tick vector, appropriate vertebrate hosts are required for enzootic maintenance of *B. burgdorferi.* There is a variety of vertebrate hosts, including small mammals and birds that might serve as reservoir hosts for *B. burgdorferi* in the U.S.A. But, in general, rodents appear to be the most common reservoir hosts in the North American LD endemic regions (7, 33, 34, 46, 46, 47, 48). Recent studies suggest that migration of infected vertebrate hosts may have a larger impact in the contemporary expansion of pathogen population than the movement of tick vectors (25, 29). The low prevalence of *B. burgdorferi* in *I. scapularis* does not necessary mean the low prevalence or absence of the pathogen in the region, taking into consideration the existence of "cryptic" maintenance cycles or impact of migrating infected reservoir hosts (25). The

311 convincing scenario showing how migrating hosts may accelerate the increasing of LD
312 risk to humans through the maintenance of *B. burgdorferi* in the absence of classic *I.*
313 *scapularis* / *P. leucopus* transmission was recently presented by Hamer and colleagues
314 (25).

315 Association between LD severity and *ospC* alleles has been reported previously (26,
316 55, 64, 68). Twenty eight *ospC* alleles have been identified in *B. burgdorferi* s.s. (8).
317 While some spirochete complexes were believed to be restricted exclusively to North
318 America (B1, C, D, F, G, H, I, J, N, U) or exclusively to Europe (B2, S, L, Q, V), three
319 *OspC* types (A, E and K) were previously detected on both continents. Further, the
320 sequences of the isolates were identical, suggesting that each group was able to thrive in
321 a new niche consisting of novel vector and host species with little or no genetic change
322 (55). To date, four *ospC* alleles, A, B, I and K, are responsible for systemic LD in
323 humans around the world (55, 65). Additional genotypes C, D, N, F, H, E, G and M have
324 been found in disseminated sites (1, 10, 18, 30). Some of *ospC* alleles that correlate with
325 human invasiveness were recently detected in non-endemic southeastern U.S.A

326 LD is increasing in incidence and is spreading geospatially. Approximately 85,000
327 Lyme disease cases are estimated in Europe every year (37). Nearly 30,000 confirmed
328 cases of LD were reported in 2009 in the U.S.A. in addition to another 8,500 probable
329 cases (http://www.cdc.gov/lyme/stats/chartstables/reportedcases_statelocality.html).
330 Taking into consideration the significant number of underreported cases, the total annual
331 number of LD cases in the world might be as many as 255,000 (15). The spreading or
332 exchange of highly pathogenic spirochete clones between the continents might be
333 supported by trans-oceanic migration of host species, especially birds.

Analysis of 53 *B. burgdorferi* s.s. strains cultivated from tick vectors and rodent hosts from the southeastern U.S.A. revealed that 30% of isolates were *ospC* allele L, a type previously considered to be exclusively European (55). Most of the samples were cultured from *I. affinis,* ticks that usually do not bite humans, thus playing little if any role in direct transmission of *B. burgdorferi* to human, as well as from ear clips or bladders of two major reservoir hosts of *B. burgdorferi* in southeastern U.S.A., *Peromyscus gossypinus* and *Sigmodon hispidus. OspC* allele L shared the frequency of distribution with *ospC* allele B strains (30.2% each) in the southeastern United States. Two other *ospC* alleles detected among the 53 *B. burgdorferi* strains were alleles G and H (28.3% and 11.3%, respectively) (57). It was believed that *ospC* allele L very rarely, if ever, causes human disease (1, 55, 65). Concerning the infectivity to non-human species it was previously found that *B. burgdorferi ospC* allele L strains are not infectious to four principal reservoir host species in the hyperendemic northeastern region, *Peromyscus leucopus* (white-footed mouse), *Tamias striatus* (eastern chipmunk), *Blarina brevicauda* (short-tailed shrew), and *Sciurus carolinensis* (gray squirrels) (11). However, analyzed in this study *B. burgdorferi ospC* allele L strains showed the ability to disseminate in two of the most common natural reservoir hosts in the south, the cotton mouse (*P. gossypinus*) and cotton rat (*Sigmodon hispidus*). It is possible, that the interspecific variation in the vertebrate immune system may provide resistance to infection by certain *ospC* alleles (57). The limited distribution of both primary reservoir hosts of *B. burgdorferi* s.s. *ospC* type L strains, the cotton mouse *P. gossypinus* and the cotton rat *S. hispidus*, and the knowledge that they are parasitized by *I. scapularis, I. affinis, I. minor, Dermacentor variabilis* and *Amblyomma maculatum* (48) suggests that globally rare *ospC* allele L might be limited largely to the southeastern U.S.A. This conclusion is indirectly supported by the previous work of Anderson and

Norris (6). Studying the genetic diversity of *B. burgdorferi* in *P. leucopus* in southern Maryland they found 5 different *ospC* types among Maryland samples: A, B, G, H and K. Southern Maryland represents the boarder between the regions of distribution of *P. leucopus* and *P. gossypinus*. While *ospC* alleles B, G and H are present in the southeastern states of the U.S.A and in southern Maryland, *ospC* allele L is restricted to southeastern part only, most probably confirming the strong host-specificity of this *ospC* allele to local rodent hosts. At the same time, *ospC* alleles A and K are present only in regions of distribution of *P. leucopus* and are absent in the southeast, where *P. gossypinus* substitute *P. leucopus*.

Detection of the invasive *ospC* type B in 30% of samples was unexpected in strains from the southeastern U.S.A. This raises the question whether LD risk to humans in this region has been overlooked, or if the geographic distribution of the LD spirochete has evolved over time. In order for LD to occur, the humans must be exposed to invasive strains via the tick bite. But, the mentioned *ospC* allele B was detected in spirochete strains isolated from either rodent hosts or tick vector that rarely bite human. The previous analysis of non-human biting *I. affinis* from southeastern United States revealed that they are heavily infected with *B. burgdorferi* (33-35%) (27, 40). Most probably, maintenance vectors, such as *I. affinis* could have significant impact in Lyme disease dynamics, helping to maintain high levels of *B. burgdorferi* in reservoir hosts that are later fed upon by bridge vectors often biting human (29, 48).

It will be also interesting to find how much the structure of *B. burgdorferi* s.s. population has changed in Europe. Is it still correct that *B. burgdorferi* s.s. is a strain of minor importance in this part of the world? How much has the pattern of distribution of

this species in Europe changed over the time and, if it has changed, what are the factors contributing to such changes?

Of 4 *ospC* alleles, B, G, H and N that have been detected in LD patients in the northeastern and Midwestern U.S.A., 3 alleles, B, G, and H at a lower rate, are widely distributed in the southeastern part of the country and associated with rodent hosts or non human biting tick. *OspC* allele B, widely distributed in the Northeast and Midwest, is commonly associated with disseminated LD around the world. Together with *ospC* alleles L and G, it became the most frequent *ospC* allele among *B. burgdorferi* s.s. strains from the southeastern U.S.A. *OspC* allele H, commonly detected in LD patients from the northeastern and Midwestern U.S.A., seems to be the fourth most frequently detected *ospC* allele in the Southeast. Isolation of *ospC* allele H strains from the secondary sites of host infection may suggest its potential to develop an invasive disease. This is in agreement with previous results obtained with human and murine isolates (1).

This is the first expanded *ospC* genotyping survey of *B. burgdorferi* s.s. strains from the southeastern U.S.A. Although *B. burgdorferi* s.s. is endemic in many foci over large areas of the southeastern U.S.A., relatively few human cases are reported from this region. The lower prevalence of LD in the southeast of the U.S.A. has been previously attributed to 1) a parallel cycle involving non-human-biting maintenance vectors, "cryptic cycle"; 2) the variations in the vertebrate immune system that provide resistance to infection by certain strains; or 3) different subsets of *B. burgdorferi* s.s. lineages that are present in different regions of the United States (35, 48). This could be determined by differences in the enzootiology of *B. burgdorferi* in the southeastern U.S.A that differs fundamentally from that reported from the northeastern U.S.A and Europe. Lyme disease in the southeastern United States might not be the public health problem, but it

deserves a closer attention as a curious natural event that has all prerequisites, pathogen, competent tick vectors and an array of reservoir hosts, to develop into the medical problem, turning from enzootic to zoonotic system. A detailed preface to an offered discussion about Lyme disease in the southeastern United States (48) is now supported by additional laboratory results. Zoonotic diseases such as LD become of concern when they spill over into the human population. It might be endemic, but not yet recognized unless humans become ill and are accurately diagnosed (48). Taking into consideration the changes that have occurred in nature and in human society, and the substantial amount of new information concerning the global distribution of LD and its growing list of causative agents, it is time to take a fresh look at LD in the southeastern U.S.A.

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Table 1 *Borrelia burgdorferi* sensu stricto reference strains used in this study: *ospC* types, strain name, GenBank accession numbers, geographic origins, sources and authors.

OspC type	Strain name	GenBank acc. #	Location	Source
A	B.b/ strain 132a	DQ437446	NE USA	human
A	B.b/ strain CS1	DQ437464	NE USA	*I. scapularis*
A	B.b/ strain CS2	DQ437465	NE USA	*I. scapularis*
A	B.b/ strain CS3	DQ437466	NE USA	*I. scapularis*
A	B.b/ strain PKa2	EF537420	Europe	human
A	B.b/ strain IP1	EF537422	Europe	human
A	B.b/ strain OC1	AF029860	New York	tick
A	B.b/ strain 132b	DQ437447	NE USA	human
A	B.b/ N/A	EU482041	New York	human
A	B.b/ strain Ip2	L42887	France	human
A	B.b/ isolate 2-1498 CA4	L81131	California	human
A	B.b/ strain HII	U91792	Italy	human
A	B.b/ strain IP3	U91797	France	human
A	B.b/ strain L5	U91798	Austria	human
A	B.b/ strain IP1	U91799	France	human
A	B.b/ strain P1F	U91801	Austria	human
A	B.b/ strain PKa	X69589	Germany	human
A	B.b/ strain TXGW	X84783	Texas	human
A	B.b/ strain B31	U01894	New York	unknown
B(nt59)	B.b/ strain SMT44	FJ932735	California	*I. pacificus*
B1	B.b/ strain MI415	EF537413	Michigan	*P. leucopus*

B2	B.b/ strain ZS7	L42868	Germany	tick
B2	B.b/ strain Lx36	EF537411	Europe	human
B	B.b/ strain OC2	AF029861	New York	tick
B	B.b/ N/A	EU482042	New York	human
B	B.b/ strain 35B808	U91794	Germany	tick
B	B.b/ strain 61BV3	U91795	Germany	human
B	B.b/ strain DK7	X73625	Denmark	human
B	B.b/ strain PBre	X81522	Germany	human
B	B.b/ strain BUR	X84765	New York	human
C	B.b/ strain OC3	AF029862	New York	tick
C	B.b/ strain JD1	DQ437462	NE USA	*I. scapularis*
C	B.b/ N/A	EU482043	New York	human
D	B.b/strain OC4	AF029863	New York	tick
D	B.b/ N/A	EU482044	New York	human
D	B.b/ strain CA-11.2A	L25413	California	unknown
E3	B.b/ strain HRPW89	FJ932732	California	*I. pacificus*
E	B.b/ strain OC5	AF029864	New York	tick
E	B.b/ strain OC7	AF029866	New York	tick
E	B.b/ strain 88a	DQ437459	New York	human
E	B.b/ N/A	EU482045	New York	human
E	B.b/ strain 28691	L42894	Pennsylvania	*I. scapularis*
E	B.b/ strain N40	U04240	Connecticut	rodent
F	B.b/ strain 27579	L42896	Connecticut	*I. scapularis*
F	B.b/ strain *B. pacificus*	X83555	California	*I. pacificus*

F	B.b/ strain OC6	AF029865	New York	tick
F	B.b/ N/A	EU482046	New York	human
F	B.b/ isolate 2-1498 Son 188	L81130	California	human
G	B.b/strain OC8	AF029867	New York	tick
G	B.b/ N/A	EU482047	New York	human
G	B.b/ strain 72a	DQ437456	New York	human
H	B.b/ strain OC9	AF029868	New York	tick
H	B.b/ strain MI411	EF537400	Michigan	*T. striatus*
H	B.b/ N/A	EU482048	New York	human
H3	B.b/ strain MCCP65	FJ932733	California	tick (nymph)
I	B.b/ strain OC10	AF029869	New York	tick
I	B.b/ strain 297	L42893	Connecticut	unknown
I	B.b/ strain HB19	U04281	Connecticut	human
I	B.b/ N/A	EU482049	New York	human
I3	B.b/ strain HPS6	FJ932734	California	tick
J	B.b/ strain OC11	AF029870	New York	tick
J	B.b/ strain 118a	DQ437444	New York	human
J	B.b/ N/A	EU482050	New York	human
J	B.b/ strain MIL	U91802	Slovakia	*I. ricinus*
K	B.b/ strain OC12	AF029871	New York	tick
K	B.b/ strain OC13	AF029872	New York	tick
K	B.b/ strain 28354	L42895	Maryland	*I. scapularis*
K	B.b/ strain 297	U08284	Connecticut	human
K	B.b/ strain MUL	X84779	New York	human

K	B.b/ strain KIPP	X84782	New York	human
K	B.b/ strain 272	X84785	Connecticut	human
K	B.b/ N/A	EU482051	New York	human
L	B.b/ strain Y1	EF537402	Europe	*I. ricinus*
L	B.b/ strain Bol6	EF537406	Europe	*I. ricinus*
L	B.b/ strain 21347	L42899	Wisconsin	*P. leucopus*
L	B.b/ strain T255	X81524	Germany	*I. ricinus*
M	B.b/ N/A	EU482052	New York	human
M	B.b/ strain 2591	U01892	Connecticut	*P. leucopus*
N	B.b/ strain 80a	DQ437457	New York	human
N	B.b/ strain CS8	DQ437470	New York	*I. scapularis*
N	B.b/ strain MI418	EF537430	Michigan	*P. leucopus*
N	B.b/ N/A	EU482053	New York	human
N	B.b/ strain 26815	L42897	Connecticut	chipmunk
O	B.b/ N/A	EU482056	New York	human
O	B.b/ strain DUNKIRK	X84778	New York	human
P	B.b/ strain 20006	U91796	France	*I. ricinus*
Q	B.b/ strain Bol15	EF537398	Europe	*I. ricinus*
Q	B.b/ strain 212	U91790	France	*I. ricinus*
R	B.b/ strain Esp1	U91791	Spain	*I. ricinus*
R	B.b/ strain NE56	U91800	Switzerland	*I. ricinus*
S	B.b/ strain Bol26	EF537417	Europe	*I. ricinus*
S	B.b/ strain Z136	U91793	Germany	*I. ricinus*
T	B.b/ N/A	EU482054	New York	human

U	B.b/ strain 94a	DQ437460	New York	human
U	B.b/ strain CS5	DQ437467	New York	*I. scapularis*
U	B.b/ N/A	EU482055	New York	human
V	B.b/ strain Bol29	EF537407	Europe	human
V	B.b/ strain Bol30	EF537408	Europe	human
W	B.b/ strain Ri5	EF537414	Finland	*I. ricinus*
X	B.b/ strain SV1	EF537427	Finland	*I. ricinus*

Table 2 The experiment group of the 127 *B. burgdorferi* s.s. samples derived from vector ticks, rodent hosts and humans in selected European countries, southeastern U.S.A. and California and analyzed in this study.

OspC type	Isolate name	Accession #	Location/ date of collection	Host/ vector	DNA isolation source
			Europe		
Q	NE5220	JQ253799	Switzerland/ N/A	*I. ricinus*	spirochete culture
L	NE5222	JQ353800	Switzerland/ N/A	*I. ricinus*	spirochete culture
V	NE5248	JQ352801	Switzerland/ N/A	*I. ricinus*	spirochete culture
R	NE5261	JQ352802	Switzerland/ N/A	*I. ricinus*	spirochete culture
B	NE5264	JQ352803	Switzerland/ N/A	*I. ricinus*	spirochete culture
L	NE5266	JQ352804	Switzerland/ N/A	*I. ricinus*	spirochete culture
R	NE5267	JQ352805	Switzerland/ N/A	*I. ricinus*	spirochete culture
L	SKT-2	AY597021	Slovakia/2004	*I. ricinus*	spirochete culture

B	SKT-9	AY597028	Slovakia/2004	*I. ricinus*	spirochete culture
L	SLV 1	JQ236853	Slovenia/2006	human	spirochete culture
B	SLV 2	JQ236854	Slovenia/2006	human	spirochete culture
R*	S277(+2)	JF754968	Czech Rep./ 2010	*I. ricinus*	whole tick
R	B4/N39Aug	JF754971	Germany/2010	*I. ricinus*	whole tick
Q**	S1/11(+1)	JF754969	Czech Rep./2010	*I. ricinus*	whole tick
V	H	JQ219681	Hungary/2000	human	human skin
B***	Brno35(+8)	JQ219682	Czech Rep./ 2009	human	serum/ joint fluid
A	N 12	JQ219683	Czech Rep./2008	human	human serum
B****	N103(+20)	JQ219684	Czech Rep./2010	human	serum/ joint fluid
			USA		
B	BUL1	JF723215	GA/1994	*N. floridana*	spirochete culture
G	BUL3	JF723216	GA/1994	*P. gossypinus*	spirochete culture
B	BUL4	JF723217	GA/1994	*P. gossypinus*	spirochete culture
B	BUL5	JF723218	GA/1994	*P. gossypinus*	spirochete culture

B	BUL6	JF723219	GA/1994	*P. gossypinus*	spirochete culture
G	BUL8	JF723220	GA/1995	*P. gossypinus*	spirochete culture
B	BUL10	JF723221	GA/1997	*P. gossypinus*	spirochete culture
H	MI 1	JF723262	FL/1992	*P. gossypinus*	spirochete culture
G	SCCH 3	JF723222	SC/1995	*I. scapularis*	spirochete culture
B	SCCH 9	JF723223	SC/1995	*P. gossypinus*	spirochete culture
B	SCCH 13	JF723224	SC/1995	*P. gossypinus*	spirochete culture
B	SCCH 19	JF723226	SC/1995	*P. gossypinus*	spirochete culture
L	SCCH 24	JF723228	SC/1995	*S. hispidus*	spirochete culture
G	SCCH 25	JF723229	SC/1995	*S. hispidus*	spirochete culture
G	SCCH 28	JF723231	SC/1995	*S. hispidus*	spirochete culture
L	SCCH 30	JF723232	SC/1995	*P. gossypinus*	spirochete culture

G	SCCH 31	JF723233	SC/1995	*P. gossypinus*	spirochete culture
G	SCGT 4	JF723263	SC/1995	*P. gossypinus*	spirochete culture
G	SCGT 7	JF723264	SC/1995	*I. affinis*	spirochete culture
L	SCGT 16	JF723265	SC/1995	*P. gossypinus*	spirochete culture
H	SCGT 17	JF723266	SC/1995	*P. gossypinus*	spirochete culture
L	SCI 1	JF723234	GA/1993	*P. gossypinus*	spirochete culture
B	SCI 3	JF723235	GA/1993	*P. gossypinus*	spirochete culture
G	SCSC 2	JF723267	SC/1995	*P. gossypinus*	spirochete culture
G	SCSC 3	JF723268	SC/1995	*I. affinis*	spirochete culture
G	SCSC 5	JF723269	SC/1995	*I. affinis*	spirochete culture
G	SCSC 6	JF723270	SC/1995	*I. affinis*	spirochete culture
B	SCW 1	JF723242	SC/1994	*P. gossypinus*	spirochete culture

H	SCW 2	JF723243	SC/1994	*P. gossypinus*	spirochete culture
B	SCW 3	JF723244	SC/1994	*P. gossypinus*	spirochete culture
H	SCW 4	JF723245	SC/1994	*P. gossypinus*	spirochete culture
H	SCW 6	JF723246	SC/1994	*P. gossypinus*	spirochete culture
L	SCW 9	JF723247	SC/1994	*S. hispidus*	spirochete culture
L	SCW 12	JF723248	SC/1994	*P. gossypinus*	spirochete culture
B	SCW 25	JF723249	SC/1994	*P. gossypinus*	spirochete culture
L	SCW 43	JF723250	SC/1995	*I. affinis*	spirochete culture
L	SCW 44	JF723251	SC/1995	*I. affinis*	spirochete culture
H	SCW 47	JF723252	SC/1995	*I. affinis*	spirochete culture
L	SCW 48	JF723253	SC/1995	*I. affinis*	spirochete culture
B	SCW 53	JF723254	SC/1995	*I. affinis*	spirochete culture

L	SCW 54	JF723255	SC/1995	*I. affinis*	spirochete culture
G	SCW 57	JF723256	SC/1995	*I. affinis*	spirochete culture
B	SCW 58	JF723257	SC/1995	*I. affinis*	spirochete culture
G	SCW 59	JF723258	SC/1995	*I. affinis*	spirochete culture
L	SCW 60	JF723259	SC/1995	*I. affinis*	spirochete culture
L	SCW 61	JF723260	SC/1995	*I. affinis*	spirochete culture
B	SCW 62	JF723261	SC/1995	*I. affinis*	spirochete culture
L	SI 14	JF723236	GA/1995	*I. affinis*	spirochete culture
B	SI 15	JF723237	GA/1995	*I. affinis*	spirochete culture
L	SI 16	JF723238	GA/1995	*I. affinis*	spirochete culture
L	SI 17	JF723239	GA/1995	*I. affinis*	spirochete culture
G	SI 18	JF723240	GA/1995	*I. affinis*	spirochete culture

L	SI 19	JF723241	GA/1995	*I. affinis*	spirochete culture
D	BOR4	JQ308215	CA/2004	*I. pacificus*	spirochete culture
H	BOR53	JQ308216	CA/2004	*I. pacificus*	spirochete culture
E3	BTE68	JQ308217	CA/2004	*I. pacificus*	spirochete culture
A	BTW11	JQ308218	CA/2004	*I. pacificus*	spirochete culture
E3	BTW16	JQ308219	CA/2004	*I. pacificus*	spirochete culture
H3	BTW37	JQ308220	CA/2004	*I. pacificus*	spirochete culture
G	BTW52	JQ308221	CA/2004	*I. pacificus*	spirochete culture
M	BTW62	FJ932736	CA/2004	*I. pacificus*	spirochete culture
H3	BTW67	JQ308222	CA/2004	*I. pacificus*	spirochete culture
D	CHRW46	JQ308223	CA/2004	*I. pacificus*	spirochete culture
A	CHRW57	JQ308224	CA/2004	*I. pacificus*	spirochete culture

A	FCR13	JQ308225	CA/2004	*I. pacificus*	spirochete culture
F	HOPK32	JQ308226	CA/2004	*I. pacificus*	spirochete culture
G	HOPN45	JQ308227	CA/2004	*I. pacificus*	spirochete culture
I3	HPS6	FJ932734	CA/2004	*I.pacificus*	spirochete culture
I3	HPS61	JQ308235	CA/2004	*I. pacificus*	spirochete culture
E3	HRPW89	FJ932732	CA/2004	*I. pacificus*	spirochete culture
B	HUMB27	JQ308233	CA/2004	*T. senex*	spirochete culture
E3	HUMB150	JQ308234	CA/2004	*N. fuscipes*	spirochete culture
E3	LAG24	JQ308228	CA/2004	*I. pacificus*	spirochete culture
H3	LMSW22	JQ308229	CA/2004	*I. pacificus*	spirochete culture
H3	MCCP65	FJ932733	CA/2004	*I. pacificus*	spirochete culture
H3	SGE03-1	JQ308230	CA/2003	*S. griseus*	spirochete culture

H	SGE03-4	JQ308231	CA/2003	*S. griseus*	spirochete culture
A	SGE03-7	JQ308232	CA/2003	*S. griseus*	spirochete culture

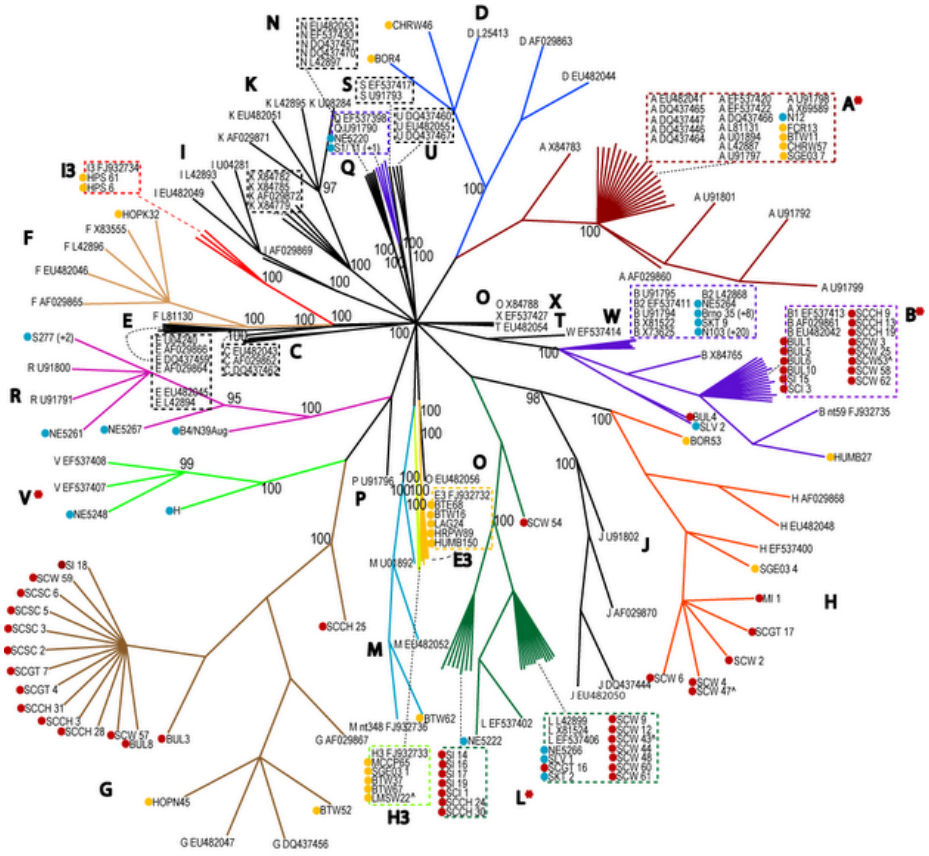

Figure legends

Figure 1. An unrooted neighbor-joining distance tree generated in BioNJ and based on nucleotide sequence alignment of 498 bp fragments of *ospC* genes. Nodes are labeled with the percentage bootstrap support when the value is 90% or higher. All clades are marked by capital letters that show the *B. burgdorferi* s.s. *ospC* type. All experimental samples in each clade are labeled to indicate the sample origin, as follows: blue dot in front of a sample name indicates European; golden dot-Californian; red dot-southeastern U.S.A. All unmarked sample names are control samples, previously identified as members of an *ospC* type and downloaded from GenBank. Samples with the ^ symbol after their name are those

that were not included in the analysis, but placed in clusters with samples whose sequences were identical over the length of the sequence fragment we were able to obtain for them (see text). Clades that include experimental *B. burgdorferi* s.s. strains isolated from European LD patients are marked with red asterisk (*).