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

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26 **Abstract**

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

46 Keywords: Symbiosis, mutualist, vector, mitochondrion, MLST, red deer.

47 **Introduction**

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

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

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

90 The role of *M. mitochondrii* in the biology of its host has not been elucidated, but multiple nonexclusive
91 hypotheses have been suggested. The increase in *M. mitochondrii* numbers in concert with feeding is
92 suggestive of a role in the metabolism of the blood meal (Sassera et al., 2008). Conversely, the loss of the
93 symbiont in populations maintained in the laboratory could be indicative of a role that is only important in
94 the wild, such as increasing survival in cold climates (Lo et al., 2006). As a precedent, the tick-borne pathogen
95 *Anaplasma phagocytophilum* has been reported to up-regulate the expression of an antifreeze protein in its
96 host, *Ixodes scapularis* (Neelakanta et al., 2010). The presence in the *M. mitochondrii* genome of a peculiar
97 proton-pumping respiratory haem-copper oxidase gene set (*cbb*$_3$) indicates that this symbiont is capable of
98 performing oxidative phosphorylation at low oxygen tensions, which could facilitate ATP production when
99 oxygen is scarce (Sassera et al., 2011); for instance, under very wet conditions when ticks may become

100 temporally submerged. Finally, the presence in the *M. mitochondrii* genome of complete metabolic pathways
101 for *de novo* B-vitamin synthesis (Sassera et al., 2011) could indicate that the symbiont provides such
102 molecules to the host, as they are absent from blood, the sole nutrient the tick consumes (Rio et al., 2016).

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

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

120 **Materials and methods**

121 **Samples for detection and quantification of *M. mitochondrii***

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

127 **Sample collection for generation of tick-symbiont MLST data**

128 *I. ricinus* questing or partially-fed adult females were collected from Powis Castle estate, Wales, as above (*n*
129 = 12); Aberdeenshire, Scotland (*n* = 12); Chizé, France (*n* = 16); Appennino tosco-emiliano, Italy (*n* = 12); and
130 the Zermatt valley, Switzerland (*n* = 12). In addition, 12 samples of *I. ricinus* genomic DNA originating from
131 Bonn, Germany, were available from an archived collection (-20˚C) used for borreliosis studies at the
132 University of Bath (Vollmer et al., 2013). Specimens were identified as *I. ricinus* using morphological criteria
133 (Hillyard, 1996). All locations sampled for the MLST analyses are displayed in Figure 1.

134 **DNA extraction**

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

139 DNA was extracted from ticks using alkaline hydrolysis as described by (Ammazzalorso et al., 2015). Briefly,
140 150 µl of 14.5 M ammonium hydroxide (Sigma-Aldrich) was added to each dissected tick, which was boiled
141 for 20 min in open tubes in a dry block heater housed in a fume cupboard. The final volume of 70 – 100 µl
142 was centrifuged for 10 min at 10,000 × *g* to remove debris. In order to increase the DNA concentration for
143 nymph samples only, 30 kDa Nanosep centrifugal devices (Pall Life Sciences) were used to reduce the volume
144 to ~20 µl. DNA concentrations were quantified by a fluorescent dye intercalation method (Quant-iT

145 PicoGreen dsDNA Assay Kit, Invitrogen) using a microplate fluorimeter (Infinite F200, Tecan) and Magellan
146 Data Analysis Software (Tecan).

147 **Quantitative PCR for *M. mitochondrii***

148 To quantify *M. mitochondrii* in tick lysates, quantitative PCR (qPCR) was applied according to a modification
149 of a published method (Sassera et al., 2008). A 146-bp fragment of the symbiont DNA gyrase subunit B gene
150 (*gyrB*) was amplified as previously described. To normalise symbiont copies between different tick stages, a
151 new qPCR assay targeting a tick single-copy nuclear gene was designed. This amplified a 77-bp fragment from
152 exon 2 of the ribosomal protein L6 gene (*rpl6*), based on the sequence from *I. scapularis* (NCBI Reference
153 Sequence: XP_002400555.1), with primers 5'-CCGGTCCAAGATTCCACA-3' (sense) and
154 5'-TGCGCTTCCTCTTCTCCTTG-3' (antisense). Standards for both assays comprised synthetic long
155 oligonucleotides representing full-length amplicons (obtained from Eurogentec for *gyrB* and Sigma-Aldrich
156 for *rpl6*).

157 The qPCR assays were performed in 20 µl reaction volumes containing final concentrations of 1× SensiMix
158 SYBR No-ROX master-mix (Bioline), 400 nM (for *gyrB*) or 200 nM (for *rpl6*) each primer, and 1 µl of tick DNA
159 (replaced with nuclease-free water in no-template control reactions). The qPCR reactions were run on a
160 CFB-3220 DNA Engine Opticon 2 System (Bio-Rad) using the published cycling conditions for *gyrB* (Sassera et
161 al., 2008) and these modifications for *rpl6*: initial denaturation at 95°C for 10 min; followed by 35 cycles of
162 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
163 from 55 - 95°C (increasing in increments of 0.5°C per cycle). Linear regression analysis based on tenfold
164 dilutions of the standards ($5 \times 10^{6} – 5 \times 10^{-1}$ copies/µl) was conducted using Opticon Monitor software version
165 3.1. All PCR assays were conducted on two dilutions of each sample, and copy number calculations were
166 performed using the dilution that lay closest to the middle of the standard curves.

167 **MLST scheme and PCR assays**

168 In order to identify candidate genes for the *M. mitochondrii* MLST scheme, 14 loci present as a single copy in
169 the symbiont genome and detected throughout the sequenced genomes of the order Rickettsiales (*gpsA,*

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

182 Conventional touchdown PCR assays were performed to amplify each gene. Reactions were performed in 25-
183 μl or 20-μl volumes containing final concentrations of 1× BioMix Red master-mix (Bioline), 1.2 μM or 1 μM
184 of each primer, and 2 μl or 1 μl of template DNA for *I. ricinus* and *M. mitochondrii*, respectively. Cycling
185 conditions for amplification of loci from *I. ricinus* are provided in supplemental table S1. Cycling conditions
186 for *M. mitochondrii* comprised initial heating at 94°C for 2 min, then 10 cycles of denaturation at 94°C for 30
187 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
188 by an amplification phase with initial heating at 94°C for 2 min, then 29 cycles of denaturation at 94°C for 30
189 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s. The final extension was performed at
190 72°C for 10 min.

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

196 **Molecular identification of Welsh and Scottish specimens**

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

202 **Sequence analysis**

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

209 For *I. ricinus* (but not *M. mitochondrii*), sequences obtained using the same MLST scheme used in the present
210 study were available from England, Scotland, Latvia, Switzerland, Germany and Portugal (Figure 1). The
211 sequences from England, Scotland and Latvia were used in a previous MLST study of *I. ricinus* population
212 structure (Dinnis et al., 2014), whereas the unpublished sequences from other locations were obtained
213 during an epidemiological analysis of Lyme borreliosis (Vollmer et al., 2013).

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

221 Minimum-spanning distance trees were drawn using PHYLOViZ (Francisco et al., 2012). Pie-charts were
222 produced in Microsoft Excel and manually transposed onto the minimum-spanning tree to indicate the origin
223 of each sample in each node. Full DNA alignments were screened for recombination using SplitsTrees4
224 (Huson and Bryant, 2006), as well as for positive and negative selection using GARD and SLAC via the
225 Datamonkey web server (Pond and Frost, 2005). In addition, the possibility that nuclear mitochondrial
226 pseudogenes (*numts*) had been amplified by the tick MLST scheme was considered by inspection of
227 translated sequences for indels or in-frame stop codons (Song et al., 2008).

228 **Statistical analysis of *Midichloria* density**

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

235 **Ethics statement**

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

245 **Results**

246 ***M. mitochondrii* prevalence and density between lifecycle stages and locations**

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

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

259 **Population structure of *I. ricinus***

260 Sequences were obtained for all six loci for 64 tick specimens. Comparison of the sequence data revealed
261 differing levels of variability at all loci. The average diversity between loci of *I. ricinus* was 11%, with a range
262 from 9.8% in *coi* and 12S to 11.9% for *coiii* (Table 3). At the amino-acid level, the average diversity between
263 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
264 study ranged from 16 (*coii*) to 43 (*cytb*) (Table 3). Sixty-three STs were identified based on the MLST allelic
265 profiles, of which none was previously reported. Of these STs, only one was observed more than once, with
266 the remainder classified as singletons.

267 These sequences were concatenated and phylogenetic analysis by maximum likelihood revealed a distinct
268 clade composed almost entirely of Scottish and Welsh sequences, in contrast with little evidence of structure
269 for continental Europe (Italy, France, Germany and Switzerland; Supplemental Figure S1). However, whereas

270 none of the Scottish sequences clustered with those from continental Europe, the Welsh samples were split
271 approximately equally between the Scottish and continental clades (Supplemental Figure S1). Phylogenetic
272 comparison of the concatenated loci produced here with those from a previous study (Dinnis et al., 2014)
273 revealed a generally low level of diversity across the 65 sequences (Supplemental Figure S2), with 63 unique
274 STs. Incorporation of the previously published sequences reinforced the picture of two major clades, with
275 the Scottish samples from both studies clustering together to the exclusion of almost all continental
276 sequences, except for some representatives from Latvia (Supplemental Figure S2). Other samples from the
277 UK displayed segregation in similar numbers between the "Scottish" and "continental" clades.

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

287 We considered two potential artefacts for the distinctiveness of the Scottish clade: misidentification of *I.*
288 *inopinatus* as *I. ricinus* (Chitimia-Dobler et al., 2018; Estrada-Pena et al., 2014), or confounding of the
289 mitochondrial MLST scheme by *numts* (Song et al., 2008). Amplification of mitochondrial 16S rRNA sequences
290 from 12 British tick specimens distributed between England, Wales and Scotland and comparison with *I.*
291 *inopinatus* 16S rRNA indicated that they are distinct from one another (Supplemental Figure S3), with a
292 nucleotide divergence of >2.1%. Moreover, translation and alignment of tick MLST sequences showed no
293 evidence for the indels or in-frame stop codons that are the hallmarks of *numts* (data not shown).

294 **Population structure of *M. mitochondrii***

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

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

311 **Co-evolution of *I. ricinus* and *M. mitochondrii***

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

319 **Discussion**

320 The population structure of *I. ricinus,* assessed now in at least seven published studies (including the current
321 work), has proved difficult to resolve. An initial small-scale study (26 ticks in total) across continental Europe
322 (Switzerland, Italy, Austria, Denmark, Sweden and Finland) using mitochondrial markers failed to find an
323 association between haplotype and geographic origin (Casati et al., 2008). A somewhat larger analysis (60
324 specimens) of both mitochondrial and nuclear markers at various scales across Europe (both on the continent
325 and in the British Isles), North Africa, and Western Asia confirmed this apparent panmixia, with the exception
326 of clearly differentiated populations in North Africa (Noureddine et al., 2011). However, these North African
327 specimens are now suspected to be from a different tick species, *I. inopinatus* (Estrada-Pena et al., 2014). In
328 contrast, analysis of microsatellite markers from ~600 ticks has provided evidence for distinct "races" of *I.*
329 *ricinus* collected from different host species in France, Belgium and Slovakia; for instance, between roe deer
330 and wild boar (Kempf et al., 2011). More recent studies using several hundred ticks have resolved clearly
331 differentiated clades between Britain and Latvia [(Dinnis et al., 2014); data also used in the present study],
332 and between western Norway and Britain *versus* more centrally located European populations (Roed et al.,
333 2016). Finally, a whole mitochondrial genome analysis of two *I. ricinus* populations in northern Italy,
334 separated by only 100 km, revealed four highly divergent lineages but no geographical structuring (Carpi et
335 al., 2016).

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

345 of birds into the UK is accompanied by >1 million immature ticks (Pietzsch et al., 2008); thus potentially
346 contributing ~10,000 adult ticks from overseas that were transported by birds as larvae or nymphs. Even
347 accounting for very high rates of attrition from the larval stage, this is likely to dwarf any contribution from
348 nymphs and adults attached to travelling pets.

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

364 Scotland has a number of biogeographical features that set it apart from the rest of the British Isles. The
365 border region between England and Scotland, the Southern Uplands, is a hilly landscape and further north,
366 the Grampian Mountains are a nontrivial barrier between the Central Lowlands and the Northwest Highlands.
367 Nevertheless, these ranges of hills and mountains do not reach the lateral extent or altitude of the Alps and
368 other mountain ranges in continental Europe where gene flow between *I. ricinus* populations appears to
369 occur unhindered. Thus, the prehistory of the region may be more important than its current topology, as
370 the locations where the Scottish ticks were sampled (Aberdeenshire and Inverness-shire) were covered by

371 ice-sheets as recently as 15,000 years ago - some 10,000 years after the British-Irish Ice Sheet began to retreat
372 at its southerly margins - and may have experienced re-advances even beyond this date (Clark et al., 2012).
373 The colonisation of post-glacial Scotland by *I. ricinus* is likely to have been slow and erratic, perhaps leading
374 to strong founder effects at this north-western extent of the species' range. Phylogeographic studies of red
375 deer (*Cervus elaphus*) suggest that populations in Western Europe, including the British Isles, derive from a
376 refugium located on the Iberian Peninsula, with little evidence that artificial introductions by humans have
377 impacted significantly on natural migrations (Skog et al., 2009). Even today, when populations of both native
378 and introduced deer species are at a record high for recent times across Britain (Putman et al., 2011),
379 densities of red deer in the Scottish Highlands greatly exceed those in most other parts of the country
380 (Edwards, 2013). It is plausible that migrations of red deer brought *I. ricinus* to Scotland after the glacial
381 retreats, and differences in the most abundant larger hosts between Scotland and other parts of Europe may
382 have acted to select a "race" of *I. ricinus* that is better adapted for feeding on *C. elaphus* (Kempf et al., 2011).
383 However, physical factors such as the longer winter at higher latitudes increasing attrition rates when ticks
384 are dormant, as well as the reduced duration of the questing season, may have also played a role in selecting
385 for certain tick and symbiont genotype combinations.

386 An alternative scenario that might explain a strong founder effect in Scottish tick populations is stochastic
387 introductions from bird migrations. Most records of *I. ricinus* on birds in the UK come from passerines such
388 as the blackbird (*Turdus merula*), the willow warbler (*Phylloscopus trochilus*), the whitethroat (*Sylvia*
389 *communis*), the song thrush (*Turdus philomelos*), and the dunnock (*Prunella modularis*) (James et al., 2011;
390 Pietzsch et al., 2005); and a link between tick infestation and a tendency to forage on the ground was
391 reported from one bird study in Scotland (James et al., 2011). While these common songbirds differ markedly
392 in their migratory habits, none has a particular predilection for Scotland. Nevertheless, during the early post-
393 glacial period, ticks introduced by migratory birds into Scotland may have had greater impacts on local
394 populations than in other parts of the British Isles, perhaps supplanting existing clades through drift. In
395 addition, some British avian *I. ricinus* records are from rare migrants that have almost never been observed
396 outside Scotland, such as the Pechora pipit (*Anthus gustavi*) (Pietzsch et al., 2005); although as most sightings
397 are from Shetland, it is very unlikely that such rarities have impacted on the mainland tick population.

398 Perhaps surprisingly, *I. ricinus* has also been recorded on Scotland's iconic raptor, the golden eagle (*Aquila*
399 *chrysaetos*) (Pietzsch et al., 2005). It is interesting to speculate whether the tick-infested, preferred
400 mammalian prey of golden eagles (rabbits, hares, sheep and deer), when carried large distances to nesting
401 sites, might on rare occasions lead to the transport of viable ticks >100 km from their site of origin. Indeed,
402 golden eagles breeding in Scotland have been frequently spotted hunting over the skies of Ireland (Watson,
403 2002).

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

420 A scenario that might resolve this discrepancy would involve a critical role for horizontal transmission in this
421 *M. mitochondrii* sweep. Indeed, we noted phylogenetic discordance in over 10% of the tick-symbiont pairs
422 analysed in this study. Infectious transmission of the symbiont, possibly by co-feeding, would explain the
423 general absence of co-cladogenesis between *Midichloria* symbionts and their tick hosts across multiple

424 genera (Cafiso et al., 2016; Epis et al., 2008). Furthermore, this is fully compatible with data indicating the
425 presence of the bacterium in the salivary glands and transmission to the host via the blood meal (Bazzocchi
426 et al., 2013; Mariconti et al., 2012). Recent horizontal transmission, coupled with a strong selective advantage
427 engendered by the presence of the bacterium, could lead to a sweep of *M. mitochondrii* in *I. ricinus*
428 populations, which would be signalled by low genetic variability of the symbiont in discordance with a high
429 diversity of mitochondrial haplotypes.

430 However, it is also possible that our study has significantly underestimated the diversity in *M. mitochondrii*
431 by focusing only on housekeeping genes that are under strong purifying selection. While Muller's ratchet
432 leads to less efficient selection on the genomes of vertically-transmitted symbionts due to extreme
433 population bottlenecks in each generation, there is evidence that at least in long-lived hosts such as filarial
434 nematodes, significant haplotype diversity can occur among symbiont genomes (in this case, *Wolbachia*)
435 within a single host (Choi et al., 2017). This probably reflects heteroplasmy caused by somatic mutations that
436 accumulate over time. Thus, a research priority for the *I. ricinus*-*M. mitochondrii* system must be to
437 understand how variation in the genome of an intramitochondrial symbiont interacts with mitochondrial
438 heteroplasmy in different tick tissues. While technically challenging, attaining such an objective could
439 radically overhaul our understanding of the biology of this resilient vector species.

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448 Scottish Government.

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

<table>
<tr><th>Stage</th><th>No. of ticks assayed</th><th>Field site</th><th>County</th><th>Country</th></tr>
<tr><td>Engorged female</td><td>20</td><td rowspan="5">Powis Castle</td><td rowspan="5">Powys</td><td rowspan="5">Wales</td></tr>
<tr><td>Semi-engorged female</td><td>25</td></tr>
<tr><td>Unengorged female</td><td>14</td></tr>
<tr><td>Male</td><td>48</td></tr>
<tr><td rowspan="6">Nymph</td><td>44</td></tr>
<tr><td>100</td><td>Salisbury</td><td>Wiltshire</td><td rowspan="5">England</td></tr>
<tr><td>100</td><td>Bentley Wood</td><td>Wiltshire</td></tr>
<tr><td>100</td><td>New Forest</td><td>Hampshire/Wiltshire</td></tr>
<tr><td>100</td><td>Exmoor</td><td>Somerset/Devon</td></tr>
<tr><td>100</td><td>Dartmoor</td><td>Devon</td></tr>
</table>

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).

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

Figure legends

Figure 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 an epidemiological study on Lyme borreliosis (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).

Figure 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).

Figure 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.

Figure 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).

Figure 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.

Supplemental figure S1. Maximum-likelihood phylogenetic tree based on concatenated mitochondrial genes from 64 *I. ricinus* ticks. The corresponding mitochondrial DNA sequences of *I. persulcatus* obtained from NCBI (accession no. KU935457) were used to root the tree. There was a total of 2,410 nucleotide positions in the final dataset.

Supplemental figure S2: A circularised maximum likelihood tree for *I. ricinus* sequences across Europe. The tree was drawn using the general time reversible model with 10,000 bootstrap iterations for the tick sequences generated as part of this study, compared to each unique tick sequence from a previous study (Dinnis et al., 2014). Sequence names were replaced with colour-coded circles corresponding to the country or countries where the tick sequences were obtained.

Supplemental figure S3. Comparison of mitochondrial 16S rRNA gene sequences from British tick specimens, *Ixodes ricinus* and *Ixodes inopinatus*. Maximum likelihood tree with 10,000 bootstraps. Sequences for British specimens were newly obtained for this study.

Supplemental figure S4. Maximum-likelihood phylogenetic tree based on concatenated *M. mitochondrii* genes from 64 *I. ricinus* ticks. There was a total of 1,853 nucleotide positions in the final dataset.

Supplemental table S1: PCR cycling conditions for amplification of loci from *I. ricinus*.

Supplemental table S2: Complete list of *I. ricinus* sequence types.

Supplemental table S3: Complete list of *M. mitochondrii* sequence types.

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