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Multi-locus sequence typing of *Ixodes ricinus* and its symbiont *Candidatus* Midichloria mitochondrii across Europe reveals evidence of local co-cladogenesis in Scotland

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ABSTRACT

Ticks have relatively complex microbiomes, but only a small proportion of the bacterial symbionts recorded from ticks are vertically transmitted. Moreover, co-cladogenesis between ticks and their symbionts, indicating an intimate relationship over evolutionary history driven by a mutualistic association, is the exception rather than the rule. One of the most widespread tick symbionts is *Candidatus* Midichloria, which has been detected in all of the major tick genera of medical and veterinary importance. In some species of *Ixodes*, such as the sheep tick *Ixodes ricinus* (infected with *Candidatus* Midichloria mitochondrii), the symbiont is fixed in wild adult female ticks, suggesting an obligate mutualism. However, almost no information is available on genetic variation in *Candidatus* M. mitochondrii or possible co-cladogenesis with its host across its geographic range. Here, we report the first survey of *Candidatus* M. mitochondrii in *I. ricinus* in Great Britain and a multi-locus sequence typing (MLST) analysis of tick and symbiont between British ticks and those collected in continental Europe. We show that while the prevalence of the symbiont in nymphs collected in England is similar to that reported from the continent, a higher prevalence in nymphs and adult males is apparent in Wales. In general, *Candidatus* M. mitochondrii exhibits very low levels of sequence diversity, although a consistent signal of host-symbiont coevolution was apparent in Scotland. Moreover, the tick MLST scheme revealed that Scottish specimens form a clade that is partially separated from other British ticks, with almost no contribution of continental sequence types in this north-westerly border of the tick's natural range. The low diversity of *Candidatus* M. mitochondrii, in contrast with previously reported high rates of polymorphism in *I. ricinus* mitogenomes, suggests that the symbiont may have swept across Europe recently via a horizontal, rather than vertical, transmission route.

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1. Introduction

In common with many other arthropods, the advent of 16S rRNA profiling using next-generation sequencing methods has revealed complex microbiomes in ticks (Bonnet et al., 2017). However, an important subset of such microbial communities, the bacterial symbionts that are maternally inherited, fall into just 10 genera. A recent meta-analysis has indicated that of these, obligate tick symbionts (*i.e.*, those present in most, if not all, individuals of a given species across both temporal and geographical scales) are restricted to five genera: *Coxiella* and *Rickettsiella* (order *Legionellales*), *Rickettsia* and *Candidatus* Midichloria (order *Rickettsiales*), and *Francisella* (order *Thiotrichales*) (Duron et al., 2017). While evidence of tick-symbiont co-cladogenesis between members of the genus *Rhipicephalus* and their *Coxiella*-like endosymbionts has been reported, this appears to be exceptional (Duron et al., 2017, 2015). Indeed, patterns of coevolution between ticks and their bacterial symbionts show dynamic evidence of losses, gains, and horizontal transfers between tick species, with these widespread *Coxiella*-like endosymbionts being replaced by members of the other four symbiont genera in multiple lineages (Duron et al., 2017).

From an applied perspective, tick symbionts are of interest for at least four reasons. First, the origin of some pathogens of humans (including *Coxiella burnetii,* the spotted-fever group rickettsiae and possibly *Francisella tularensis*) and other vertebrates can be traced to symbiotic bacteria that were originally restricted to arthropods (Duron et al., 2015; Scoles, 2004; Weinert et al., 2009). This is an ongoing process that can lead to the emergence of diseases, such as Q-fever and tularaemia, which no longer require ticks in order to be transmitted between vertebrate hosts. Second, symbionts might affect the transmission of related (*i.e.*, other intracellular bacteria) or unrelated (*i.e.*, viruses or helminths) pathogens by the vector, as has been demonstrated for *Wolbachia* infections in mosquitoes under certain conditions (Gomes et al., 2017; Kambris et al., 2009; Walker et al., 2011). Third, symbionts have the potential to affect the reproductive fitness of their hosts, and this could be exploited for vector control as has been attempted with *Wolbachia* symbionts in mosquitoes (Laven, 1967; O'Connor et al., 2012). Finally, symbionts might interfere with the diagnosis of infections caused by related bacteria, or more positively, could provide a means to identify biomarkers of tick exposure (Mariconti et al., 2012).

The family *Midichloriaceae* is a group of obligate intracellular bacteria associated with a diverse range of protistan, vertebrate, arthropod, and marine invertebrate hosts (Montagna et al., 2013). The type species, *Candidatus* Midichloria mitochondrii (hereafter *M. mitochondrii*) was originally described from the castor bean or sheep tick, *Ixodes ricinus* (Lo et al., 2006). This vector of the causative agents of Lyme borreliosis, tick-borne encephalitis, louping ill, tick-borne fever (anaplasmosis) and redwater fever (babesiosis) is the most important tick, from a medical and veterinary perspective, in Western and Central Europe (Medlock et al., 2013). Studies on wild *I. ricinus* in continental Europe have shown fixation of *M. mitochondrii* in female ticks, whereas 44% of adult males are infected (Sassera et al., 2008). Notably though, it has been observed that *M. mitochondrii* can be lost from *I. ricinus* during the process of laboratory colonisation (Lo et al., 2006). Other strains of *Midichloria* have been detected in all major tick genera of medical and veterinary importance, but often at a low prevalence (in populations) and density (in individuals) (Cafiso et al., 2016). Only a few members of the genus *Ixodes* exhibit *Midichloria* infections that have reached fixation, and the cellular tropism appears to be variable, with the symbiont colonising mitochondria in *I. ricinus* but not in those of the Australian paralysis tick, *Ixodes holocyclus* (Beninati et al., 2009). Moreover, absence of co-cladogenesis between *Midichloria* and ixodid ticks has been reported (Duron et al., 2017; Epis et al., 2008). Accordingly, *Midichloria* DNA and antigens have been detected in the blood of parasitized mammals, suggesting that this bacterium can be transmitted horizontally between ticks (Bazzocchi et al., 2013).

The role of *M. mitochondrii* in the biology of its host has not been elucidated, but multiple nonexclusive hypotheses have been suggested. The increase in *M. mitochondrii* numbers in concert with feeding is suggestive of a role in the metabolism of the blood meal (Sassera et al., 2008). Conversely, the loss of the symbiont in populations maintained in the laboratory could be indicative of a role that is only important in the wild, such as increasing survival in cold climates (Lo et al., 2006). As a precedent, the tick-borne pathogen *Anaplasma phagocytophilum* has been reported to up-regulate the expression of an antifreeze protein in its host, *Ixodes scapularis* (Neelakanta et al., 2010). The presence in the *M. mitochondrii* genome of a peculiar proton-pumping respiratory haem-copper oxidase gene set (cbb_3) indicates that this symbiont is capable of performing oxidative phosphorylation at low oxygen tensions, which could facilitate ATP production when oxygen is scarce (Sassera et al., 2011); for instance, under very wet conditions when ticks may become temporarily submerged. Finally, the presence in the *M. mitochondrii* genome of complete metabolic pathways for *de novo* B-vitamin synthesis (Sassera et al., 2011) could indicate that the symbiont provides such molecules to the host, as they are absent from blood, the sole nutrient the tick consumes (Rio et al., 2016).

Defining the phylogeography of *I. ricinus* across its range has proved controversial, in large part because the substantial size and repeat content of its genome has precluded genome-wide population studies. Early studies based on mitochondrial markers suggested that even distant populations across continental Europe were genetically indistinguishable (Casati et al., 2008), or that only populations separated by the Mediterranean basin were significantly different (Noureddine et al., 2011) [although North African "*I. ricinus*" may in fact be a different species, *Ixodes inopinatus* (Estrada-Pena et al., 2014)]. However, other workers have reported that population structure is clearly detectable in *I. ricinus*, sometimes even at relatively fine physical scales, depending on the precise genetic markers used and the geographic regions sampled (Carpi et al., 2016; Dinnis et al., 2014; Roed et al., 2016). Furthermore, one microsatellite-based study provided evidence that *I. ricinus* can be divided into host-adapted "races" (Kempf et al., 2011).

Here, we present the first combined population genetic analysis of *I. ricinus* and *M. mitochondrii* across a wide swathe of its geographic range using multi-locus sequence typing (MLST) of tick mitochondrial markers and bacterial housekeeping genes. We show that although *M. mitochondrii* exhibits a remarkably low level of genetic diversity, tick and symbiont populations in Great Britain, and particularly in Scotland, have a distinct signature that provides evidence of local coevolution in isolation from continental Europe. This has potential implications for the natural history of *I. ricinus* in the United Kingdom and its role in the spread of disease.

2. Materials and methods

2.1. Samples for detection and quantification of M. mitochondrii

Adult and nymphal ticks in various states of engorgement were removed from three freshly-culled fallow deer (*Dama dama*) belonging to the Powis Castle estate (Table 1). Questing nymphs were collected using the flagging method from five field sites in southern England (Table 1). All ticks were placed in 70% ethanol on collection and stored at 4 °C. Morphological identifications were conducted using a dissecting microscope with reference to Hillyard (1996).

2.2. Sample collection for generation of tick-symbiont MLST data

I. ricinus questing or partially-fed adult females were collected from Powis Castle estate, Wales, as above (n = 12); Aberdeenshire, Scotland (n = 12); Chizé, France (n = 16); Appennino tosco-emiliano, Italy (n = 12); and the Zermatt valley, Switzerland (n = 12). In addition, 12 samples of *I. ricinus* genomic DNA originating from Bonn, Germany, were available from an archived collection (-20 °C) used for *Borrelia*

Table 1
British *I. ricinus* specimens used for the estimation of *Midichloria* density.

Stage	No. of ticks assayed	Field site	Geolocation (decimal degrees)	County	Country
Engorged female	20	Powis Castle	52.650244, -3.161398	Powys	Wales
Semi-engorged female	25				
Unengorged female	14				
Male	48				
Nymph	44				
	100	Salisbury	50.993133, -1.754662	Wiltshire	England
	100	Bentley Wood	51.057250, -1.637326	Wiltshire	
	100	New Forest	50.924299, -1.662935	Hampshire	
	100	Exmoor	51.170772, -3.624800	Somerset	
	100	Dartmoor	50.571832, -3.920656	Devon	

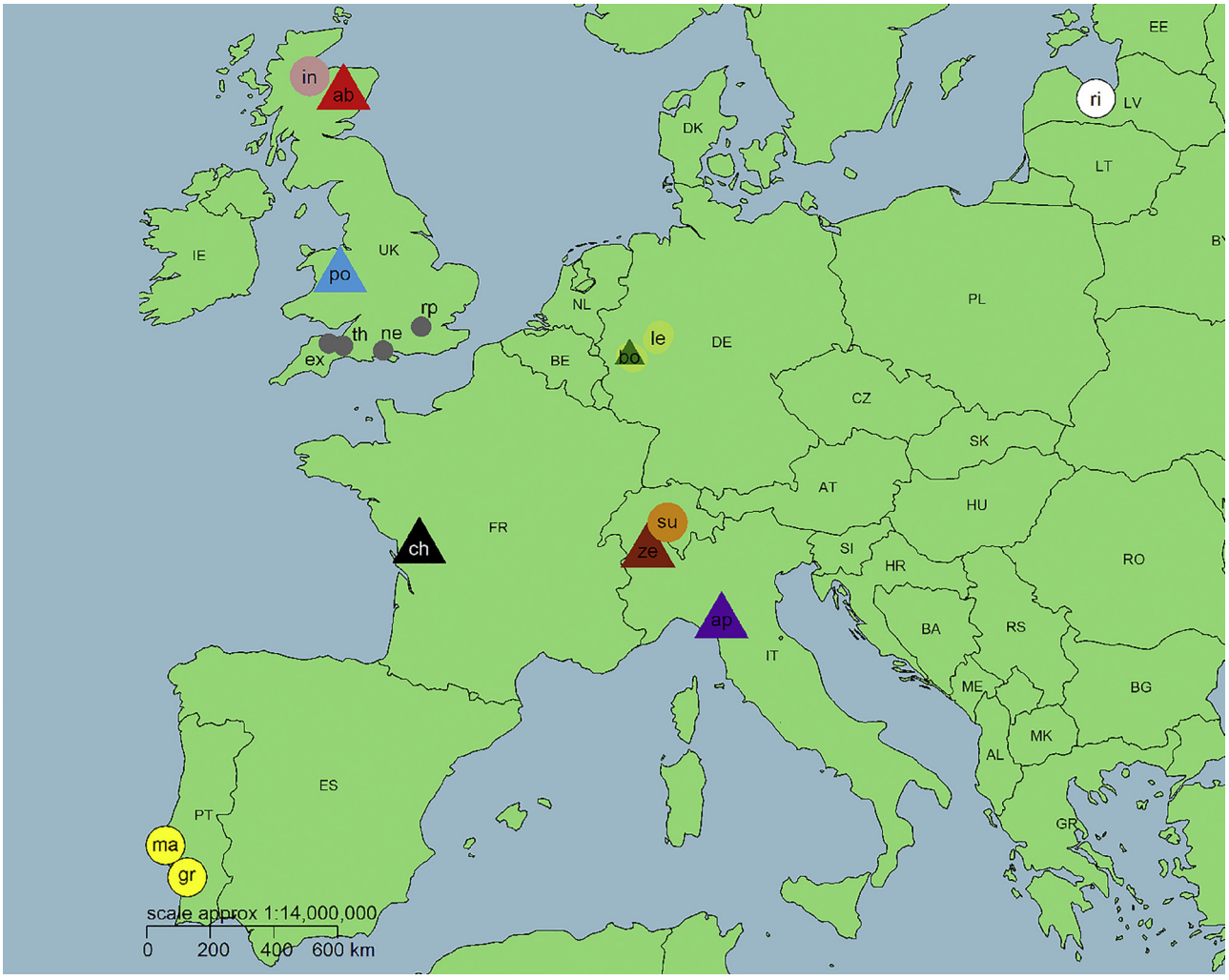

Fig. 1. Tick sampling locations used in the MLST analysis. Sites marked by triangles were newly sequenced for this study for both *M. mitochondrii* and *I. ricinus*. Sites marked by circles represent locations where sequences were obtained previously for a MLST study of the tick only (Dinnis et al., 2014) or during a population analysis of *Borrelia burgdorferi* sensu lato (Vollmer et al., 2013). Of the "circle" sites, only those from Scotland, England and Latvia were analysed in the study of Dinnis et al. (2014). Key: *ab* – Aberdeenshire and *in* - Inverness (Scotland); *po* - Powis Castle (Wales); *ch* - Chizé (France); *bo* – Bonn and *le* - Lennestadt-Meggen (Germany); *ap* - Appennino tosco-emiliano (Italy); *ze* - Zermatt valley and *su* – Susten (Switzerland); *ri* - Riga (Latvia); *ex* – Exmoor, *ne* - New forest, *rp* - Richmond Park and *th* - Thurlbear Woods (England); gr – Grândola and ma – Mafra (Portugal).

studies at the University of Bath (Vollmer et al., 2013). Specimens were identified as *I. ricinus* using morphological criteria (Hillyard, 1996). All locations sampled for the MLST analyses are displayed in Fig. 1.

2.3. DNA extraction

Ticks were rinsed with distilled water to remove ethanol. Each specimen was dissected from the palps to the anal groove using a scalpel blade under a dissecting microscope. Quadrisection was applied to the larger stages (engorged females and semi-engorged females) and bisection for smaller specimens (unengorged females, males and nymphs).

DNA was extracted from ticks using alkaline hydrolysis as described by Ammazzalorso et al. (2015). Briefly, 150 µl of 14.5 M ammonium hydroxide (Sigma-Aldrich) was added to each dissected tick, which was boiled for 20 min in open tubes in a dry block heater housed in a fume

cupboard. The final volume of 70–100 µl was centrifuged for 10 min at 10,000 × *g* to remove debris. In order to increase the DNA concentration for nymph samples only, 30 kDa Nanosep centrifugal devices (Pall Life Sciences) were used to reduce the volume to ~20 µl. DNA concentrations were quantified by a fluorescent dye intercalation method (Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen) using a microplate fluorimeter (Infinite F200, Tecan) and Magellan Data Analysis Software (Tecan).

2.4. Quantitative PCR for M. mitochondrii

To quantify *M. mitochondrii* in tick lysates, quantitative PCR (qPCR) was applied according to a modification of a published method (Sassera et al., 2008). A 146-bp fragment of the symbiont DNA gyrase subunit B gene (*gyrB*) was amplified as previously described. To normalise symbiont copies between different tick stages, a new qPCR assay targeting a tick single-copy nuclear gene was designed. This amplified a 77-bp fragment from exon 2 of the ribosomal protein L6 gene (*rpl6*), based on the sequence from *I. scapularis* (NCBI Reference Sequence: XP_002400555.1), with primers 5'-CCGGTCCAAGATTCCACA3′ (sense) and 5'-TGCGCTTCCTCTTCTCCTTG3′ (antisense). Standards for both assays comprised synthetic long oligonucleotides representing full-length amplicons (obtained from Eurogentec for *gyrB* and Sigma-Aldrich for *rpl6*).

The qPCR assays were performed in 20 µl reaction volumes containing final concentrations of 1 × SensiMix SYBR No-ROX master-mix (Bioline), 400 nM (for *gyrB*) or 200 nM (for *rpl6*) each primer, and 1 µl of tick DNA (replaced with nuclease-free water in no-template control reactions). The qPCR reactions were run on a CFB-3220 DNA Engine Opticon 2 System (Bio-Rad) using the published cycling conditions for *gyrB* (Sassera et al., 2008) and these modifications for *rpl6*: initial denaturation at 95 °C for 10 min; followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 15 s; with a melt-curve from 55 to 95 °C (increasing in increments of 0.5 °C per cycle). Linear regression analysis based on tenfold dilutions of the standards (5×10^6 – 5×10^{-1} copies/µl) was executed using Opticon Monitor software version 3.1. All PCR assays were conducted on two dilutions of each sample, and copy number calculations were performed using the dilution that lay closest to the middle of the standard curves.

2.5. MLST scheme and PCR assays

In order to identify candidate genes for the *M. mitochondrii* MLST scheme, 14 loci present as a single copy in the symbiont genome and detected throughout the sequenced genomes of the order Rickettsiales (*gpsA, mdh, nrdB, nuoF, ppdk, sucD, sucB, adk, lepB, lipA, lipB, secY, sodB, sucA*) were evaluated. Following primer design and initial PCR attempts for these 14 genes using five adult *I. ricinus* specimens each from England, Scotland, Latvia, Germany, Switzerland and Portugal, all genes except *sucB* and *lepB* were amplified successfully. However, only five loci [*nuf2* (kinetochore protein), *adk* (adenylate kinase), *ppdk* (pyruvate orthophosphate dikinase), *lipA* (lysosomal acid lipase), and *secY* (protein translocase subunit)] were found to exhibit any sequence variation. These were also widely distributed on the *M. mitochondrii* genome and showed no evidence of recombination when sequence alignments were evaluated with RDP4 (v. Beta 4.95) using all of the available tests within the software package (Martin et al., 2015). Therefore, these five loci were selected for the MLST scheme (Table 2). For the tick host, a previously described mitochondrial MLST scheme (Dinnis et al., 2014) was used to allow a direct comparison with existing datasets. This scheme utilises six housekeeping genes: *coi* (cytochrome oxidase I), *coii* (cytochrome oxidase II), *coiii* (cytochrome oxidase III), *atp6* (ATPase 6), *12S* (small RNA subunit) and *cytB* (cytochrome B).

Conventional touchdown PCR assays were performed to amplify each gene. Reactions were performed in 25-µl or 20-µl volumes containing final concentrations of 1 × BioMix Red master-mix (Bioline), 1.2 µM or 1 µM of each primer, and 2 µl or 1 µl of template DNA for *I. ricinus* and *M. mitochondrii*, respectively. Cycling conditions for amplification of loci from *I. ricinus* are provided in supplemental table S1. Cycling conditions for *M. mitochondrii* comprised initial heating at 94 °C for 2 min, then 10 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s (decreasing by 1 °C per cycle), and extension at 72 °C for 30 s. This was followed by an amplification phase with initial heating at 94 °C for 2 min, then 29 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The final extension was performed at 72 °C for 10 min.

Despite optimisation attempts, in some cases it was not possible to produce a single amplicon band. Hence, the target band was excised and DNA was obtained using a PureLink Quick Gel Extraction Kit (Invitrogen) according to the manufacturer's instructions. The PCR amplicons were visualised using a Safe Imager transilluminator (Invitrogen) and purified with a QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions.

2.6. Molecular identification of Welsh and Scottish specimens

To confirm morphological identification of the Welsh and Scottish specimens, a conventional PCR assay targeting the mitochondrial 16S rRNA gene was applied (Black and Piesman, 1994). Following gel electrophoresis, PCR products were purified using a Monarch PCR & DNA Cleanup Kit (New England Biolabs) according to the manufacturer's instructions. Sanger sequencing of the PCR products in both directions was performed by Source BioScience.

2.7. Sequence analysis

Purified PCR products were sequenced using Sanger chemistry by Eurofins MWG or Macrogen and chromatograms were verified and assembled using BioEdit [Ibis Bioscience (Hall, 1999)]. Gene sequences were aligned using CLUSTALW as implemented in Mega 6.0 (Tamura et al., 2013). Alleles and sequence types (STs) were assigned manually and analyzed using eBURST [(Feil et al., 2004) data not shown]. Individual loci from *M. mitochondrii* and *I. ricinus* ticks were manually concatenated separately. All gene sequences have been submitted to NCBI (Tables 3 and 4).

For *I. ricinus* (but not *M. mitochondrii*), sequences obtained using the same MLST scheme used in the present study were available from England, Scotland, Latvia, Switzerland, Germany and Portugal (Fig. 1). The sequences from England, Scotland and Latvia were used in a previous MLST study of *I. ricinus* population structure (Dinnis et al., 2014), whereas the unpublished sequences from other locations were obtained during a population analysis of *Borrelia burgdorferi* sensu lato (Vollmer et al., 2013).

To infer phylogeny for all individual loci, the sequence alignment was subjected to Modeltest as implemented in TOPALi (Milne et al., 2009), which indicated that the best-fit model for phylogeny was TN93 (Tamura and Nei, 1993). In order to analyse the phylogenetic relationships of each gene for the bacterial endosymbiont and the tick host, nucleotide and amino acid maximum-likelihood phylogenetic trees (with bootstrap values based on 10,000 iterations) were produced. Individual gene alignments were concatenated and realigned. Phylogenetic inferences were made as described above using the same TN93 model as predicted by TOPALi, and maximum-likelihood trees were again drawn using 10,000 bootstrap iterations.

Minimum-spanning distance trees were drawn using PHYLOViZ (Francisco et al., 2012). Pie-charts were produced in Microsoft Excel and manually transposed onto the minimum-spanning tree to indicate the origin of each sample in each node. Full DNA alignments were screened for recombination using SplitsTrees4 (Huson and Bryant, 2006), as well as for positive and negative selection using GARD and SLAC via the Datamonkey web server (Pond and Frost, 2005). In

Table 2
Oligonucleotide primers designed to amplify housekeeping genes from *M. mitochondrii*.

Locus	Encoded protein	Predicted product size (bp)	Genome co-ordinates[a]	Sense primer (5' - 3')	Antisense primer (5' - 3')
nuf2	Kinetochore protein	648	968094 - 968609	CTTTATGGACAAGATAGTGCTG	CAGTACGCCTCATAATGGC
ppdk	Pyruvate orthophosphate dikinase	532	219742 - 220086	GTAAATCCATTCTAGGAGGCAA	ACCAGCATGTTGTAAGACGA
adk	Adenylate kinase	510	27193 - 27540	GCGAAATACTTAGGAATGAGGT	AAATCAATCGTCTTATCTCCATCA
lipA	Lysosomal acid lipase	550	393462 - 393707	GATATTAGGAAGTGTCTGCAC	GCTGTAGATATTGTCCAATCG
secY	Protein translocase subunit	550	26532 - 26870	AAAGTTTATGCAGGAGATTCAAC	GTGAGGAAATAGGTTTGGATTC

[a] Gene positions are relative to those from *M. mitochondrii* strain IricVA (GenBank accession number NC_015722).

addition, the possibility that nuclear mitochondrial pseudogenes (*numts*) had been amplified by the tick MLST scheme was considered by inspection of translated sequences for indels or in-frame stop codons (Song et al., 2008).

2.8. Statistical analysis of Midichloria density

Midichloria copy numbers normalised against *rpl6* were compared between tick stage (for the Welsh samples) and collection location (for the English nymphs) in IBM SPSS Statistics v. 24 (IBM Corp.). Data were log-transformed and Levene's homogeneity of variance test was run to verify that variances were not significantly different between groups. Where variances were equal, a one-way ANOVA was performed with Tukey's post-hoc test. If log-transformation failed to equalise the variances, a non-parametric ANOVA (Kruskal-Wallis test) was conducted. The critical probability was set as $P < 0.05$.

2.9. Ethics statement

Culling of fallow deer in Wales prior to tick removal was performed for routine population management at the Powis Castle estate, Powys, by qualified deerstalkers. French ticks were collected from roe deer (*Capreolus capreolus*) in the Chizé Forest in strict accordance with the recommendations of the French National Charter on the Ethics of Animal Experimentation, and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocol was approved by the "Comité d'Ethique en Expérimentation Animale de l'Université Claude Bernard Lyon 1" (CEEA-55; DR2014-09). The capture of roe deer was conducted only by competent persons using methods that do not cause the animals avoidable pain, suffering, distress or lasting harm. All other tick specimens used in this study were unfed individuals collected from the environment.

3. Results

3.1. M. mitochondrii prevalence and density between lifecycle stages and locations

The ticks collected from deer in Wales exhibited a very high prevalence of infection with *M. mitochondrii*, with only four specimens (three adult males and one semi-engorged female) testing negative for the symbiont. In all cases, the tick nuclear gene *rpl6* was successfully amplified. The density of *M. mitochondrii* was significantly higher in female ticks of every engorgement stage compared with both males and nymphs (one-way ANOVA, $P < 0.01$; Fig. 2a), whereas levels between males and nymphs were not statistically distinguishable. Neither was the apparent positive trend between symbiont density and female tick engorgement stage statistically significant (Fig. 2a).

In contrast with the apparent fixation of *M. mitochondrii* in the partially-fed Welsh nymph samples, only approximately three-quarters of questing nymphs in England were positive for the symbiont, and this prevalence was remarkably consistent between locations (Fig. 2b). While the range of symbiont densities in the English samples was very wide, the median was similar between locations and not significantly different (Kruskal-Wallis test, $P = 0.092$).

3.2. Population structure of I. ricinus

Sequences were obtained for all six loci for 64 tick specimens. Comparison of the sequence data revealed differing levels of variability at all loci. The average diversity between loci of *I. ricinus* was 11%, with a range from 9.8% in *coi* and 12S to 11.9% for *coiii* (Table 3). At the amino-acid level, the average diversity between loci was 22%, with a range from 11.2% in *coii* to 26.8% in *coiii* (Table 3). The number of new alleles within this study ranged from 16 (*coii*) to 43 (*cytb*) (Table 3). Sixty-three STs were identified based on the MLST allelic profiles, of which none was previously reported. Of these STs, only one was observed more than once, with the remainder classified as singletons.

These sequences were concatenated, and phylogenetic analysis by maximum likelihood revealed a distinct clade composed almost entirely of Scottish and Welsh sequences, in contrast with little evidence of structure for continental Europe (Italy, France, Germany and Switzerland; Supplemental Figure S1). However, whereas none of the Scottish sequences clustered with those from continental Europe, the Welsh samples were split approximately equally between the Scottish and continental clades (Supplemental Figure S1). Phylogenetic comparison of the concatenated loci produced here with those from a previous study (Dinnis et al., 2014) revealed a generally low level of diversity across the 65 sequences (Supplemental Figure S2), with 63 unique STs. Incorporation of the previously published sequences reinforced the picture of two major clades, with the Scottish samples from both studies clustering together to the exclusion of almost all continental sequences, except for some representatives from Latvia (Supplemental Figure S2). Other samples from the UK displayed segregation in similar numbers between the "Scottish" and "continental" clades.

To assess the relatedness between different STs, a minimum-spanning tree was generated to compare the sequences obtained in the current study (Fig. 3a). Sequences close to each other on the minimum-spanning tree are generally only different at a single locus, whereas the more distant sequences have fewer loci in common. Interestingly, the only ST that we observed more than once (ST 478) was the founder sequence of a large clade composed predominantly of continental sequences. With the exception of ST472 from Switzerland, a second clade that we identified (founded by ST461 from Wales) was composed exclusively of British STs (Fig. 3a). As for the maximum-likelihood trees, incorporation of previously derived tick STs into the minimum-spanning tree lent further support for a Scottish clade clustering with other British, and some Latvian, STs (Fig. 4). However, this tree exhibited somewhat more structure in the continental STs than was previously detected (Fig. 4).

We considered two potential artefacts for the distinctiveness of the Scottish clade: misidentification of *I. inopinatus* as *I. ricinus* (Chitimia-Dobler et al., 2018; Estrada-Pena et al., 2014), or confounding of the mitochondrial MLST scheme by *numts* (Song et al., 2008). Amplification of mitochondrial 16S rRNA sequences from 12 British tick specimens distributed between England, Wales and Scotland and comparison with *I. inopinatus* 16S rRNA indicated that they are distinct from one another (Supplemental Figure S3), with a nucleotide divergence of > 2%. Moreover, translation and alignment of tick MLST sequences showed no

Table 3
Comparison of variation at different loci for the 64 *I. ricinus* ticks sequenced as part of this study. Analyses at the nucleotide and amino acid levels are shown.

	DNA									Amino acid (AA)				
Gene name	GenBank accession	Number of alleles[a]	Number of new alleles*	Length (bp)	Gene product	GC content (%)	Polymorphic sites (%)	Parsimony informative sites (%)	d_N/d_S	No. of AA STs*	Number of new AA STs*	Length (AA)	Polymorphic sites (%)	Parsimony informative sites (%)
atp6	MH334375 - MH334945	127	29	411	ATPase 6	21.1 (87/411)	11.4 (47/411)	4.1 (17/411)	0.29602	62	22	137	22.6 (31/137)	10.2 (14/137)
coi	MH336088 - MH336658	132	29	447	Cytochrome oxidase I	29.7 (133/447)	9.8 (44/447)	4.5 (20/447)	0.92570	114	33	149	24.2 (36/149)	12.0 (18/149)
coii	MH334946 - MH335516	106	16	399	Cytochrome oxidase II	29.3 (117/399)	13.0 (52/399)	5.8 (23/399)	0.70581	33	7	133	11.3 (15/133)	4.5 (6/133)
coiii	MH336659 - MH337229	131	23	504	Cytochrome oxidase III	26.9 (136/504)	11.9 (60/504)	5.4 (27/504)	0.65842	87	14	168	26.8 (45/168)	13.0 (22/168)
12S	MH333804 - MH334374	171	19	285	Small RNA subunit	20.0 (57/285)	9.8 (28/285)	3.9 (11/285)	1.30853	–	–	–	–	–
cytb	MH335517 - MH336087	154	43	371	Cytochrome B	24.5 (91/371)	10.0 (37/371)	4.0 (15/371)	0.149389	21	27	123	25.2 (31/123)	11.3 (14/123)

[a] Indicates total STs including those previously reported (Dinnis et al., 2014) and new STs sequenced as part of this study.

Table 4
Comparison of variation at different loci for the 64 *M. mitochondrii* samples sequenced as part of this study. Analyses at the nucleotide and amino acid levels are shown.

Gene name	DNA								Amino acid (AA)			
	GenBank accession	Number of DNA alleles	Length (bp)	Gene product	GC content (%)	Polymorphic sites (%)	Parsimony informative sites (%)	d_N/d_S	No. of AA alleles	Length (AA)	Polymorphic sites (%)	Parsimony informative sites (%)
adk	MH295309 - MH295373	3	349	Adenylate kinase	36.7 (128/349)	0.6 (2/349)	0	n/a	3	116	1.7 (2/116)	0
lipA	MH295374 - MH295438	1	247	Lysosomal acid lipase	38.7 (106/247)	0	0	n/a	1	82	0	0
nuf2	MH295439 - MH295503	4	516	Kinetochore protein	42.2 (218/516)	0.6 (3/516)	0.4 (2/516)	5.00E-09	1	172	0	0
ppdk	MH295504 - MH295568	8	337	Pyruvate orthophosphate dikinase	38.3 (129/337)	1.2 (4/337)	0.6 (2/337)	0.780116	4	112	2.7 (3/112)	1.8 (2/112)
secY	MH295569 - MH295633	2	339	Protein translocase subunit	34.5 (117/339)	0.3 (1/339)	0	n/a	1	113	0	0

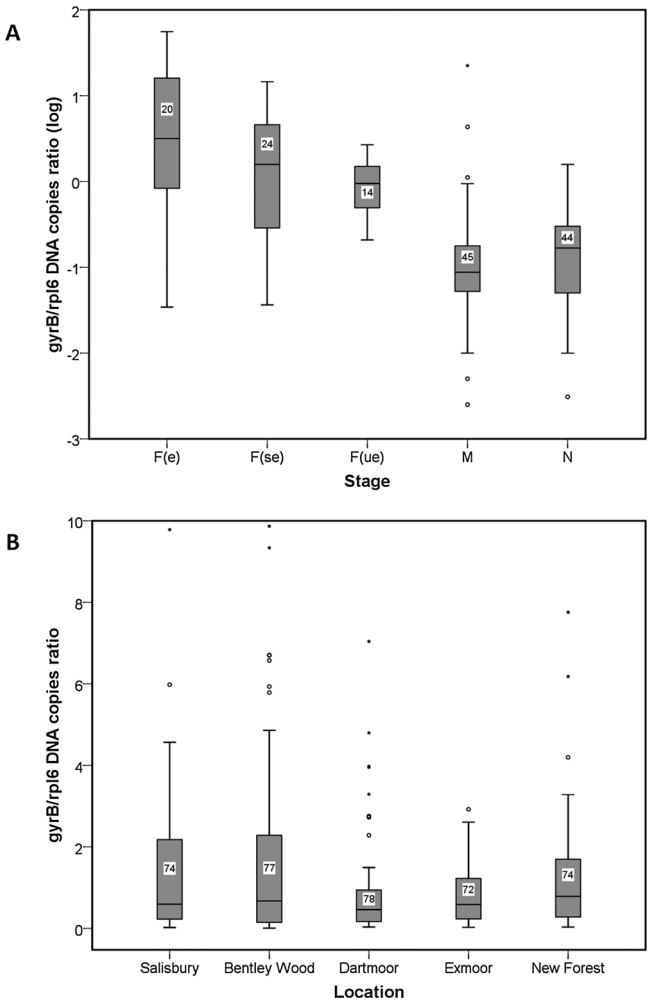

Fig. 2. Density of *M. mitochondrii* in *I. ricinus* from Wales and England. Boxplots display the median, 25th and 75th percentiles, 1.5 × interquartile range (whiskers), outliers (circles) and extreme outliers (asterisks). A: Density of the symbiont in different stages of *I. ricinus* collected from deer at Powis Castle estate, Wales. Note that the copy number ratio has been subjected to $\log_{10}$ transformation. Numbers within boxplots refer to *n*. F(e), female (engorged); F (se), female (semi-engorged); F(ue), female (unengorged); M, male; N, nymph. B: Density of the symbiont in questing nymphs from field sites in southern England. Numbers within boxplots refer to the number of positive samples (n = 100 per location).

evidence for the indels or in-frame stop codons that are the hallmarks of *numts* (data not shown).

3.3. Population structure of M. mitochondrii

For all 64 ticks, a full complement of five gene sequences from *M. mitochondrii* was obtained. Comparison of the sequence data revealed differing levels of variability at all loci, except for *lipA* which was wholly conserved throughout all sequences. The average diversity between loci of *M. mitochondrii* was very low at 0.54%, with a maximum level of 1.2% for *ppdk* (Table 4). At the amino-acid level, only two loci (*adk* and *ppdk*) showed any amino acid diversity, indicating that these genes are highly conserved and that the majority of DNA mutations are synonymous (Table 4).

The number of DNA alleles within this study ranged from one (*lipA*) to eight (*ppdk*) (Table 4). On the basis of these MLST allelic profiles, 14 STs were identified, of which six were seen more than once. The most common ST was ST 8, which contained 28 (44%) of the 64 endosymbiont sequences, with STs 3 and 7 containing 14 and eight sequences, respectively. A maximum-likelihood tree, while clearly reflecting this low level of sequence diversity, did nevertheless resolve two clades with good bootstrap support. These were composed of all sequences of Scottish origin and approximately half of the Welsh samples, juxtaposed against a “continental” clade, containing poorly-resolved subclades with dispersion of the remaining Welsh samples between them (Supplemental Figure S4). Applying a minimum-spanning phylogenetic analysis to the *M. mitochondrii* data revealed that three STs (8, 3 and 7) were founder sequences, with STs 7 and 3 representing those which gave rise to the cluster of Scottish sequences (Fig. 3b).

3.4. Co-evolution of I. ricinus and M. mitochondrii

To investigate potential patterns of co-cladogenesis, maximum-likelihood trees for *I. ricinus* and its symbiont were aligned to compare topologies. In total, 57 of 64 sequences showed similar positions on both trees, which is compatible with co-cladogenesis. However, extensive polytomies in the continental branches of the *M. mitochondrii* tree only allowed a distinct signal to be observed for the Scottish samples, although these were allied to a subset of the Welsh sequences (Fig. 5). The seven incongruent pairs in the tree included one specimen from Scotland, two from Wales and three from Switzerland, constituting a signal of potential horizontal transmission (Fig. 5).

4. Discussion

The population structure of *I. ricinus,* assessed now in at least seven published studies (including the current work), has proved difficult to resolve. An initial small-scale study (26 ticks in total) across continental Europe (Switzerland, Italy, Austria, Denmark, Sweden and Finland) using mitochondrial markers failed to find an association between haplotype and geographic origin (Casati et al., 2008). A somewhat larger analysis (60 specimens) of both mitochondrial and nuclear markers at various scales across Europe (both on the continent and in the British Isles), North Africa, and Western Asia confirmed this apparent panmixia, with the exception of clearly differentiated populations in North Africa (Noureddine et al., 2011). However, these North African specimens are now suspected to be from a different tick species, *I. inopinatus* (Estrada-Pena et al., 2014). In contrast, analysis of microsatellite markers from ~600 ticks has provided evidence for distinct “races” of *I. ricinus* collected from different host species in France, Belgium and Slovakia; for instance, between roe deer and wild boar (Kempf et al., 2011). More recent studies using several hundred ticks have resolved clearly differentiated clades between Britain and Latvia [(Dinnis et al., 2014); data also used in the present study], and between western Norway and Britain versus more centrally located European populations (Roed et al., 2016). Finally, a whole mitochondrial genome analysis of two *I. ricinus* populations in northern Italy, separated by only 100 km, revealed four highly divergent lineages but no geographical structuring (Carpi et al., 2016).

In the context of these previous findings, our study is consistent with the conclusions of Dinnis et al. (2014) and Roed et al. (2016) in identifying the marine barrier around the British Isles as a significant impediment to gene flow from continental Europe. Moreover, our data strongly corroborate the asymmetric nature of this barrier highlighted by Roed et al. (2016), in which STs of British origin are much more common among the continental European clade than vice-versa, reflecting greater easterly than westerly gene flow. This may be a result of spring migrations of birds from the British Isles seeding continental Europe with engorged immature stages, whereas the autumn migrations in the opposite direction are less likely to lead to successful tick establishment due to winter attrition. While the success rate of moulting to the next stage in the lifecycle has been estimated to be only 10% for *I. ricinus* (Randolph et al., 2002), it is possible that the spring migration of birds into the UK is accompanied by > 1 million immature

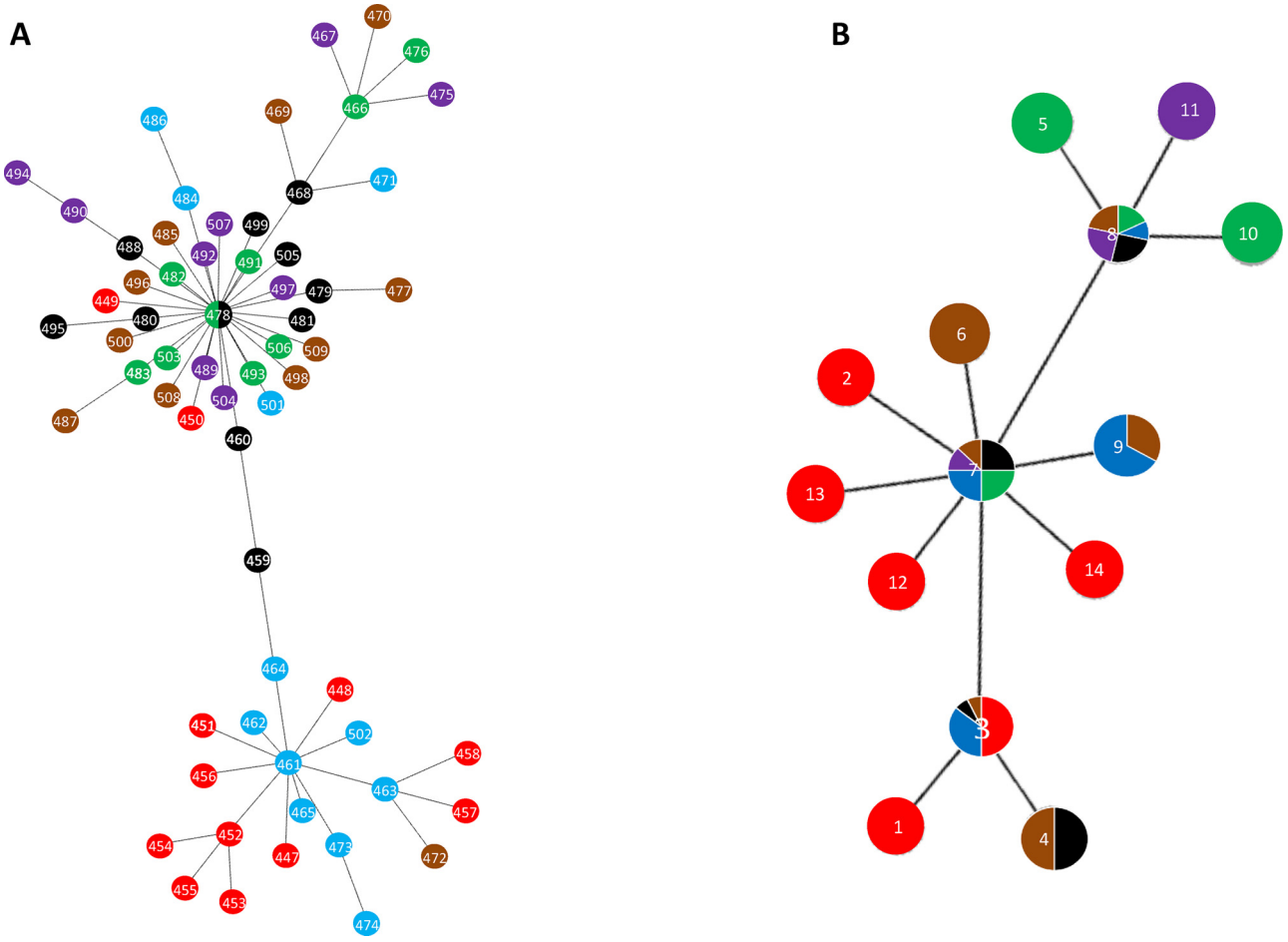

Fig. 3. Minimum-spanning distance tree of 64 *I. ricinus* specimens and their *M. mitochondrii* symbionts. A: *I. ricinus* sequences. B. *M. mitochondrii* sequences. The ST numbers are shown inside the nodes as pie charts, representing the different country of origin of those sequences. France – black, Italy – purple, Germany – green, Switzerland – brown, Wales - blue, Scotland - red. A full list of STs is provided in Supplemental Tables S2 and S3 for the tick and symbiont, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ticks (Pietzsch et al., 2008); thus potentially contributing ~10,000 adult ticks from overseas that were transported by birds as larvae or nymphs. Even accounting for very high rates of attrition from the larval stage, this is likely to dwarf any contribution from nymphs and adults attached to travelling pets.

Here, we conducted the first survey of *Midichloria* prevalence and density in the British Isles. Although we were unable to screen nymphs or male adults from Scotland for *Midichloria* in this study, there was a very high prevalence of the symbiont in nymphs and male adults from Wales (100% and 94% infected, respectively) in contrast with a prevalence of only ~75% in English nymphs. This supports the hypothesis that evolutionary forces experienced by *I. ricinus* in the UK may differ significantly to those in its heartland in mainland Europe. Thus, the prevalence in the English nymphs tallies perfectly with what would be anticipated if 100% of females and ~50% of male nymphs were infected (assuming a 1:1 sex ratio), as observed in continental Europe (Lo et al., 2006); whereas the Welsh data do not conform to these expected frequencies. Moreover, our data indicate that the ticks of Scotland form a discrete clade that contains STs from other parts of Britain, but very little contribution from continental Europe. Importantly, the only clear evidence for structure in our *M. mitochondrii* dataset was observed in STs from Scotland, and to a lesser extent Wales, relative to the locations in continental Europe. This indicates that the evolutionary history of the tick-symbiont relationship in these parts of the British Isles has been subject to either genetic drift caused by a population bottleneck, or selection on ticks and their symbionts for traits that are important for reproductive success in certain locales.

Scotland has a number of biogeographical features that set it apart from the rest of the British Isles. The border region between England and Scotland, the Southern Uplands, is a hilly landscape and further north, the Grampian Mountains are a nontrivial barrier between the Central Lowlands and the Northwest Highlands. Nevertheless, these ranges of hills and mountains do not reach the lateral extent or altitude of the Alps and other mountain ranges in continental Europe where gene flow between *I. ricinus* populations appears to occur unhindered. Thus, the prehistory of the region may be more important than its current topology, as the locations where the Scottish ticks were sampled (Aberdeenshire and Inverness-shire) were covered by ice-sheets as recently as 15,000 years ago - some 10,000 years after the British-Irish Ice Sheet began to retreat at its southerly margins - and may have experienced re-advances even beyond this date (Clark et al., 2012). The colonisation of post-glacial Scotland by *I. ricinus* is likely to have been slow and erratic, perhaps leading to strong founder effects at this northwestern extent of the species' range. Phylogeographic studies of red deer (*Cervus elaphus*) suggest that populations in Western Europe, including the British Isles, derive from a refugium located on the Iberian Peninsula, with little evidence that artificial introductions by humans have impacted significantly on natural migrations (Skog et al., 2009). Even today, when populations of both native and introduced deer species are at a record high for recent times across Britain (Putman et al., 2011), densities of red deer in the Scottish Highlands greatly exceed those in most other parts of the country (Edwards and Kenyon,

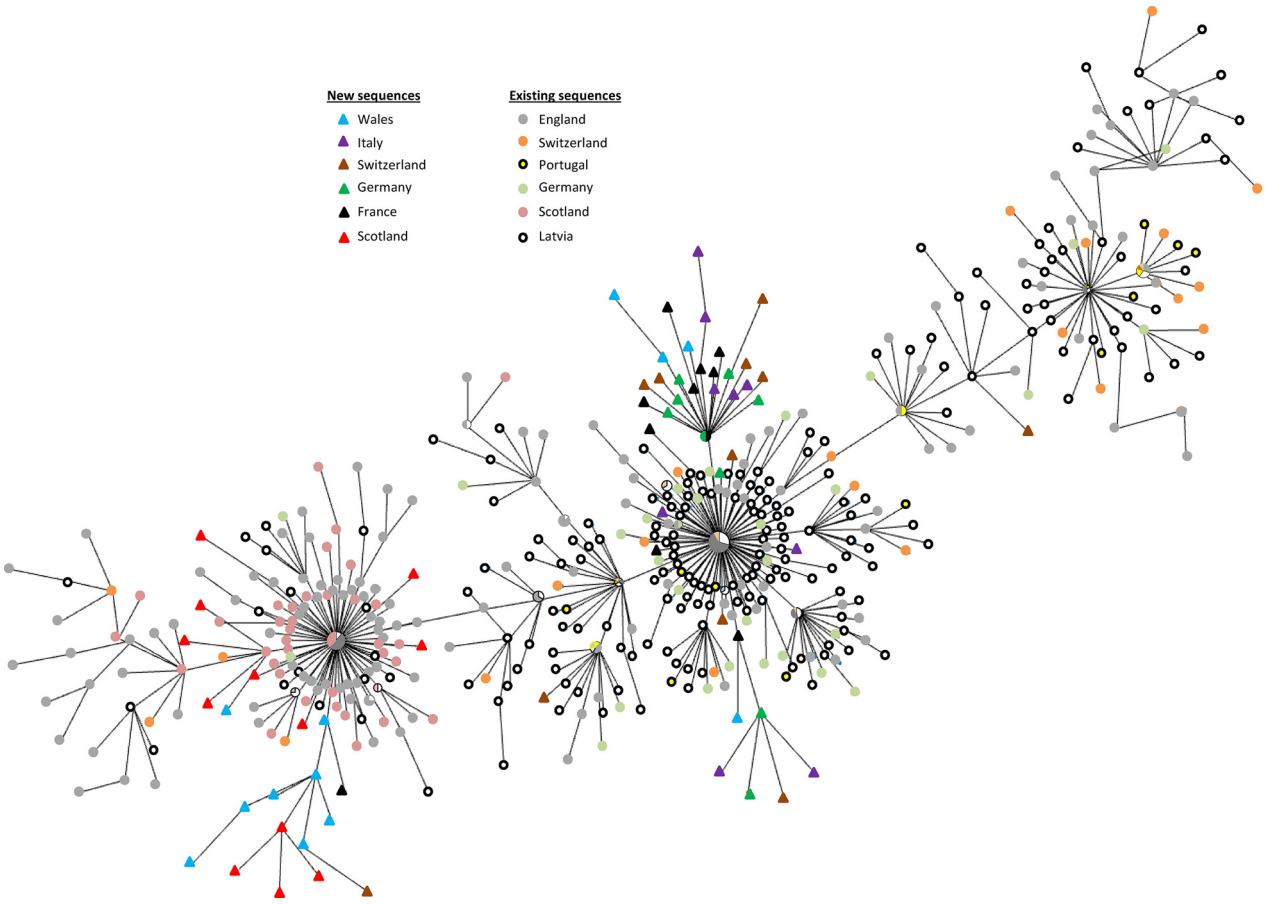

Fig. 4. Minimum-spanning distance tree of *I. ricinus* mitochondrial sequences. Previously-acquired data (circles), some of which have been published (Dinnis et al., 2014), are incorporated into the tree alongside newly-obtained sequences (triangles).

2013). It is plausible that migrations of red deer brought *I. ricinus* to Scotland after the glacial retreats, and differences in the most abundant larger hosts between Scotland and other parts of Europe may have acted to select a "race" of *I. ricinus* that is better adapted for feeding on *C. elaphus* (Kempf et al., 2011). However, physical factors such as the longer winter at higher latitudes increasing attrition rates when ticks are dormant, as well as the reduced duration of the questing season, may have also played a role in selecting for certain tick and symbiont genotype combinations.

An alternative scenario that might explain a strong founder effect in Scottish tick populations is stochastic introductions from bird migrations. Most records of *I. ricinus* on birds in the UK come from passerines such as the blackbird (*Turdus merula*), the willow warbler (*Phylloscopus trochilus*), the whitethroat (*Sylvia communis*), the song thrush (*Turdus philomelos*), and the dunnock (*Prunella modularis*) (James et al., 2011; Pietzsch et al., 2005); and a link between tick infestation and a tendency to forage on the ground was reported from one bird study in Scotland (James et al., 2011). While these common songbirds differ markedly in their migratory habits, none has a particular predilection for Scotland. Nevertheless, during the early post-glacial period, ticks introduced by migratory birds into Scotland may have had greater impacts on local populations than in other parts of the British Isles, perhaps supplanting existing clades through drift. In addition, some British avian *I. ricinus* records are from rare migrants that have almost never been observed outside Scotland, such as the Pechora pipit (*Anthus gustavi*) (Pietzsch et al., 2005); although as most sightings are from Shetland, it is very unlikely that such rarities have impacted on the mainland tick population. Perhaps surprisingly, *I. ricinus* has also been recorded on Scotland's iconic raptor, the golden eagle (*Aquila chrysaetos*) (Pietzsch et al., 2005). It is interesting to speculate whether the tick-infested, preferred mammalian prey of golden eagles (rabbits, hares, sheep and deer), when carried large distances to nesting sites, might on rare occasions lead to the transport of viable ticks > 100 km from their site of origin. Indeed, golden eagles breeding in Scotland have been frequently spotted hunting over the skies of Ireland (Watson et al., 2002).

Classically, as for *Buchnera aphidicola* in aphids, obligate mutualist symbionts that are transmitted vertically down the maternal line are expected to show strict co-cladogenesis with their host (Funk et al., 2000). While our study is compatible with such a pattern of coevolution between *I. ricinus* and *M. mitochondrii*, the poor phylogenetic signal in the latter hampered efforts to clearly identify co-cladogenesis in the populations from continental Europe. Moreover, the phylogenetic mirroring of symbiont and tick population structures between the Scottish and continental clades is not sufficient evidence to conclude that co-speciation occurred between the two parties at an earlier stage of evolution. Neither does it indicate, as discussed above, that the observed co-cladogenesis is necessarily a result of adaptive selection. The apparent low diversity in the symbiont suggests a recent selective sweep through *I. ricinus* populations, at least on the mainland, consistent with a previously report involving just two molecular markers (Lo et al., 2006). However, this scenario would conflict with complete mitogenome data obtained in northern Italy, in which the four identified lineages were estimated to coalesce around 427,000 years ago, suggesting that *I. ricinus* has maintained a large population size since the Pleistocene (Carpi et al., 2016). Since mitochondria and vertically-transmitted symbionts are generally considered to be almost exclusively transmitted by the same route, a symbiont-mediated selective sweep would be expected to markedly reduce mitochondrial polymorphism (Cariou et al., 2017).

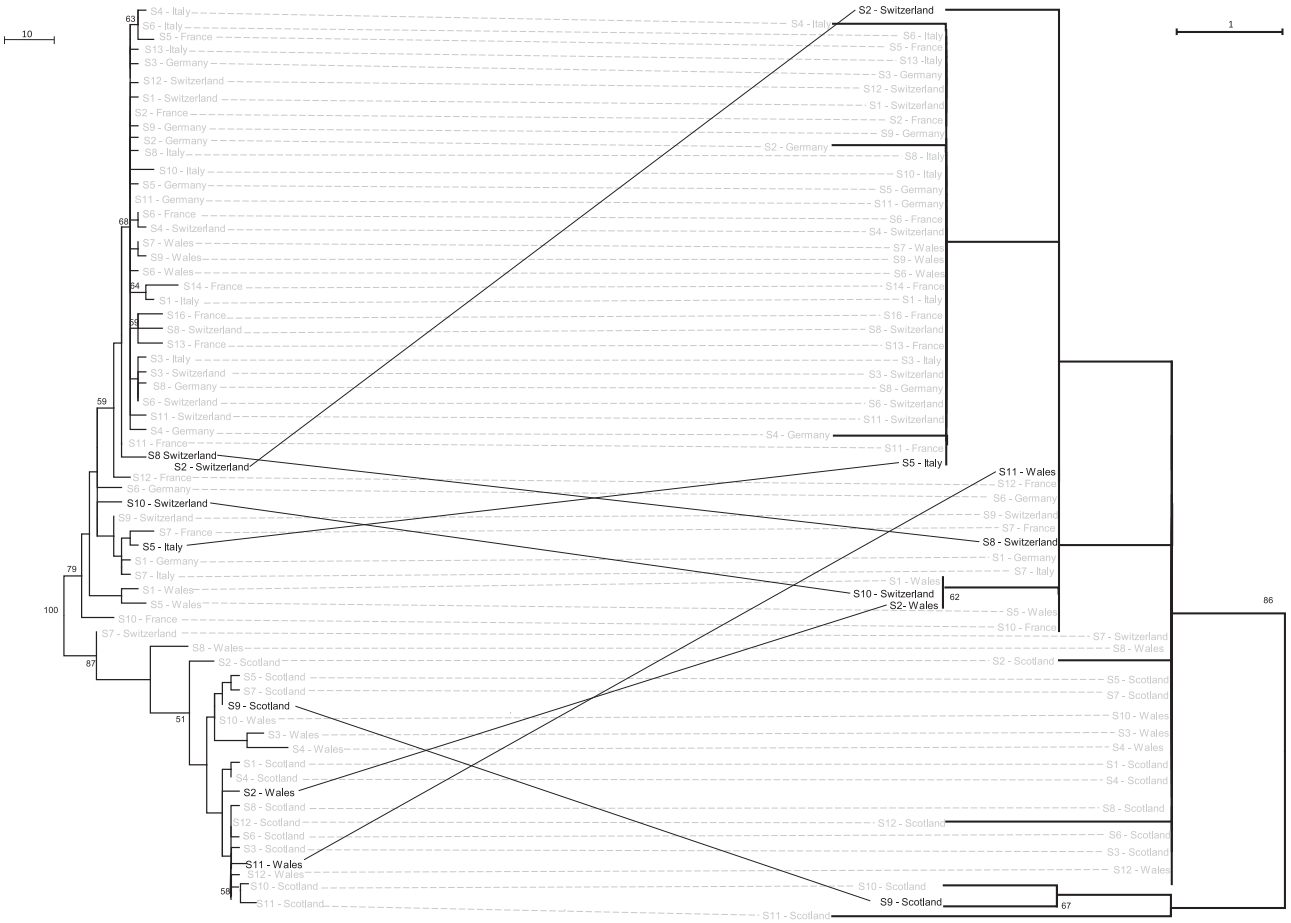

Fig. 5. Comparison of the phylogenetic reconstructions of 64 *I. ricinus* tick samples (left) and their *M. mitochondrii* endosymbionts (right). Dashed lines join endosymbionts and tick hosts on the respective trees. Scale bar indicates number of substitutions per site.

A scenario that might resolve this discrepancy would involve a critical role for horizontal transmission in this *M. mitochondrii* sweep. Indeed, we noted phylogenetic discordance in over 10% of the tick-symbiont pairs analysed in this study. Infectious transmission of the symbiont, possibly by co-feeding, would explain the general absence of co-cladogenesis between *Midichloria* symbionts and their tick hosts across multiple genera (Cafiso et al., 2016; Epis et al., 2008). Furthermore, this is fully compatible with data indicating the presence of the bacterium in the salivary glands and transmission to the host via the blood meal (Bazzocchi et al., 2013; Mariconti et al., 2012). Recent horizontal transmission, coupled with a strong selective advantage engendered by the presence of the bacterium, could lead to a sweep of *M. mitochondrii* in *I. ricinus* populations, which would be signalled by low genetic variability of the symbiont in discordance with a high diversity of mitochondrial haplotypes.

However, it is also possible that our study has significantly underestimated the diversity in *M. mitochondrii* by focusing only on housekeeping genes that are under strong purifying selection. While Muller's ratchet leads to less efficient selection on the genomes of vertically-transmitted symbionts due to extreme population bottlenecks in each generation, there is evidence that at least in long-lived hosts such as filarial nematodes, significant haplotype diversity can occur among symbiont genomes (in this case, *Wolbachia*) within a single host (Choi et al., 2016). This probably reflects heteroplasmy caused by somatic mutations that accumulate over time. Thus, a research priority for the *I. ricinus-M. mitochondrii* system must be to understand how variation in the genome of an intramitochondrial symbiont interacts with mitochondrial heteroplasmy in different tick tissues. While technically challenging, attaining such an objective could radically overhaul our understanding of the biology of this resilient vector species.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.08.016.

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