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Host migration impacts on the phylogeography of Lyme Borreliosis spirochaete species in Europe

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Summary

The geographic patterns of transmission opportunities of vector-borne zoonoses are determined by a complex interplay between the migration patterns of the host and the vector. Here we examine the impact of host migration on the spread of a tick-borne zoonotic disease, using Lyme Borreliosis (LB) spirochaetal species in Europe. We demonstrate that the migration of the LB species is dependent on and limited by the migration of their respective hosts. We note that populations of *Borrelia* spp. associated with birds (*Borrelia garinii* and *B. valaisiana*) show limited geographic structuring between countries compared with those associated with small mammals (*Borrelia afzelii*), and we argue that this can be explained by higher rates of migration in avian hosts. We also show the presence of *B. afzelii* strains in England and, through the use of the multi-locus sequence analysis scheme, reveal that the strains are highly structured. This pattern in English sites is very different from that observed at the continental sites, and we propose that these may be recent introductions.

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Introduction

Lyme Borreliosis (LB) is the most prevalent vector-borne disease in the Northern Hemisphere, and the number of LB cases diagnosed each year in the UK is increasing (HPA, 2010). LB is a tick-borne zoonosis caused by the LB group of spirochaetes, which comprises 17 named species (Margos *et al*., 2009; Rudenko *et al*., 2009a,b). The three most abundant species in Europe are *Borrelia afzelii*, *B. garinii* and *B. valaisiana*, although the relative dominance of these species varies strikingly between sample sites (Kurtenbach *et al*., 2001). *Borrelia burgdorferi sensu stricto* (s.s.) is more commonly encountered in North America than in Europe (Piesman and Gern, 2004).

The principal LB vector in Europe is the sheep tick, *Ixodes ricinus* (Burgdorfer, 1984), which transmits the spirochaetes between vertebrate hosts, including both avian and mammalian species (Kurtenbach *et al*., 2002a). LB species tend to vary in terms of host specificity, and many are associated with specific disease symptoms. For example, *B. afzelii* is most frequently linked with skin manifestations (Canica *et al*., 1993), *B. garinii* with neuroborreliosis (Rijpkema *et al*., 1997; Ornstein *et al*., 2001; Ruzic-Sabljic *et al*., 2001) and *B. burgdorferi* s.s. with arthritic symptoms (van Dam *et al*., 1993; Ornstein *et al*., 2001; van Dam, 2002). Other species, such as *B. valaisiana*, are very rarely associated with human disease (Wang *et al*., 1999).

Host specialization is an important factor in vector-borne disease, and different pathogens show varying levels and patterns of host specialization which may impact the spread of pathogens. For example, western tick-borne encephalitis virus is only transmissible via rodent hosts (Randolph *et al*., 1999), while West Nile virus transmission depends on birds (Granwehr *et al*., 2004). In the LB group of spirochaetes, *B. garinii* and *B. valaisiana* are transmitted by avian species while *B. afzelii* is associated with rodents and certain insectivore species (Kurtenbach *et al*., 2002b; Hanincova *et al*., 2003a,b). *Borrelia burgdorferi* s.s. is a generalist species, known to infect both rodent and avian species, as well as other hosts (Ginsberg *et al*., 2005; Hanincova *et al*., 2006). The diversity in host specialisms in the LB group of

spirochaetes makes this an ideal system to examine the interplay between the ecology of the host and the epidemiology of the bacteria. As ticks do not move over large distances independently (Falco and Fish, 1991), the spread of LB spirochaetes is likely to be linked to the migration of their hosts (Kurtenbach *et al.*, 2002b). Species that are maintained by rodents are therefore predicted to show more limited migration than those associated with birds. In addition to being of public health importance, the delineation and monitoring of the geographic ranges of the different LB species also provides an opportunity to examine in more general terms the role of host ecology in the epidemiology of vectored zoonoses. Here we test the prediction that host migration determines spirochaete biogeography by characterizing different LB species from sites in Great Britain, Latvia, Germany and France using multi-locus sequence analysis (MLSA).

Results

Prevalence of Borrelia *infections in field collected* I. ricinus *nymphs*

In total, 1910 nymphs (the intermediate immature tick stage) were collected by blanket dragging at sites in Great Britain in 2006 and 2007, and 5% ($n = 96$) of these were positive for *Borrelia* infection. In 2008 and 2009 an additional 827 nymphs were collected from a broader range of sites in Great Britain, and 7.7% ($n = 64$) nymphs were positive. In Latvia, a total of 951 nymphs were collected in 2006 and 2007, and 10.6% ($n = 101$) were positive. The infection prevalence of the nymphs at individual sites in Britain ranged from 0% at Rhossili Down, South Wales, to 12.4% at Bathampton Woods, Bath (in south-western England). The infection prevalence at Latvian sites ranged from 4% in Kemeri to 16.4% in Babite (Fig. 1). A full list of the infection prevalence found at individual sites is given in the supplementary information (Table S1). Adult ticks from Britain were also screened and included in the MLSA analyses, but they were not included in the prevalence studies as there were too few to draw meaningful conclusions.

Multi-locus sequence analysis (MLSA)

The sequences from the eight MLSA housekeeping genes were analysed from 212 *Borrelia*-positive ticks from Britain ($n = 118$), Latvia ($n = 73$) and Germany ($n = 21$) (Tables S2 and S3). These data were supplemented with 46 French strains sequenced previously

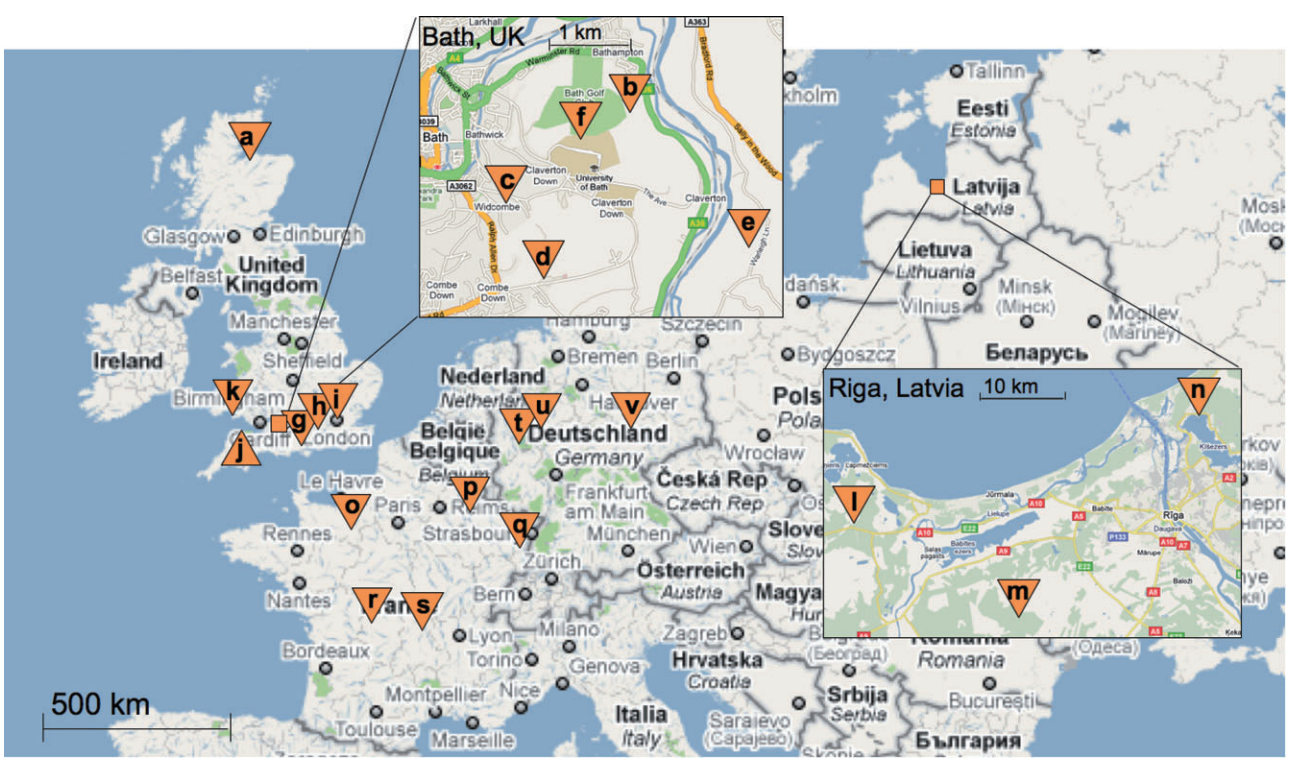

Fig. 1. Google map of Europe showing tick collection sites. Labels a to v represent the following collection sites: (a) Inverness, (b) Bathampton, (c) Widcombe Hill, (d) Rainbow Wood, (e) Warleigh Wood, (f) Campus, (g) New Forest, (h) Hazeley Heath, (i) Richmond Park, (j) Exmoor, (k) Rhossili Downs, (l) Kemeri, (m) Babite, (n) Jaunciems, (o) Normandie, (p) Meuse, (q) Alsace, (r) Limousin, (s) Auvergne, (t) Kottenforst and Siebengebirge, (u) Lennestadt-Meggen, (v) Wichmar.

(Margos *et al*., 2009). Although *B. afzelii* has not previously been observed in questing ticks in England, this species was found in five English sites (Table S1). The 72 *B. afzelii* strains were resolved by MLSA into 40 sequence types (STs) (0.56 STs per isolate), the 112 *B. garinii* into 46 STs (0.41 STs per isolate) and the 75 *B. valaisiana* isolates into 26 STs (0.34 STs per isolate). No alleles were found in more than one species and the mean pairwise distances (p-distance) also differed between the species (Table S4). *Borrelia garinii* had the highest p-distance of the three species, while *B. afzelii* and *B. valaisiana* showed a similar level of diversity (Table S4). All three species had similarly low dN/dS ratios for the concatenated housekeeping genes (Table S4) and the amino acid conversion sequences can be found in Table S5.

Distribution of STs between countries

The majority of STs in *B. garinii* (26/46; 57%) were only found once, but of those STs found at least twice, 13/20 (65%) were noted in more than one country (Fig. 2A, Table S6).

In *B. valaisiana* a similar pattern was observed; 13/26 (50%) of the STs were found only once, but of those found at least twice, 9/13 (69%) were recorded in at least two countries (Fig. 2B, Table S6). For neither *B. garinii* nor *B. valaisiana* was there any suggestion from the data that STs are more likely to be shared between countries in close proximity. For *B. afzelii*, 30/40 (75%) of the STs were found only once, but of those found more than once, 2/10 (20%) were recorded in at least two countries (Fig. 2C, Table S6).

The two *B. afzelii* STs found in more than one country were ST80 (found in Alsace, France and Siebengebirge, Germany) and ST204 (found in Sauerland, Germany and Kemeri, Latvia). The first two sites are in close geographical proximity (Fig. 1). The five *B. afzelii* STs in England show a particularly high level of localized clustering. ST164 corresponded to 13 infected ticks, all of which were sampled at Widcombe Hill, Bath. STs 240 and 265 were unique to one and three infected ticks, respectively, from the Exmoor site, and the only example of ST241 was encountered at the New Forest site, Hampshire. Only one *B. afzelii* ST was found at more than one site in England, and the two sites were very close; ST250 was noted in four ticks at Rainbow wood, Bath and in one tick at Warleigh forest, near Bath (Fig. 1). A higher degree of *B. afzelii* diversity was noted within individual sites in continental Europe than in each

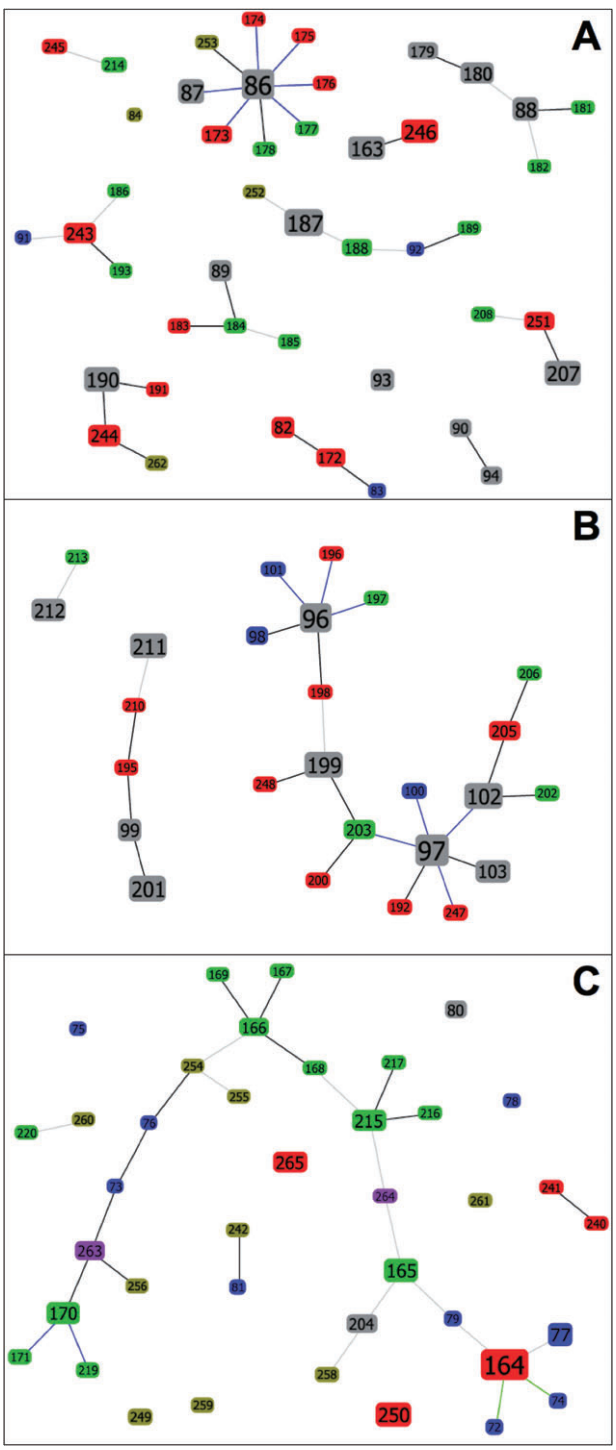

Fig. 2. goeBURST diagrams based on the multi-locus allelic profiles for *B. afzelii*, *B. garinii* and *B. valaisiana*. Each coloured box represents an ST. The colour and size of the boxes corresponds to country and the number of that ST found. STs unique to a particular country were coloured as follows: England, France, Germany, Latvia, Scotland. Those STs that were found in more than one country are . STs connected by black or blue lines are single-locus variants (SLVs) and STs connected by grey or green lines are double-locus variants (DLVs). (A) *Borrelia garinii* goeBURST, (B) *B. valaisiana* goeBURST and (C) *B. afzelii* goeBURST.

Table 1. Pairwise F_{ST} values of the concatenated housekeeping genes for pairs of populations with values where *P* is less than 0.05 shown in bold.

		England		France		Latvia	
		F_{ST}	*P*-value	F_{ST}	*P*-value	F_{ST}	*P*-value
England	*B. afzelii*						
	B. garinii						
	B. valaisiana						
France	*B. afzelii*	**0.109**	**0.015**				
	B. garinii	0.035	0.058				
	B. valaisiana	**0.065**	**0.027**				
Latvia	*B. afzelii*	**0.364**	**0.000**	**0.22**	**0.000**		
	B. garinii	0.021	0.091	−0.007	0.546		
	B. valaisiana	0.012	0.216	**0.0895**	**0.009**		
Germany	*B. afzelii*	**0.256**	**0.000**	**0.118**	**0.001**	**0.08**	**0.017**
	B. garinii	N/A	N/A	N/A	N/A	N/A	N/A
	B. valaisiana	N/A	N/A	N/A	N/A	N/A	N/A

Analyses that were not completed due to too few strains are indicated N/A.

English site. For example, all 12 German *B. afzelii* strains investigated were different STs and came from only three different sites. Furthermore, continental *B. afzelii* STs were not always unique to particular sites, as was most often the case in England. For example, the same *B. afzelii* STs were found at different sites within Latvia (albeit mostly exclusively in that country) and a similar pattern was observed with *B. afzelii* STs exclusive to France.

Population differentiation

We computed pairwise F_{ST} values for the three species, comparing populations from England, Germany, France and Latvia using the concatenated housekeeping gene sequences in ARLEQUIN 3.1. F_{ST} scores were defined as being 'low' when between 0.00 and 0.05, 'medium' between 0.05 and 0.25 and 'high' above 0.25 (Freeland, 2005). German samples were not included in the analyses of *B. valaisiana* and *B. garinii*, since no *B. valaisiana* and only nine *B. garinii* strains were found in Germany. Overall, we found low to moderate differentiation in *B. valaisiana* (F_{ST} 0.0477, $P = 0.0323$) but no statistically significant differentiation in *B. garinii* populations. For a more detailed picture, we then computed pairwise F_{ST} scores for pairs of populations as indicated in Table 1. Again, for *B. garinii* no significant differentiation was found. In *B. valaisiana* two of the three pairs of populations showed statistically significant moderate differentiation; these were France/Latvia (F_{ST} 0.0895, $P = 0.009$) and France/England (F_{ST} 0.0653, $P = 0.027$).

Borrelia afzelii was found to have the highest overall F_{ST} value of the three species of 0.222 ($P = 0.000$). All comparisons of pairs of populations were found to be statistically significant but these F_{ST} values showed no significant trend to increase with the geographical distance between the countries (Table 1). All the continental European populations, when compared pairwise, showed moderate levels of differentiation ranging from 0.08 (Germany/Latvia) to 0.22 (France/Latvia). Only population pair comparisons that included England fell into the high differentiation category; these were England/Germany (F_{ST} 0.256) and England/Latvia (F_{ST} 0.364) (Table 1).

Phylogenetic relationship of B. afzelii *populations*

The PhyML tree in Fig. 3 was created using the concatenated housekeeping genes for all *B. afzelii* STs. This tree suggests that there is no single common ancestor for all *B. afzelii* strains found in England; instead these STs are scattered throughout the tree. The goeBURST image in Fig. 2C shows that the majority of *B. afzelii* STs collected in England are not associated with the main clonal complex of *B. afzelii*, appearing instead as lone STs, meaning that they differ by more than two loci from all Latvian, French, Scottish and German strains included in this study. The PhyML tree suggests that their closest relatives in this data set are always strains that originated in France or Germany but never in Latvia. This is in contrast to the two STs identified from Scotland, which appear more closely associated with Latvian strains. The only ST from England associated with the large *B. afzelii* clonal complex is ST164 and appears as a founder to the French STs 72, 74, 77.

The PhyML trees for *B. garinii* and *B. valaisiana* can be found in *Supporting information* (Figs S1 and S2). Strains obtained from the different countries do not show clustering in accordance with geographic location, but strains from different countries appear in the same cluster/terminal nodes. The mixing of populations apparent from these phylogenetic relationships would be expected to have arisen from a high level of migration.

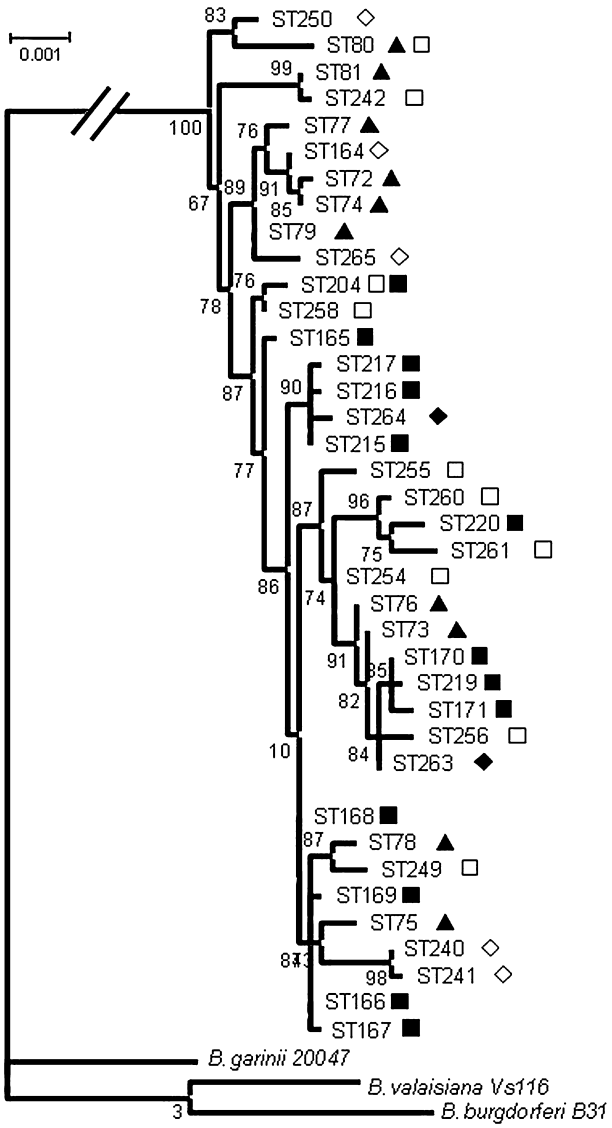

Fig. 3. PhyML phylogenetic inference of the concatenated housekeeping genes of *B. afzelii* STs. The symbols indicate the country the ST was found in: ▲ France, ◇ England, ◆ Scotland, □ Germany and ■ Latvia. The tree is rooted with three other LB species: *B. garinii*, *B. valaisiana*, *B. burgdorferi*, but the branch lengths of the outgroups are not to scale as indicated by the slashes. (Scale bar: 0.1% divergence.)

Discussion

The discovery of B. afzelii *in England, its distribution and its origin*

To date there have been a limited number of published studies concerning the infections found within tick populations in England and Wales. To the best of our knowledge the last conclusive study investigating LB infections in English questing ticks focused solely on a woodland in Southern England and found only *B. garinii* and *B. valaisiana* in questing ticks and in reservoir hosts (Kurtenbach *et al*., 1998). In Continental Europe field studies have indicated that *B. afzelii* is common and, in many cases, the dominant species (Etti *et al*., 2003; Rauter and Hartung, 2005; van Overbeek *et al*., 2008). In the Scottish highlands *B. afzelii* also appears to be the predominant species (Ling *et al*., 2000). It has been suggested previously that tick questing behaviour resulting in a lack of nymphal infestation of mice may prevent the existence of *B. afzelii* in England and Ireland (Gray *et al*., 1999; Randolph and Storey, 1999; Randolph *et al*., 1999), but it has also been a consideration that the lack of sites investigated in England and Wales make it difficult to speculate on.

Here we report *B. afzelii* in questing ticks from England. Just over half the sites were found to be supporting *B. afzelii* strains, and the MLSA scheme revealed that the infections were highly focal, with four of the five STs from England each found exclusively and often repeatedly at a single site. Although it is possible that isolated *B. afzelii* populations have been maintained in England for long periods of time, and may have diverged from each other by genetic drift, we consider it more likely that the genetically distinct localized populations represent independent and recent introductions from outside the UK. This view is supported by the PhyML tree, which reveals that the English STs are polyphyletic, hence there is no single common ancestor unique to English STs (Fig. 3). However, at present it is only possible to speculate on these strains' geographical origins. The goeBURST reveals that most English STs are not linked to the major clonal complex of the species (Fig. 2C), suggesting they have not recently spread from France, Germany or Latvia. The exception to this is ST164, which was observed at the Widcombe Hill site in England and is assigned as founder to three French STs in Fig. 2C. This infers a French origin of this strain, although the exact ST was not present in our sample from France. In general, the PhyML tree in Fig. 3 suggests that the English STs are more closely related to French and German STs than to the Latvian and Scottish STs.

Genomic regions with more genetic variation are required, such as whole genome intergenic SNP analysis, to allow for an estimate on the period since *B. afzelii* introduction to the English sites. Furthermore, a more extensive survey of European sites may reveal the origins of the English *B. afzelii* strains and also shed light on potential methods of entry for these rodent-associated strains.

Phylogeographic structuring of LB species

Host specialization is a key process in the ecology and evolution of tick-borne zoonotic diseases. In this study we aimed to further our understanding of the impact of host association on the spread of zoonotic vector-borne patho-

gens by analysing three species of the LB group of spirochetes that are specialized to either avian or rodent hosts. MLSA on housekeeping genes has revealed differences in the level of geographic structuring of populations of LB species that are consistent with patterns of migration of their different vertebrate hosts. Both bird-related species investigated, *B. valaisiana* and *B. garinii*, showed evidence of spatial mixing of STs between countries, while the rodent-related *B. afzelii* showed evidence of differentiation of populations from each of the four countries. This differentiation was pronounced to the extent that only two *B. afzelii* STs were found in more than one country.

This finding was statistically supported using an F_{ST} test for pairwise differentiation between populations. Interestingly, while in *B. garinii* we found no significant differentiation of populations in different countries, suggesting entirely free movement of strains, *B. valaisiana* showed low to moderate differentiation, suggesting there is not complete homogenization of *B. valaisiana* strains within Europe. This was surprising because both species appear to be transmitted by similar species of avian hosts (Taragel'ova *et al*., 2008; Dubska *et al*., 2009). However, our results suggest that subtle ecological differences may exist between these species and it has been known for a long time that, apart from transmission cycles in terrestrial birds, *B. garinii* populations are also maintained by seabirds and their associated tick, *Ixodes uriae* (Olsen *et al*., 1995; Bunikis *et al*., 1996; Larsson *et al*., 2007). It is interesting to note that Comstedt and colleagues (2009) reported an overlap of marine and terrestrial *B. garinii* populations, although whether or not this may play a role in the population structure observed in the present study remains speculative, since no *B. garinii* from marine transmission cycles have been analysed by MLSA to date.

Of the three species analysed in the present study, *B. garinii* was found to be the most diverse. The finding that *B. garinii* showed a higher genetic diversity than *B. valaisiana* has been reported previously and it has been proposed that this might be due to a more recent adaptation of *B. garinii* to avian hosts (Margos *et al*., 2009). This may also contribute to subtle ecological differences in host associations, and therefore to the differences in population patterns observed here. In the pairwise F_{ST} comparisons, the only population pair showing no significant differentiation in *B. valaisiana* was England/Latvia, which is the most geographically distant pair. Currently, it is not known whether the differentiation observed between the LB strains from France and England is due to bias created by the culturing of the LB strains from France. Further sampling of environmental LB strains from France would be required to confirm these findings.

The *B. afzelii* F_{ST} score was markedly higher than *B. garinii* or *B. valaisiana*, suggesting that movement of strains between countries is more restricted. In *B. afzelii* there is no significant trend to suggest that F_{ST} increases with geographical distance, which may implicate a role of geographical barriers in determining population structure. For example, it is likely that the English Channel acts as a barrier to the movement of *B. afzelii* strains between Great Britain and continental Europe.

The *B. afzelii* population in Latvia, where it is more common for the same ST to be found in different sites, is less clearly differentiated than in England, where different sites tend to be associated with specific genotypes. The three Scottish strains included in this study appear to be more closely related to Latvian STs than to English ones (Fig. 3), suggesting that there is limited, or potentially no, migration between north and south in the UK. This is interesting in light of a study by Searle and colleagues (2009) investigating three species of small mammals (including the field vole *Microtus agrestis*, bank vole *Myodes glareolus* and pygmy shrew *Sorex minutus*) in Great Britain. In each case, two geographically distinct phylogroups were found. All the species showed a clear north/south divide between the phylogroups, which occurred in northern England, in the case of *M. agrestis*, and as far north as central Scotland in the case of *S. minutus*. The marked differentiation between English and Scottish *B. afzelii* samples may therefore be a result of limited north–south rodent migration.

Taken together, the findings reported in this article support the hypothesis that the movement and spread of LB species is dependant on the host, not the vector, but further investigations on a finer genetic scale, and much denser sampling, are required to fully understand the subtle ecological differences between hosts and to reconstruct fully the migration patterns of the different LB species. Given that the migration of some LB species is limited by the propensity for their vertebrate hosts' ranges to shift, landscape genetic analysis would be an appropriate approach to determine barriers to migration (Manel *et al*., 2003). This is particularly important for understanding the migration and emergence patterns of *B. afzelii*, which is associated with rodent hosts. Such future investigations would be facilitated by (i) identification of the rodent hosts of *B. afzelii* in England and (ii) the use of neutral genetic markers.

Experimental procedures

Tick and bacterial samples

Questing ticks were collected by blanket dragging between March and November of 2006 and 2007 in Latvia and England. Three woodland sites in north-east Somerset, UK, and three located around the Latvian capital, Riga, were visited repeatedly over this 2-year period, with a median of 5.5 visits per site per year in England and 4.5 visits per site per year in Latvia. Additional ticks were collected from 2007

to 2009 from a broader geographical range of British sites, including sites from England, Scotland and Wales. All ticks were preserved in 1.5 ml microcentrifuge tubes containing 70% ethanol. A list of samples included in this study, as well as the coordinates of all collection sites, can be found in *Supporting information* (Tables S2 and S3), as well as on the MLST website (http://borrelia.mlst.net).

In addition to the ticks from Latvia and the UK, 21 *Borrelia*-positive questing ticks from Germany and 46 isolates from the France were also included in the phylogenetic and population analyses (Table S2). The German ticks were collected in 2008 and 2009 by blanket dragging and the French isolates were described previously by Margos and colleagues (2009).

DNA extraction and PCR

Total genomic DNA was extracted from all ticks by alkaline hydrolysis (Guy and Stanek, 1991). Briefly, ticks were placed individually in Eppendorf safelock tubes (Eppendorf, UK) in 120 µl (nymphs) or 200 µl (adults) of 1.25% aqueous ammonia (NH_4OH) and homogenized with a disposable sterile pipette tip. The tick homogenate was incubated (lid closed) on a heat block for 20 min at 100°C. Homogenates were removed from the block for 2 min before they were opened and replaced onto the heat block at 100°C until approximately half the liquid had evaporated. Samples were stored at –20°C. Several tubes in which the tick material was omitted were included to serve as negative controls for cross-contamination.

All DNA samples were subjected to nested PCR amplification of 5S–23S (*rrf–rrl*) intergenic spacer (IGS), which is specific for LB spirochetes, as described previously (Rijpkema *et al.*, 1995). Tick samples found positive for *Borrelia* infection were used in nested PCR reactions to amplify each of the eight MLSA gene loci. *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *rplB* and *uvrA* were all amplified as described previously (Margos *et al.*, 2008; 2009) (see also http://borrelia.mlst.net). For *nifS* a new outer reverse primer was designed (nifS1049R) for the hemi-nested PCR. The sequence is: nifS1049R: 5′-GATATTATTGAATTTCTTTTAAG-3′.

The original outer reverse primer for *nifS* (nifS719R) (Margos *et al.*, 2008) was used as the inner reverse primer, while the forward primer (nifS1F) remained the same. PCR conditions were the same as published previously (Margos *et al.*, 2008).

Sequence analyses

The PCR products of the MLSA genes *clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB* and *uvrA* were sequenced in both forward and reverse directions. The sequences and their traces were then viewed, compared manually and sorted using Seqman (DNASTAR, Madison, USA). The IGS was sequenced in a single direction only and sequences were searched against sequences available in the GenBank database using the NCBI BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the species of the spirochaetal strain. Mixed infections were detected by double peaks in the chromatograms and were excluded from further analyses.

MLSA, alignments and constructing phylogenies

All sequences were compared with those in the MLST database (http://borrelia.mlst.net). Unique alleles and allelic profiles were given new allele and sequence type numbers respectively. As there was no allele over-lap between species, the MLSA data were split into species for analysis. *Borrelia burgdorferi* strains were excluded from analysis as there were too few samples in the data set for population studies.

Sequence alignments were generated using MUSCLE Multiple Sequence Alignment Software (Edgar, 2004). Mega 4.0 (Kumar *et al.*, 2004) was used to visualize 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 using the ATGC Montpellier bioinformatics platform (Guindon and Gascuel, 2003). The evolutionary model used in the phylogenic 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 tree was a BIONJ tree and the type of tree improvement was subtree pruning and regrafting (SPR) and nearest neighbour interchange (NNI). The branch support values were estimated using approximate likelihood ratios (aLRT) and Shimodaira–Hasegawa-like (SH-like) method. All other settings remained as the default settings.

Data analysis

Allelic profiles, generated by MLST, were separated into species and entered into the comparative goeBURST algorithm (Francisco *et al.*, 2009). goeBURST was used to identify founder STs and their relationship to other STs through single- and double-locus variants (SLVs and DLVs).

Pairwise F_{ST} values were used to compare genetic polymorphism between LB populations in France, Germany, England and Latvia in each of the three species using ARLEQUIN 3.1 (Excoffier *et al.*, 2005), 1000 permutations were run to assess the significance of the F_{ST} value.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PhyML phylogenetic inference of the concatenated housekeeping genes of *B. garinii* STs.
The symbols indicate the country the ST was found in: France (black triangle), England (white diamond), Latvia (black square) and Germany (white square). The tree is rooted with three LB species: *B. valaisiana*, *B. afzelii* and *B. burgdorferi*, but the branch length is not to scale as indicated by the slashes. (Scale bar: 0.5% divergence.)
Fig. S2. PhyML phylogenetic inference of the concatenated housekeeping genes of *B. valaisiana* STs. The tree is rooted with three LB species: *B. garinii*, *B. afzelii* and *B. burgdorferi*, but the branch length is not to scale as indicated by the slashes. (Scale bar: 0.1% divergence.)
Table S1. LB prevalence of nymphs collected at sites in Britain and Latvia.
Table S2. List of strains, their sequence type (ST) and geographic origin.
Table S3. Sequence types (STs) identified in strains in this study.
Table S4. Mean nucleotide p-distance (π) and mean dN/dS ratio of individual genes and concatenated sequence.
Table S5. Amino acid sequences of the concatenated housekeeping genes: *clpA* 1–193, *clpX* 194–402, *nifS* 403–590, *pepX* 591–780, *pyrB* 781–981, *recG* 982–1198, *rplB* 1199–1406, *uvrA* 1407–1595. (A) *B. afzelii* ST amino acid sequences, (B) *B. garinii* ST amino acid sequences, (C) *B. valaisiana* ST amino acid sequences.
Table S6. Frequency of all STs where multiple examples were identified and the number of countries the ST was identified in. Abbreviations for countries are as follows: France (FRA), Germany (GER), Great Britain (GB), Latvia (LAT).

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