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Spatial spread and demographic expansion of Lyme borreliosis spirochaetes in Eurasia

Stephanie A. Vollmer [a,*], Edward J. Feil [a], Chen-Yi Chu [b,c], Stephen L. Raper [a], Wu-Chun Cao [b], Klaus Kurtenbach [a,1], Gabriele Margos [d]

[a] Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom
[b] Beijing Institute of Microbiology and Epidemiology, State Key Laboratory of Pathogen and Biosecurity, Beijing 100071, China
[c] PLA Institute of Disease Control and Prevention, Beijing 100071, China
[d] Department of Infectious Diseases and Zoonosed, Ludwig-Maximilians-University of Munich and German National Reference Centre for Borrelia at the Bavarian Health and Food Safety Authority, Veterinärstrasse 2, 85764 Oberschleißheim, Germany

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ABSTRACT

The Lyme borreliosis (LB) group of spirochaetes currently comprises 18 named species that vary in their geographic distribution, host specificity and ability to cause disease in humans. In Europe three species are most abundant, *Borrelia afzelii*, *Borrelia garinii* and *Borrelia valaisiana* but only two of these (*B. garinii* and *B. afzelii*) are regularly found in Asia as well. A recently published study has shown that *Borrelia* species associated with birds, such as *B. garinii*, showed limited geographic structuring between European countries while, the rodent associated species, *B. afzelii*, showed extensive spatial structuring in Europe. Here, we use multilocus sequence analysis to show that when the wider, inter-continental, distribution is considered, there is evidence of spatial structuring even in the bird-associated species *B. garinii*. Furthermore, our investigations into historical LB populations provided evidence for range expansions of *B. garinii* and *B. afzelii* populations in Europe in the distant past. We propose that the expansion of *B. afzelii* in Europe may be linked to rodent population expansions after the last glacial maximum.

1. Introduction

Lyme borreliosis (LB) is the most prevalent vector-borne disease in the temperate zones of the Northern Hemisphere and is caused by spirochaetal bacteria. The bacteria form a species complex, termed *Borrelia burgdorferi* sensu lato or the Lyme borreliosis group of spirochetes, currently comprising 18 named species that vary in their geographic distribution, host specificity and ability to cause disease in humans (Margos et al., 2010; Margos et al., 2009; Rudenko et al., 2009a; Rudenko et al., 2009b). In China the primary vector species of *Borrelia garinii* and *Borrelia afzelii* is *Ixodes persulcatus* while, in Europe it is *Ixodes ricinus* (Masuzawa, 2004; Piesman and Gern, 2004).

Transmission of spirochaetes between ticks and hosts is essential for the survival of the bacteria because transovarial transmission of LB spirocheates to questing larvae is very rare. Furthermore, direct transmission among hosts or among vectors does not occur (Kurtenbach et al., 2006). As ticks do not move actively over long distances (Falco and Fish, 1991) nor are they dispersed by the wind, as many insect vectors are (Purse et al., 2005), ticks of the genus *Ixodes* migrate passively when attached to hosts which may occur on a number of spatial scales.

In Europe three *Borrelia* species are most abundant, *B. afzelii*, *B. garinii* and *B. valaisiana* but only two of these (*B. garinii* and *B. afzelii*) are regularly found in Asia as well (Etti et al., 2003; Kurtenbach et al., 2001; Masuzawa, 2004). Although *B. valaisiana*-like isolates have been recorded in China, these comprise a rodent-associated ecotype that differ genetically from bird-associated *B. valaisiana* in Europe and a new species, *B. yangtze*, has been defined (Chu et al., 2008). A fourth species, *B. bavariensis* (previously known as *B. garinii* OspA serotype 4) (Margos et al., 2009), has a patchy distribution in Europe, is transmitted by rodents, and genetically closely related strains are also present in Asia (NT29 strains) (Takano et al., 2011). There are many species unique to specific areas of Asia and these have been reviewed in more detail by Masuzawa (Masuzawa, 2004).

LB species differ clinically, having been associated with different disease symptoms. *B. afzelii* is most frequently linked with skin manifestations (Canica et al., 1993) while, *B. garinii* and *B. bavariensis* are most often associated with neuroborreliosis (Ornstein et al., 2001; Rijpkema et al., 1997; Ruzic-Sabljic et al., 2001). *B. valaisiana*, are very rarely associated with human disease (Wang et al., 1999). It is, therefore, of epidemiological relevance to improve the understanding of the geographic distribution range of the different LB species.

* Corresponding author. Tel.: +44 1225 385116.
E-mail address: Stephanie_vollmer@hotmail.com (S.A. Vollmer).
[1] Deceased March 2009 – this paper is dedicated to his memory.

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The LB spirochaetal species differ in patterns and levels of vertebrate host specialisation and it had been suggested that this has important implications for dispersal and spread of these species. We have recently shown that the migration of the LB species is commensurate with that of their respective hosts (Vollmer et al., 2011). The bird associated species, *B. garinii* and *B. valaisiana*, showed limited geographic structuring between European countries including England, France and Latvia compared with the rodent associated species, *B. afzelii*. However, the study does not shed light on geographical structuring over a larger scale, and no studies to date have compared *B. garinii* or *B. afzelii* populations in Europe and Asia. The ecology of the hosts would predict far greater geographical heterogeneity within *B. afzelii* than within the bird associated species, as no putative host species of *B. afzelii* covers the entire *B. afzelii* distribution range which in turn may lead to a higher level of fragmentation of *B. afzelii* in China but it is difficult to predict without a better understanding of the exact host range of *B. afzelii* in Asia. In particular Chinese mouse (*Apodemus*) and vole (*Microtus* and *Myodes*) species are highly fragmented (IUCN, 2010). However, known hosts of *B. garinii*, such as *Turdus merula* (common blackbird) are distributed throughout Eurasia. Here, we investigated for the first time population structuring of two species of LB spirochetes that are prevalent across a continent (*B. afzelii* and *B. garinii*) and that are specialised to hosts that differ fundamentally in their dispersal pattern, i.e. rodent or avian hosts, respectively. We combine our previous European data set with novel data from China and through this combined dataset we can also consider the deeper evolutionary histories of the LB species within the context of these host-pathogen relationships.

2. Experimental procedures

2.1. Samples

Here, we use a multi-locus sequence typing scheme (MLST) based on eight chromosomal housekeeping genes (clpA, clpX, nifS, pepX, pyrG, recG, rplB, and uvrA) which are subject to purifying selection and slow evolution (Margos et al., 2008). All eight loci have been found to be under purifying selection (Margos et al., 2008; Vollmer et al., 2011) but evolve differently from other commonly used genetic markers such as non coding regions and outer surface proteins (Margos et al., 2008).

Sequences obtained for this MLST scheme are submitted to, and maintained at the *Borrelia* MLST database (http:// borrelia.mlst.net). This database was searched for *B. afzelii* and *B. garinii* strains from Europe. 41 strains obtained from *I. persulcatus* ticks from Northeastern China were sequenced and analysed for this paper using methods described previously. Briefly, genomic DNA was extracted and purified from tick material (*I. persulcatus*) as described earlier (Margos et al., 2008). Primers and PCR conditions have been described in detail previously and can be found on the MLST website (Margos et al., 2008; Margos et al., 2009). A map of all collection sites is available as an online figure. A list of Chinese strains can be found in the (Appendix A (Table A.1). A proportion of strains ($n = 18$) that were found to be *B. bavariensis* (formerly *B. garinii* serotype 4) were excluded from the analysis because they differ ecologically from *B. garinii*.

2.2. Pairwise mismatch distribution and F_{ST} values

The frequency distributions of pairwise sequence mismatches were calculated in order to investigate the past population changes. These were calculated using ARLEQUIN 3.0 (Excoffier et al., 2005) for demographic and spatial population expansion models. Model fit was calculated by minimising the sum of squared deviations. The constant population expansion model was calculated using DnaSP (Librado and Rozas, 2009).

Pairwise F_{ST} values were calculated to investigate spatial structuring of LB populations. The putative populations were defined by country of origin as it has been observed previously that there is significant differentiation between populations defined in this way (Vollmer et al., 2011). Using ARLEQUIN 3.1 and 100 permutations were run to assess the significance of the F_{ST} value. AMOVA (Analysis of Molecular Variance) (Cockerham and Weir, 1984; Excoffier et al., 1992) was performed to address variation at the different levels. In this approach individuals were combined into two groups; a group of populations from European countries (English, French, German and Latvian populations) and China. A neighbour-joining tree was then created using a distance matrix of the F_{ST} values using Mega 4.0 to visualise the relationship between populations based on the F_{ST} values.

2.3. Geographic and genetic distance correlations

A Spearman's rank correlation was carried out to determine if there was a linear correlation between pairwise genetic differences, calculated using Mega 4.0, and geographic distance between collection sites. The analysis was carried out for *B. afzelii* and *B. garinii* populations in Europe and China using Minitab 16 statistical package.

2.4. Alignments and constructing phylogenies

Sequence alignments were generated using MUSCLE Multiple Sequence Alignment Software (Edgar, 2004). Mega 4.0 (Kumar et al., 2004) was used to visualise the alignments and ensure the alignment remained in frame. Mean p-distance (π) and non-synonymous to synonymous substitutions (dN/dS) of genes were also determined using MEGA 4.0 for each species. dN/dS ratio was determined using the modified Nei–Gojobori method and Jukes–Cantor model.

Phylogenetic trees were constructed for the concatenated housekeeping genes using PhyML 3.0 via the ATGC Montpellier bioinformatics platform (Guindon and Gascuel, 2003). The evolutionary model used in the phylogenetic analysis was determined using FindModel (Tao et al., 2009). For all phylogenies constructed, the general time reversible (GTR) model with gamma-distributed rate variation across sites was selected. The starting BIONJ tree was improved using the SPR (subtree pruning and regrafting), and NNI (nearest neighbour interchange) methods. The branch support values were estimated using approximate likelihood ratios (aLRT) and Shimodaira–Hasegawa-like (SH-like) method. All other settings were default.

3. Results

3.1. Comparison of European and Chinese populations

All available MLSA data for European and Asian *B. afzelii* and *B. garinii* strains were obtained from the public MLST database, http://borrelia.mlst.net. After excluding Chinese isolates classified as *B. garinii*, which were found to be closely related to *B. bavariensis* (see below), 89 *B. afzelii* and 121 *B. garinii* strains were included. In total, 210 *Borrelia* isolates from England ($n = 78$), Scotland ($n = 3$), Latvia ($n = 51$), Germany ($n = 23$), Switzerland ($n = 1$), France ($n = 31$) and China ($n = 23$) were analysed. A full list of strains investigated, their species assignment and their geographic origin can be found in Appendix A (Table A.2)). The vast majority of European strains were from the study by Vollmer and colleagues (Vollmer et al., 2011) while the Chinese strains were sequenced