Host dispersal shapes the population structure of a tick-borne bacterial pathogen

Running title: Population structure of a tick-borne pathogen

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1 **Abstract**

2 **Keywords:** birds; ticks; host-parasite interactions; migration; *Borrelia garinii;* Lyme
3 borreliosis

4

1. INTRODUCTION

Wild birds are relevant for public health because of their role in the spread of emerging zoonotic pathogens that cause newly recognised diseases or diseases which are rapidly increasing in incidence or geographical range (Reed, Meece, Henkel, & Shukla, 2003). Some birds act as reservoirs of pathogens such as *Borrelia burgdorferi* sensu lato (s.l.), enterobacteria, flavivirus and influenza A virus, being significantly involved in the direct infection of humans or arthropod vectors that transmit the disease agents to humans (Thomas, Hunter, & Atkinson, 2007). Wild birds, especially migratory species, may also carry the arthropod vectors (e.g. ticks) to different geographic areas creating new foci of disease (Reed et al., 2003). Studies that monitored tick infestation of birds during migration estimated that birds are responsible for the transport of 6.8 - 175 million ticks each spring between wintering and breeding areas (Ogden et al., 2008; Olsen, Jaenson, & Bergstrom, 1995), which may greatly impact the distribution and population structure of ticks and their associated pathogens. The life cycle of tick-borne pathogens is complex and their evolutionary ecology is shaped by the interactions with vertebrate hosts and tick vectors (Kurtenbach et al., 2006). This study focused on the ecology and genetic diversity of *Borrelia burgdorferi* s.l. as a model to investigate the drivers of the population structure and to understand the role of host-associated dispersal on the evolution of tick-borne pathogens. This represents a consequential question in the ecology and evolution of any pathogen.

Borrelia burgdorferi s.l. is a bacterial complex of over 20 known genospecies, including the etiologic agents of Lyme borreliosis (Casjens et al., 2011; Margos et al., 2015), whose main vectors are ticks of the genus *Ixodes* (Eisen & Lane, 2002). These bacteria are widespread in Europe, Asia and North America and are also present in North Africa (Margos,

Vollmer, Ogden, & Fish, 2011; Zhioua et al., 1999). Different *Borrelia* genospecies have different patterns of association with vertebrate reservoir hosts (Humair & Gern, 2000; Kurtenbach, Peacey, et al., 1998) because of the immunological host response, mediated by the action of the host's complement system (Kurtenbach et al., 2002). While *B. burgdorferi* sensu stricto (s.s.) is a generalist genospecies, *B. afzelii* is mostly associated with mammalian hosts such as rodents, whereas *B. valaisiana*, *B. garinii* and *B. turdi* are mostly associated with birds (Heylen, 2016; Margos et al., 2011). Because tick vectors cannot move large distances independent of hosts, it has been suggested that host specialization determines the spread and dispersal of *B. burgdorferi* s.l. genospecies (Kurtenbach et al., 2010; Sonenshine & Mather, 1994). Because birds are both important hosts for some *Borrelia* genospecies and for various species of vector ticks, they act as a driving force shaping *B. burgdorferi* s.l. distribution and phylogeographical patterns (Margos et al., 2011; Vollmer et al., 2011).

Here, we assessed the role of passerine birds as hosts and dispersers of *B. burgdorferi* s.l. We tested the hypothesis that infection prevalence with *Borrelia* genospecies would differ among bird species due to differences in their ecological niche occupancy and reservoir competence for *B. burgdorferi* s.l. We also evaluated whether the avian-associated and human pathogenic genospecies *B. garinii* would show lack of geographical structuring, because of the large distance range of movements of its avian hosts and potential for dispersal and consequent spatial mixing of strains. To achieve this, we collected ticks feeding on common passerine species with different ranges of migratory movements in a coordinated study covering a broad geographical area in Europe (11 countries), and assessed the infection status of these ticks with *B. burgdorferi* s.l. The diversity of the common avian-associated *B. garinii* genospecies and potential phylogeographical patterns were determined using a multilocus sequence typing scheme (MLST) of eight house-keeping genes (Margos et al., 2008).

Analyses based on these conserved genetic markers have been previously used to estimate and describe the degree of population genetic structure over the largest geographical range studied so far, and have the potential to reveal signatures of demographic processes, dispersal and migration (Hoen et al., 2009; Margos et al., 2012; S. Vollmer et al., 2013).

2. METHODS

2.1 Birds and ticks

Birds were captured in collaboration with ornithologists and ringers in 11 European countries (Czech Republic, Estonia, Finland, Germany, Greece, Hungary, Netherlands, Portugal, Slovenia, Spain and Sweden) covering an area from 8°23'W to 24°57'E and from 40°35'N to 62°14'N. Capturing effort directed to tick collection was concentrated during the year of 2015, but additional data (including data from the years 2005-2008, 2013-2014 and 2016) was included for 5 of the 21 study sites (Sup. Mat 1). These collection sites correspond to ringing stations or sites where bird populations of particular species have been studied in a long-term perspective and with which we could establish a collaborative protocol. Therefore, some European areas are missing from this study due to sampling limitations. Birds were captured using mist-nets or, when breeding in nest boxes: incubating females were captured by hand (in Estonia, in Gotland, Sweden and in Harjavalta, Finland), and both parents were caught when feeding the nestlings using spring or wire traps. Nestlings were sampled in the nest between 8 and 15 days of age. Countries, collection sites, range of capturing dates and bird species sampled are detailed in Fig.1 and Sup. Mat. 1. Although classification of collection

sites according to country has no biological meaning, for convenience, and because countries are related to geographical positioning, we refer to country of collection when reporting some results for an easy identification of sample origin, for comparability with previous localised studies in different countries, and for an integration with sequence data available in public databases. Birds were carefully inspected for attached ticks with special attention to the head (around the eyes, beak, ears, chin and crown) and neck, where ticks are most often attached. We removed infesting ticks with fine forceps and collected them into tubes containing 70 - 99% ethanol according to each individual host. Because we were interested in *B. burgdorferi* s.l. infection prevalence in ticks feeding on birds, our analyses use ticks as sampling units. Therefore, we did not collect data on non-infested birds nor on tick infestation intensity. Our statistical analyses take into account sampling site, bird life cycle stage and month, to account for differences in sampling effort and uneven sample distribution across sites and time of year. The ticks were identified morphologically using identification keys (Estrada-Peña, Bouattour, Camicas, & Walker, 2004; Estrada-Peña, Nava, & Petney, 2014; Pérez-Eid, 2007).

2.2 Molecular analysis

We extracted tick DNA in a subset of *Ixodes* spp. ticks (n = 656 ticks; mean ticks ± SE = 65.6 ± 11.5 per country, 38.6 ± 14.28 per bird species, 1.54 ± 0.03 per bird), using a column DNA extraction kit (DNeasy, Qiagen, Hilden, Germany). Tick (nymph and adult) exoskeleton was broken by piercing followed by incubation with proteinase K, for 24h. We tested a sub-sample of these *Ixodes* spp. (n = 58, mean ticks ± SE = 5.8 ± 0.79 randomly selected per country) using a conventional PCR targeting the mitochondrial 16S rRNA gene of ticks using the primers described by (Mangold, Bargues, & Mas-Coma, 1998) and an annealing

temperature of 56ºC, to confirm morphological identification of ticks by BLASTn search (https://blast.ncbi.nlm.nih.gov/). For specimens (n = 4) for which the % of identity in BLASTn search was less than 98% we built a Maximum Likelihood phylogenetic tree, together with reference sequences (Chitimia-Dobler et al., 2018; Estrada-Peña et al., 2014) retrieved from Genbank, and confirmed the species identification with the obtained clustering patterns. We assessed infection of ticks by a nested PCR targeting the *flaB* gene of *B. burgdorferi* s.l. using the primers described in (Johnson, Happ, Mayer, & Piesman, 1992), with an annealing temperature of 52ºC. We used the Invitrogen PCR Reagent System mix (Life Technologies, Waltham, USA), according to manufacturer's instructions, and a positive and a negative control were used in all PCR runs. *Borrelia* genospecies were identified by sequencing. The procedures listed above were performed at the Portuguese National Institute of Health Doutor Ricardo Jorge, Portugal.

A sub-sample of *B. garinii*-positive specimens were tested using MLST targeting eight housekeeping genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, *uvrA*) according to Margos et al. (2008) at the German National Reference Centre for *Borrelia*, Bavarian Health and Food Safety Authority, Germany. Those isolates were selected from common bird species (*Turdus* spp. and hole-nesting birds) that covered the geographical range in which *B. garinii* was detected in our study. All samples were analysed following the same protocol (see https://pubmlst.org/borrelia/sequencing for protocol and primer information). We compared the obtained sequences with those available in the *Borrelia* MLST database (https://pubmlst.org/borrelia/) located at the University of Oxford, to obtain allele and sequence type (ST) numbers for each isolate (Jolley & Maiden, 2010). Novel alleles or STs were given novel numbers. Samples that contained mixed infections were discarded from

123 further MLST/MLSA analyses because allele numbers and sequence types could not be
124 identified for these samples. We submitted all data to the *Borrelia* MLST database.

125 A novel *Borrelia* genospecies was detected and characterised by PCR amplification of
126 the 5S-23S rRNA intergenic spacer (Rijpkema, Molkenboer, Schouls, Jongejan, &
127 Schellekens, 1995), and subsequent sequencing of PCR amplicons of the 16S rRNA locus
128 (Radulović, Milutinovic, Tomanovic, & Mulenga, 2010) and of the *clpX* gene (Margos et al.,
129 2008).

130

131 **2.3 Statistical analyses**

132

133 Factors affecting the prevalence of *B. burgdorferi* s.l. in ticks from birds (each tick feeding on
134 a bird was used as a sampling unit) were tested using a Generalized Linear Mixed Model
135 (GLMM) with a binomial error distribution (logit function). The full model included bird
136 species (10 levels), latitude, longitude, tick stage (larvae or other), tick species (4 levels), bird
137 life cycle stage (breeding or non-breeding) and month (12 levels) as fixed effects. Bird
138 identity (bird_ID, to control for ticks tested from the same individual), nested within
139 collection site, was included as a random effect. We ran a set of models with different fixed
140 effects structures and used the Akaike Information Criterion (AIC) to select the best model
141 (best model selection table is presented in Sup. Mat 2). After ranking the models using AIC,
142 we used those with a ΔAIC <2 with respect to the top model to calculate conditional model-
143 averaged parameter estimates. Significance level was defined at $P = 0.05$.

144 In these models, only bird species for which we had information on *Borrelia* infection
145 for at least eight ticks (from different individuals) were used. Tick stage was considered in

146 statistical analyses and was divided into larvae and other tick stages because nymphs
147 represented the majority whilst adults were too infrequently found to justify their own group
148 for statistical analysis. This procedure was adopted because transovarial transmission (i.e. the
149 acquisition of *Borrelia* by larvae via vertical transmission from the parent) is considered to
150 be very low in *Ixodes* spp. (Eisen & Lane, 2002). For this reason, an infection of a larva is
151 highly unlikely if they were collected from an uninfected or incompetent reservoir bird host.
152 On the other hand, nymphs/adults may have acquired an infection during a previous blood
153 meal on an infected host. In such case *Borrelia* DNA may be detected in the tick independent
154 of the bird hosts they were collected from. Tick species was also included in the models
155 because within the genus *Ixodes*, different species differ in their vector competence for *B.*
156 *burgdorferi* s.l. (Eisen & Lane, 2002; Heylen, Krawczyk, et al., 2017; Heylen, Sprong, et al.,
157 2014). We controlled for the effects of timing of tick collection throughout the year including
158 month of collection as explanatory variable and also by grouping those ticks collected during
159 the birds' breeding season (April – July) and those collected outside the breeding season
160 (August – March), because breeding is one of the most stressful periods in the birds' life cycle
161 and the stress associated with breeding duties may suppress their immune system potentially
162 leading to spirochetemia. Therefore, this could affect the probability of infection of the ticks
163 feeding on the birds. We used the same statistical approach to test factors affecting prevalence
164 of infection by the most prevalent *Borrelia* genospecies in our study (*B. garinii*). The models
165 were run in R (R core team, 2013) using the packages lme4, lmerTest, bbmle, MuMin and
166 arm (Bates, Mächler, Bolker, & Walker, 2015; Kuznetsova, Brockhoff, & Christensen, 2017).

167

168 **2.4. Multilocus Sequence Typing and Multilocus Sequence Analysis (MLST/MLSA)**

169

170 In order to study the population structure and the phylogenetic relationships of *B. garinii* in a
171 global context, we selected a subset of 82 *B. garinii*- positive samples, as to include isolates
172 from all European countries sampled in this study and represent all bird species sampled with
173 *B. garinii*- infected ticks (n ≥ 5 infected ticks), and tested those by MLST.

174 Complete allelic profiles of *B. garinii*- positive specimens obtained in our study were
175 analysed with goeBURST analysis using Phyloviz (Francisco, Bugalho, Ramirez, & Carriço,
176 2009) together with other *B. garinii* genotypes detected worldwide (identification of the
177 isolates used for MLST/MLSA is given in Sup. Mat. 3; we included each ST only once for
178 each country). Relationships among STs were evaluated through triple locus variants (TLV),
179 and founder clonal complexes were identified to infer patterns of descent.

180 We estimated nucleotide diversity (π; Nei, 1987) and Tajima D (Tajima, 1989) for
181 each gene using R packages pegas v. 0.9 (Paradis, 2010) and ape v. 3.5 (Paradis, Claude, &
182 Strimmer, 2004) on each continent for a sample set including 304 isolate sequences (198 from
183 Europe, 85 from Asia and 21 from North America), and on each country (n = 11) for which
184 more than five isolates were available (see Sup. Mat. 3 for identification of the isolates
185 included in this analysis). The sequences for gene *clpX* were realigned using MAFFT v7.205
186 (Katoh & Standley, 2013) as there was a deletion of three bases in some isolates.
187 An ancestry recombination graph for the 85 STs present in Europe was reconstructed with
188 BEAST2 software v. 2.5 (Bouckaert et al., 2019) and package bacter v. 2.2 (Didelot, Lawson,
189 Darling, & Falush, 2010; Vaughan et al., 2017) using sequences of the eight housekeeping
190 genes. BEAST2 was run three times with a unique tree and substitution model for the eight
191 loci but with a lognormal-relaxed clock model for each locus. We used the following priors:

HKY substitution model (Hasegawa, Kishino, & Yano, 1985), Gamma site heterogeneity model with four gamma categories, Tree prior: Coalescent Constant Populations. Due to the high number of STs and the complex model including recombination, the chain was slow to converge (as was shown by Tracer v. 1.6 (Rambaut, Suchard, Xie & Drummond, 2014) and we thus extended the original 10M states to 17M for one run and 19M for the other two. A consensus tree was reconstructed with the tool ACGAnnotator present in the bacter package after removing 40% to 70% burn-in depending on the run.

3. RESULTS

3.1 Ticks collected from birds

In total, 2,308 ticks were collected from 843 infested birds, belonging to 28 bird species (Sup. Mat. 1). Ticks collected from these birds belonged to three genera: *Haemaphysalis* (n = 3), *Hyalomma* (n = 48) and *Ixodes* (n = 2,255). Two ticks could not be identified to genus by morphological criteria as they were damaged. We identified four species of *Ixodes*: *I. ricinus* (n = 1,779), *I. arboricola* (n = 214), *I. frontalis* (n = 164), and *I. ventalloi* (n = 24) but 74 *Ixodes* ticks could not be identified to species because they lacked critical body structures needed for morphological identification (Sup. Mat. 4). The vast majority of collected ticks were immatures (2,175 out of 2,255 *Ixodes* spp.), and from these, 63% were nymphs. Adults belonged to *I. arboricola* (n = 63), *I. frontalis* (n = 11), *I. ricinus* (n = 1) and *I. ventalloi* (n = 4). Amplification and sequencing of the ribosomal 16S rRNA gene of ticks (Mangold, Bargues, & Mas-Coma, 1998) confirmed tick morphological identification in 84% of the cases, corresponding to a misidentification rate of 16%.

The blackbird *Turdus merula*, the song thrush *T. philomelos*, the redwing *T. iliacus*, the great tit *Parus major*, the collared-flycatcher *Ficedula albicollis*, and the Eurasian jay *Garrulus glandarius* presented co-infestations by ticks of different species (Sup. Mat. 4).

3.2 Prevalence of *Borrelia burgdorferi* s.l. in ticks collected from birds

Out of 656 *Ixodes* ticks collected from birds and analysed for *B. burgdorferi* s.l. infection, 244 (37.2%) were positive. Of these, 22 were larvae (prevalence of *B. burgdorferi* s.l. in larvae = 20%, 22/110), and 214 were nymphs (prevalence of *B. burgdorferi* s.l. in nymphs = 41%, 214/521). *Ixodes ricinus* was the most infected tick species (40.2%, 210/522), followed by *I. ventalloi* (31.3%, 5/16), *I. arboricola* (29.7%, 14/47) and *I. frontalis* (20.5%, 9/44).

Borrelia burgdorferi s.l. prevalence differed significantly between ticks collected from different bird species and was affected by latitude ($\chi_{10,616} = 90.10$, $P < 0.0001$; Fig. 2, Fig. 3). Longitude, tick stage, tick species, month and the birds' life cycle stage did not affect *B. burgdorferi* s.l. prevalence. The model selection table is presented in Sup. Mat. 2 and the conditional model averaged coefficients parameters obtained from the generalized linear mixed models (GLMMs) that best explained the prevalence of *B. burgdorferi* s.l. in ticks collected from birds are presented in Table 1. In comparison with the reference species (the blue tit *Cyanistes caeruleus*), ticks collected from *T. merula* (estimate ± SE = 2.50 ± 0.91, z = 2.76, P = 0.006) and *Turdus pilaris* (estimate ± SE = 4.29 ± 1.51, z = 2.84, P = 0.005) showed higher infection rates, whereas those collected from the robin *E. rubecula* had the lowest infection rates (estimate ± SE = -2.41 ± 1.25, z = -1.93, P = 0.054). *B. burgdorferi* s.l. prevalence increased with latitude (estimate ± SE = 0.08 ± 0.03, z = 2.28, P = 0.022). The

239 fieldfare *T. pilaris* was the bird species that carried ticks with the highest *Borrelia* prevalence
240 (92%), followed by the blackbird *T. merula* (58%). Only two out of 53 (3.8%) ticks feeding
241 on the robin *E. rubecula* were positive for *B. burgdorferi* s.l. (Fig. 2).

242 The genospecies of 193 positive samples was identified by sequencing the *flaB* gene.
243 The most prevalent genospecies was *B. garinii* (60.7%, 116/191), followed by *B. valaisiana*
244 (23.6%, 45/191), *B. afzelii* (9.4%, 18/191) and *B. turdi* (5.2%, 10/191). *B. lusitaniae* (0.5%,
245 1/191) and a novel genospecies (0.5%, 1/191) were also detected (Fig. 2 and 3; Sup. Mat. 5).

246 The most abundant genospecies associated with *T. pilaris*, *T. philomelos*, *P. major* and
247 *F. albicollis* was *B. garinii* with a prevalence varying between 100% (13/13) in *T. pilaris* and
248 53% (8/15) in *T. philomelos* (Fig. 2), whereas *B. valaisiana* was the most prevalent
249 genospecies detected in ticks collected from *T. merula* (50%, 30/60; Fig. 2). The model
250 explaining the variation in *B. garinii* prevalence, the most common genospecies detected in
251 ticks feeding on birds, was identical to the one explaining *B. burgdorferi* s.l. prevalence, with
252 the exception that the prevalence of *B. garinii* in ticks feeding on *T. merula* and *E. rubecula*
253 was not significantly different from the reference bird species. Under the assumption that
254 there was no co-feeding transmission (i.e. when larvae acquire the infection due to feeding in
255 close proximity to other infected tick stages; Randolph, Gern, & Nuttall, 1996), data on larval
256 infection suggested that *F. albicollis* and *T. merula* may act as reservoirs for *B. garinii* and *B.*
257 *valaisiana*, *T. iliacus* for *B. garinii*, *B. valaisiana* and *B. turdi*, *P. major* for *B. garinii*, and the
258 willow warbler *P. trochilus* and *E. rubecula* for *B. garinii* (Fig. 2; Sup. Mat. 5).

259 *Borrelia afzelii* DNA was detected only in nymphs, mostly feeding on *P. major*, and
260 to a lesser extent on other bird species (*C. caeruleus*, *F. albicollis*, *T. iliacus* and *T. merula*).
261 *Borrelia lusitaniae* DNA was detected in one *I. ricinus* nymph feeding on *P. major*. *Borrelia*

turdi DNA was detected in all stages of two tick species, *I. frontalis* (66.7%; 6 out of 9 positive ticks), and *I. ventalloi* (80%; 4 out of 5 positive ticks), feeding on *T. iliacus*, *T. merula*, *T. philomelos* and *P. major*.

DNA of the new *Borrelia* genospecies (*Candidatus* Borrelia aligera) was detected in an *I. ventalloi* nymph feeding on a *T. iliacus* in Portugal. Its *flaB* sequence was 100% identical to a *flaB* sequence previously detected in one *I. ricinus* nymph feeding on a Sardinian warbler *Sylvia melanocephala* in Portugal (isolate T794A; accession number KT207789; Norte et al., 2015). The PCR targeting the 5S-23S rRNA intergenic spacer was positive showing that this genospecies belongs to the *B. burgdorferi* s.l. group. Its 16S rRNA sequence showed only 97% similarity to several *B. burgdorferi* genospecies, including *B. bissettiae* and *B. mayonii*. The sequence of the housekeeping gene *clpX* showed 36 nucleotide differences from all previously detected alleles available at the MLST database (http://pubmlst.org/borrelia). Detailed information on specimens from which different *Borrelia* genospecies were detected in this study is presented in Sup. Mat. 5.

3.3 Multilocus Sequence Typing / Multilocus Sequence Analysis (MLST/MLSA)

Twenty-nine complete allelic profiles with sequences for all eight genes were obtained from a subset of 82 *B. garinii*- positive samples selected as to include isolates from all European countries sampled in this study. Some samples (n = 25) were excluded because they represented *B. garinii* mixed infections, and, therefore, allelic profiles could not be determined. These complete 29 profiles represented all countries from which *B. garinii* was detected in ticks from birds in this study, apart from Greece, for which we did not obtain any

286 complete profiles. Comparison of alleles from an incomplete ST (i.e. not obtaining sequences
287 for all alleles) from a tick feeding on a bird in Greece showed that they were identical to
288 samples previously reported from the UK.

289 The 29 *B. garinii* allelic profiles were resolved into 20 STs, nine of which were new.
290 These sequence data were supplemented with sequences of *B. garinii* isolates available at the
291 MLST database (see Sup. Mat. 3 for identification of isolates included in this analysis) and
292 used for goeBURST (n = 172; Fig.4) and phylogenetic analyses (n = 110; Fig. 5).

293 At a global scale, out of the 201 *B. garinii* isolates (137 STs) analysed (downloaded
294 from the MLST database and our own data), 2% (three STs: ST244, ST86 and ST575) were
295 found in more than one continent, 21% (29/137) were found in more than one country and
296 9.5% (13/137) were found in three or more countries. When a ST was detected in more than
297 one country, those countries were generally distant (i.e. did not border each other, 96.6%).
298 Eleven STs (out of the 20) found in ticks feeding on birds, and typed during the course of this
299 study, were detected in more than one country, and included two STs that were found in more
300 than one continent and four widespread STs (detected in 5 to 9 countries; Fig. 4). Among
301 these 20 STs described as part of this study, two were shared between migrant bird species
302 and species with both resident and mixed populations.

303 In the goeBURST analysis of the global collection (137 STs), using TLV as
304 parameter, 16 major clonal complexes and 4 minor clonal complexes (consisting of only two
305 associated STs) were found. Thirty-three isolates formed singletons (Fig 4). In five out of the
306 16 major clonal complexes, a clonal founder could be identified - those were ST86, ST88,
307 ST184, ST244 and ST251. The goeBURST analysis further revealed that STs from different
308 continents belonged to different clonal complexes, with only a few exceptions: seven out of

309 the 137 STs were shared between continents, or clustered together (e.g. ST364, ST694; Fig.
310 4). Two of the STs found in more than one continent were also detected in ticks from
311 European birds investigated during the course of this study.

312 Focusing on European STs, the pattern of clonal complexes was not related to
313 geographical distribution – there was no evidence that STs from different countries formed
314 separate clonal complexes, except for four STs detected only in Norway (ST487, ST488,
315 ST498 and ST516; Tveten, 2013) - Sup. Mat. 6, marked with an *). There was also no
316 indication of clustering according to bird species (Sup. Mat. 6).

317 STs detected in ticks from birds worldwide did not cluster tightly as clonal complex
318 but were distributed amongst clonal complexes, including those from migrant and birds which
319 have both resident and mixed populations (Sup. Mat. 7).

320 The averaged nucleotide diversity of *B. garinii* for all eight genes together was of the
321 same order for the three continents and all countries (ranging from 0.007 to 0.010), except
322 Norway and Sweden which presented the lowest nucleotide diversity (π = 0.005; Table 2).
323 Tajima´s *D* was close to zero for most countries, showing that there is no specific sign of
324 selection or expansion in these genes. However, the population in Norway showed a
325 comparatively high Tajima´s *D* of 1.085, which could be a sign of a bottleneck, being in
326 agreement with the low genetic diversity observed in this population.

327 At the European scale, *B. garinii* showed no spatial structuring in goeBURST analysis
328 (Sup. Mat. 6): STs for which more than one isolate has been obtained (e.g. ST86, ST187,
329 ST251, ST94, ST82) were not regionally restricted, but originated from distant countries such
330 as UK, Latvia, Slovenia, Hungary or Austria.

This is also what we observed on the ancestry recombination graph reconstructed using sequences of the 85 European STs and generated in BEAST2 (Bouckaert et al., 2019); Fig.5). This method was used because we suspected recombination between housekeeping loci. Indeed 12 occurrences of recombination (dashed lines) were identified in at least 50% of the sampled graphs in the phylogeny showing that recombination does occur but that there is a global clonal frame. These recombination events concerned six out of the eight loci. The ancestry recombination graph shows no evident geographical clustering for four main European regions (Northern Europe - Estonia, Finland, Latvia, Norway and Sweden; Eastern Europe - Czech Republic, Hungary, Serbia, Slovenia and Yugoslavia; Central Europe - France, Germany, Italy, Netherlands; and British islands - United Kingdom) except for the 11 STs present in Norway (marked with square brackets on Fig.5) that cluster into three monophyletic groups and one isolated ST. Out of the 85 STs present in Europe, 25% (n = 22) were present in at least two of the geographical regions defined (Northern, Eastern, Central Europe and the British islands). *Borrelia garinii* STs can thus disperse very far at the continental scale. STs detected in birds (this study) were dispersed among other STs isolated from ticks or humans.

4. DISCUSSION

In this study *I. ricinus* was the most abundant tick collected from common passerine birds across a large geographical area in Europe. Overall, *B. burgdorferi* s.l prevalence in ticks collected from birds was 37%. Thrushes (*Turdus* spp.) were the most important carriers of infected *Ixodes* spp., supporting the notion that different bird species contribute differently to *B. burgdorferi* s.l. genospecies complex maintenance and dispersal. Our genetic

characterization of the most prevalent genospecies detected in ticks feeding on birds, *B. garinii*, showed that this tick-borne pathogen presents little overlap of STs among continents, but no geographical population structuring was detected in Europe, or according to isolation source (bird-derived ticks or questing ticks/ human isolates). Taken together this provides supporting evidence that birds act as important reservoirs for *B. garinii* and are a main source of infection of this genospecies to ticks and ultimately humans (through the bite of an infected tick). Given the importance of birds as main hosts of this tick-borne pathogen, they have the potential to modulate its phylogeography by homogenising the distribution of STs within the European continental range through dispersal and migratory movements. Studying the different factors in action driving this complex host-vector-parasite system is important for a full understanding of *B. burgdorferi* s.l. enzootic cycle and potentially other (not only tick-borne) bird-associated zoonotic pathogens.

Ixodes ricinus is a generalist tick and birds are known to be important hosts for its immature stages (Norte et al., 2012; Santos-Silva et al., 2011). The other tick species (*I. frontalis*, *I. arboricola* and *I. ventalloi*) and genera (*Haemaphysalis* and *Hyalomma*) collected from birds in this study have also been previously reported on birds (Diakou et al., 2016; Norte et al., 2012; Pérez-Eid, 2007) and differ in vector competence for *Borrelia* (Eisen & Lane, 2002; Heylen, Krawczyk, et al., 2017; Heylen, Sprong, et al., 2014). Some bird species such as hole-nesting birds (*P. major* and *F. albicollis*), *T. merula* and *T. philomelos* were hosts for different tick species, however, the opportunities for co-feeding transmission of *B. burgdorferi* s.l. between different tick species are limited by spatial and temporal tick species distribution. The misidentification rate of ticks based on morphological features in our study was similar to that reported for the genus *Ixodes* (14%; Estrada-Peña et al., 2017). Although *I. persulcatus* occurs in part of the geographic range included in our study (e.g. Finland and

Estonia; ECDC 2018; Laaksonen et al., 2017), and its morphological distinction from *I. ricinus* in immature stages is difficult, none of the tested samples were identified as *I. persulcatus* by 16 rRNA sequencing.

Overall, the prevalence of *B. burgdorferi* s.l. (37%) was within the range reported for ticks collected from hosts in The Netherlands and Belgium (34%; Heylen, Fonville, et al., 2017). However, it was higher than in ticks collected from birds migrating through Italy (30.7%; Toma et al., 2014) and Sweden (26.6%; Olsen et al., 1995), birds from central Europe (25.8 – 28%; Dubska, Literak, Kocianova, Taragelova, & Sychra, 2009; Hanincova et al., 2003; Taragel'ova et al., 2008), Germany (25%; Kipp, Goedecke, Dorn, Wilske, & Fingerle, 2006), Poland (13.3%; Michalik, Wodecka, Skoracki, Sikora, & Stanczak, 2008), Switzerland (19.6-22.5%; Lommano, Bertaiola, Dupasquier, & Gern, 2012; Poupon et al., 2006), Spain (9.2%; Palomar et al., 2016) and Portugal (7.3%; Norte et al., 2015). We cannot exclude the possibility that this may be related to different methodologies used for *B. burgdorferi* s.l. detection in different studies (real-time PCR *versus* conventional PCR and target genes). Our results, using the same detection method across samples from different geographical origins revealed that prevalence of *B. burgdorferi* s.l. in ticks from birds varied significantly according to latitude. The fact that prevalence increased with latitude is in accordance with Scandinavian countries such as Finland and Sweden showing relatively high prevalence when compared with other European countries, as reported in previous studies on Lyme borreliosis incidence and infection loads of questing ticks (Hubalek, 2009; Rauter & Hartung, 2005; Wilhelmsson et al., 2013). A meta-analysis for Europe revealed an overall prevalence of 13.7% in questing ticks, higher in central Europe and Sweden, but with a significant increase with longitude, rather than latitude, as in our study (Rauter & Hartung, 2005). Nonetheless, it is also known that Lyme borreliosis presents a focal pattern of distribution, determined by the

heterogeneous spatial distribution of vector ticks, and also that the north-south gradient has a greater influence on disease incidence at its distributional range limits (Hubalek, 2009). A heterogeneous geographical distribution of *Borrelia* genospecies was also reported by Olsen et al. (1995). These authors showed that *Borrelia* infections in ticks collected from birds arriving to Sweden from the south or southeast in spring were mainly caused by *B. garinii*, whereas the genospecies distribution was more heterogeneous in ticks from birds coming from the southwest, and included *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s.

The higher prevalence of *B. burgdorferi* s.l. in ticks removed from birds than that reported from questing ticks is in accordance with birds acting as reservoirs for some *Borrelia* genospecies and transmitting the infection to feeding ticks (Heylen, Matthysen, Fonville, & Sprong, 2014; Humair, Postic, Wallich, & Gern, 1998b; Kurtenbach, Carey, Hoodless, Nuttall, & Randolph, 1998; Norte, Lopes de Carvalho, Núncio, Ramos, & Gern, 2013). Additionally, *Borrelia* starts dividing in feeding ticks and may be more readily detected by PCR (Schwan & Piesman, 2002). Our study revealed a non-homogeneous distribution of *Borrelia* among bird species in bird-derived ticks. Thus, our data corroborate previous reports that not all bird species contribute equally to the *Borrelia* enzootic cycle, as it is also known for different mammal species (LoGiudice, Ostfeld, Schmidt, & Keesing, 2003; Talleklint & Jaenson, 1994), and suggested by studies including different lizard species (Norte, Alves da Silva, Alves, da Silva, Nuncio, et al., 2014; Szekeres, Majláthová, Majláth, & Földvári, 2016). Contributing factors may not only be different infestation rates with vector ticks, which may be related to foraging behaviour and consequent probability of exposure (Norte et al., 2012), but also to other intrinsic factors related to host competence, including the host's adaptive and innate immune system (Kurtenbach et al., 2006). *Turdus* species have been identified as main reservoirs for *B. garinii* and *B. valaisana* in Europe (Dubska et al., 2011; Mannelli et al.,

2005; Michalik et al., 2008) and Asia (Miyamoto & Masuzawa, 2002). In addition, *T. merula* has also been proven as competent reservoir for *B. turdi* through xenodiagnosis (Heylen, Krawczyk, et al., 2017; Humair, Postic, Wallich, & Gern, 1998a; Norte et al., 2013). In an experimental setup in which migratory restlessness was induced, latent *B. garinii* infections were re-activated in *T. iliacus* (Gylfe, Bergstrom, Lunstrom, & Olsen, 2000). Although *B. burgdorferi* s.l. infected ticks, including larvae, have occasionally been collected from *E. rubecula* (this study; Poupon et al., 2006), our results suggest that this bird species, although often infested by vector ticks (Norte et al., 2012), plays a minor role in *B. burgdorferi* s.l. enzootic cycle because of the very low prevalence of *B. burgdorferi* s.l. in its ticks.

Ticks associated with hole-nesting birds such as *P. major* and *F. albicollis* presented infection rates of 33.8 - 36.4%. The most prevalent genospecies was also *B. garinii*, which made up 64 - 77.9% of infections in these bird species. *Parus major* has been shown experimentally to selectively amplify *B. garinii* and *B. valaisiana*, whereas *B. afzelii* prevalence in moulted adult ticks that fed as nymphs on this bird species tended to decrease in successive infestations of the birds with wild questing nymphs (Heylen, Matthysen, et al., 2014). The finding of this mammal-associated genospecies in attached ticks derived from birds has been suggested to result from previously acquired infection from another (mammal) host because these spirochetes were found to be unviable by culturing (Heylen et al., 2017). In our study, all *B. afzelii*- positive ticks were nymphs and we cannot comment on birds' reservoir competence for *B. afzellii* with these findings because PCR does not allow distinguishing between viable and non-viable bacteria. We cannot rule out that nymphs acquired the infection during a previous blood meal as larvae from a mammalian host, or that larvae were infected via transovarial transmission (Bellet-Edimo, Betschart, & Gern, 2005), because larvae of *I. ricinus* have been shown to transmit *B. afzelii* and *B. miyamotoi* to

vertebrate hosts (van Duijvendijk et al., 2016). In Europe, the role of transovarial transmission for different tick and *Borrelia* species has not been resolved (Bellet-Edimo et al., 2005; Eisen & Lane, 2002; Humair & L. Gern, 2000; van Duijvendijk et al., 2016). Thus, the role of birds in *B. afzellii* transmission needs to be further scrutinized.

Borrelia turdi, originally described in Japan (Fukunaga et al., 1996), has been increasingly detected in Europe, often in association with the ornithophilic tick *I. frontalis* and its bird hosts (Heylen, Tijsse, Fonville, Matthysen, & Sprong, 2013; Norte et al., 2015). In this study, it was detected only in Portugal, although it is known to be present in Spain, Belgium and Norway (Hasle, Bjune, Midthjell, Røed, & Leinaas, 2011; Heylen et al., 2013; Palomar et al., 2016). In our study it has been detected in *I. frontalis* and *I. ventalloi* only, which are host- specialised tick species (to birds and rabbits, respectively; Hillyard, 1996). *Ixodes frontalis* has been proven to be a competent vector for *B. turdi* (Heylen, Krawczyk, et al., 2017) but vector competence of *I. ventalloi* remains unknown. This *Borrelia* genospecies may have been overlooked in the past in questing ticks such as *I. ricinus* probably because of its low prevalence (Heylen, Krawczyk, et al., 2017). Because *B. turdi* prevalence in our study was relatively low, we were unable to evaluate statistically its bird host and vector species' associations. Furthermore, the small sample size for tick species other than *I. ricinus*, may have hampered the detection of significant associations between *Borrelia* and tick species, to infer tick vector competence. Such relationships may be better evaluated in experimental transmission studies (Heylen, Fonville, et al., 2017; Heylen, Sprong, et al., 2014).

Besides the avian-associated genospecies *B. valaisiana*, *B. garinii* and *B. turdi*, we also detected DNA of a new *Borrelia* genospecies that has not been previously described. Although PCR amplification and sequencing of 16S rRNA, *flaB* and *clpX* was possible and clearly indicated the genetic distinction of the isolate from other *Borrelia* species, it is

conceivable that its genetic dissimilarity precluded a deeper characterisation involving other house-keeping genes (which could not be amplified). This finding adds to the growing evidence of the diversity of genospecies in circulation in cryptic cycles in bird hosts. Specificities of reservoir host and/or vector competence may explain why this novel *Borrelia* sp. genospecies was not detected before.

Borrelia lusitaniae, a genospecies whose main reservoirs are lizards (De Sousa et al., 2012; Dsouli et al., 2006; Norte, Alves da Silva, Alves, da Silva, Núncio, et al., 2014), has been occasionally detected in ticks feeding on birds, including larvae (Poupon et al., 2006). In our study, only one tick feeding on a bird was positive for *B. lusitaniae*. However, the paucity of these findings suggests that birds, at most, have a minor role as reservoirs for this genospecies. These infections could be the result of a previous incomplete blood meal on a lizard, transovarial or co-feeding transmission. Surveys in endemic areas in Italy and Portugal in which hundreds of bird-derived ticks were tested revealed no *B. lusitaniae* positive specimens and thus, did not provide evidence that birds may serve as reservoir hosts for *B. lusitaniae* (Amore et al., 2007; Norte, Alves da Silva, Alves, da Silva, Núncio, et al., 2014; Norte, Ramos, Gern, Núncio, & Lopes de Carvalho, 2013).

Focusing on the genetic diversity and geographical distribution of the most prevalent genospecies detected in ticks feeding on birds, the avian-associated *B. garinii*, we found that its STs clustered according to continent showing some spatial structuring at this very wide geographical scale. However, there was one ST shared between Europe and Asia, one ST shared between Europe and North America, and one ST shared between Europe, Asia and North America providing evidence of overlap among distant areas at a global scale. One would expect that finding identical STs on continents separated by the Atlantic would be less likely than that between adjacent continents whose geographical barriers may be easily

crossed by migrating birds. The movement of long-distance migratory birds, such as seabirds, which can travel thousands of miles and between hemispheres may be responsible for the spread of some *B. garinii* STs to distant geographical regions. *Borrelia garinii* is known to circulate in a marine cycle involving the ornithophilic tick *I. uriae* that infests seabirds at their colonies (Comstedt, Jakobsson, & Bergström, 2011; Gómez-Díaz et al., 2011). Migratory shorebirds such as the black-tailed godwit *Limosa limosa*, the common redshank *Tringa totanus*, and the little stint *Calidris minuta* were also reported to carry *B. garinii* (Lopes de Carvalho et al., 2012). To this point, *B. garinii* isolates sharing the same *flaB* sequence have been found in both Campbell Island (New Zealand), the Crozet Islands, and in the northern hemisphere (Egg and St. Lazaria Islands, USA; Comstedt et al., 2011). In our study, two of the transcontinental *B. garinii* STs were indeed found in ticks feeding on birds. One of these (ST244) was found in *I. uriae* on a Canadian island (Munro et al., 2017), in questing *I. ricinus* in Europe and *I. persulcatus* in Russia (https://pubmlst.org/borrelia/), in human isolates in Germany (https://pubmlst.org/borrelia/), and in *Ixodes* spp. feeding in terrestrial birds in Finland and Germany (this study). Some passerine birds (e.g. the northern wheatear *Oenanthe oenanthe*) can also perform long distance migrations across the Atlantic (Bairlein et al., 2012). An overlap and exchange of strains between the marine and terrestrial cycles is, therefore, likely, as suggested by previous studies (Comstedt et al., 2011; Gómez-Díaz et al., 2011). However, Goméz-Díaz et al. (2011) reported a population division of *B. garinii* from seabirds between the Atlantic and Pacific basins. These researchers did not use the same MLST as employed in our study, thus, immediate comparison of the results is not possible.

When evaluating European *B. garinii* STs only, no pattern of geographical clustering was noticeable in our analysis, probably due to *B. burgdorferi* s.l./or ticks' dispersal promoted by the birds. Similarly, although seabird species show high fidelity to breeding colonies

524 (Schreiber & Burger, 2001), and their main tick *I. uriae* occurs in seabird populations with
525 strong host species associations (McCoy et al., 2005), no geographic structuring was observed
526 in *B. garinii* within the Atlantic and Pacific Oceans (Gómez-Díaz et al., 2011). In contrast, the
527 mammal-associated *B. afzelii* STs were shown to have much less geographical overlap in
528 studies which compared geographical patterns and population structure of the avian-
529 associated *B. garinii* and this mammal-associated genospecies (*B. afzelii*), using the same
530 MLST scheme as this study (Vollmer et al., 2013; Vollmer et al., 2011). Their results
531 illustrated that *B. garinii* showed higher spatial mixing than *B.afzelii* but that *B. garinii*
532 presented population differentiation over a large geographical scale (Europe and China).
533 However, Vollmer et al. (2013) included fewer strains from a smaller geographical range in
534 Europe and China.

535 Although, in general, no overall apparent structure was found for European strains of
536 *B. garinii*, some Norwegian samples were divergent. This could be due to a relative isolation
537 of the study area in Norway, located in the northwest of the country (Tveten, 2013), or a
538 recent invasion event, which would explain the lower diversity of these strains. Recent
539 invasion would be consistent with the reported recent expansion of *I. ricinus* tick populations
540 to northern latitudes in Norway (Gray, Dautel, Estrada-Peña, Kahl, & Lindgren, 2009), which
541 could have caused a population bottleneck. This may also explain the evidence for selection
542 and expansion on MLST genes revealed by the relatively high Tajima's D in this *B. garinii*
543 population.

544 The uniform distribution of *B. garinii* STs among ticks collected from various bird
545 species, and other sources (e.g. questing ticks), does not suggest specialization of certain *B.*
546 *garinii* STs to certain hosts, contrary to the hypothesis of multiple niche polymorphism
547 associated with OspC variation (Dustin Brisson, Drecktrah, Eggers, & Samuels, 2012) found

548 for *Borrelia burgdorferi* sensu stricto (Brisson & Dykhuizen, 2004; Vuong et al., 2014), but
549 not for *B. afzelii* (Raberg et al., 2017). Our results are consistent with birds being the main
550 reservoir hosts of *B. garinii*: they maintain its natural transmission cycle and are the source of
551 infection for questing vector ticks. The lack of clustering of *B. garinii* STs regarding country
552 of origin or isolation source at a finer scale (i.e. Europe), was also supported by the ancestry
553 recombination graph. The clustering pattern between goeBURST (Sup. Mat 6) and that of the
554 ancestry recombination graph was generally similar with only a few exceptions showing
555 recombination among strains that could also be promoted by the avian-associated dispersal,
556 which may increase chances of encounter between different strain types and mixing of strains.

557 We should acknowledge that for migrant bird species, and for those which have both
558 resident and short-distance migrant populations, one cannot be completely confident that the
559 *B. burgdorferi* s.l. infections which the bird-infesting ticks carried were acquired in the
560 geographical area where the birds were captured. Birds (or their ticks) may have acquired the
561 bacteria in a different area where they remained or stopped-over during migration. This may
562 bias prevalence estimates and sequence type origin classification according to geographical
563 location.

564 The results presented in this study demonstrate how *B. burgdorferi* s.l.- vector- host
565 associations and the behaviour of hosts may shape and impact the spread and dispersal, and
566 ultimately the evolutionary biology of *B. burgdorferi* s.l., used here as a model for tick-borne
567 pathogens. Our data which includes *B. garinii* MLST characterization from bird-derived ticks
568 from the largest geographical range investigated so far substantiates that bird migration and
569 dispersal movements appear to be one of the main driving forces to shape *B. garinii*
570 populations, one of the most genetically heterogeneous Lyme borreliosis- causing
571 genospecies (Jacquot et al., 2014; Margos et al., 2008). Because birds are highly mobile and

572 the main reservoir hosts not only for *B. garinii,* but also for other pathogens, they contribute
573 to frequent, fast and long-range spatial mixing of strains and populations. Our study
574 underlines that understanding pathogen variability and spatial distribution, and consequent
575 modulation of transmission rates and evolution of new variants, is essential to understand
576 disease risk.
577

578 **PUBLIC DATABASES ACCESSION NUMBERS**

579

580 *Borrelia* sp. "*Candidatus* Borrelia aligera" 16S rRNA and *clpX* gene partial sequences
581 obtained in this study have been deposited in GenBank with the accession numbers
582 MH068784 and MH157920, respectively. *Borrelia garinii* MLST sequences have been
583 deposited in *Borrelia* MLST database (https://pubmlst.org/borrelia/) with the isolate id
584 numbers 2451 to 2479.
585

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587

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Table 1. Conditional model averaged coefficient parameters from the generalized linear mixed models (GLMMs) that best explained (lowest AICc, ΔAICc < 2, see Sup. Mat. 2) the prevalence of *B. burgdorferi* s.l. in ticks collected from birds.

	Conditional averaged model coefficients				
Parameter	**Estimate**	**SE**	**Adjusted SE**	**Z Value**	**P**
Intercept	-5.72	2.07	2.07	2.76	0.0058
Bird_species_*E.rubecula*	-2.41	1.25	1.25	1.93	0.054
Bird_species_*F.albicollis*	1.27	0.88	0.88	1.44	0.15
Bird_species_*P.major*	1.12	0.82	0.83	1.35	0.18
Bird_species_*P.trochilus*	0.11	1.24	1.24	0.09	0.93
Bird_species_*P.modularis*	-0.30	1.27	1.27	0.24	0.81
Bird_species_*T.iliacus*	0.89	1.05	1.05	0.84	0.40
Bird_species_*T.merula*	2.50	0.91	0.91	2.76	0.006
Bird_species_*T.philomelos*	1.11	0.95	0.95	1.17	0.24
Bird_species_*T.pilaris*	4.29	1.51	1.51	2.84	0.0046
Tick_species_*I.frontalis*	0.47	1.03	1.03	0.45	0.65
Tick_species_*I.ricinus*	1.18	0.70	0.71	1.66	0.096
Tick_species_*I.ventalloi*	0.96	1.22	1.22	0.78	0.44
Latitude	0.079	0.03	0.03	2.28	0.022
Latitude*Longitude	0.0008	0.0005	0.0005	1.56	0.12

Table 2. Nucleotide diversity (π) and Tajima´s D averaged over the eight MLST genes (*clpA*, *clpX*, *pepX*, *pyrG*, *nifS*, *recG*, *rplB*, *uvrA*) of *B. garinii* strains (strain IDs included in this analysis are available in Sup. Mat. 3).

Population	N strains	Mean π	Mean Tajima´s *D*
Continent			
Europe	227	0.008	-0.222
Asia	85	0.009	-1.184
North America	21	0.008	0.617
Country			
Canada	21	0.008	0.617
China	8	0.009	0.022
Finland	12	0.007	0.157
France	18	0.007	-0.090
Germany	55	0.007	-0.326
Japan	21	0.010	-0.865
Latvia	30	0.010	-0.192
Norway	16	0.005	1.085
Russia	54	0.008	-1.026
Sweden	6	0.005	-0.205
UK	70	0.009	0.219

Figure captions

Fig. 1. Map of countries and sampling locations where birds were screened for infesting ticks. Sampling locations closer than 40 km apart are represented under the same pin and are numbered according to study site listed in Sup. Mat. 1. Light grey pins – sites where birds were screened for ticks but no ticks were found; Dark grey pins – sites where ticks were collected feeding on birds. Details on sampling locations, range of bird capturing dates, bird species, number of bird individuals infested and number of *Ixodes* spp. ticks collected at each location are detailed in Sup. Mat 1. Adapted from the Cartographic Research Lab of the University of Alabama.

Fig. 2. *Borrelia burgdorferi* s.l. prevalence (%) and genospecies in *Ixodes* spp. ticks collected from different bird species. Bird species from which less than 10 ticks were tested were included in the category "other bird spp.". Numbers at the top of the bars represent the number of ticks tested. L – larva; N + A – nymph and adult.

Fig. 3. *Borrelia burgdorferi* s.l. prevalence (%) and genospecies in *Ixodes* spp. ticks collected feeding on birds per country of collection. Numbers at the top of the bars represent the number of ticks tested. L – larva; N + A – nymph and adult.

Fig. 4. *Borrelia garinii* STs distribution between countries in Europe (blue/green), Asia (red) and North America (purple). GoeBURST analysis included 137 STs, using TLV as parameter. Sixteen major clonal complexes and 4 minor clonal complexes (consisting of only two associated STs) were found. Thirty-three isolates formed singletons. A clonal founder was identified in five out of the 16 major clonal complexes (ST86, ST88, ST184, ST244 and ST251), in red. N refers to the number of *B. garinii* isolates used in the analyses.

Fig. 5. Ancestry Recombination Graph of 85 European Sequence Types reconstructed with BEAST2 v. 2.5 and package bacter v. 2.2. Labels are coloured by geographic origin: green - Northern Europe (Estonia, Finland, Latvia, Norway and Sweden), orange - Eastern Europe (Czech Republic, Hungary, Serbia, Slovenia and Yugoslavia), red - Central Europe (France, Germany, Italy, Netherlands) and blue - British islands (United Kingdom). Branches leading to taxa found in one geographic region only show the corresponding colour. Black labels refer to STs present in several geographic regions indicated in coloured rectangles and corresponding in coloration to the regions defined (North, East, Central Europe and British islands). STs isolated from birds (this study) are marked with a bird next to the label name. Dashed lines show recombination events present in at least 50% of all posterior graphs and stars mark high confidence nodes (present in at least 80% of all posterior graphs).

Sup. Mat. 1 Details on sampling locations, range of bird capturing dates, bird species, number of bird individuals infested and number of *Ixodes* spp. ticks collected at each location.

Sup. Mat. 2 Best model selection using Akaike Information Criterion (AIC) to explain (a) *B. burgdorferi* s.l. and (b) *B. garinii* prevalence in ticks collected from birds. Models were fitted using logit function for binomial error distributions. For each model we present: Maximum Likelihood, AICc = Akaike information criterion corrected for sample size, ΔAIC = difference in AIC to the best ranked model and df = degrees of freedom.

Sup. Mat. 3. List of *B. garinii* isolates retrieved from MLST database and used (a) for goeBURST (n = 172), (b) for phylogenetic analysis (n = 110), and (c) to calculate nucleotide diversity and Tajima's D (n = 304), including ST information and MLST database (https://pubmlst.org/borrelia/) ID numbers.

Sup. Mat. 4 *Borrelia burgdorferi* s.l. (Bb) prevalence in different tick species and development stages collected feeding on bird species in which > 10 individuals were infested.

Sup. Mat. 5 Data on specimen detailed source of the *B. burgdorferi* s.l. genospecies detected in our study.

Sup. Mat. 6. Figure caption - *Borrelia garinii* STs distribution in European countries. STs detected in ticks feeding on birds are symbolised with a bird icon and the bird species is indicated by the icon colour. GoeBURST analysis included 85 STs, using TLV as parameter. Thirteen major clonal complexes were found and 5 isolates formed singletons. A founder was identified in five out of the 13 major clonal complexes (ST86, ST88, ST184, ST244 and ST251), these are indicated in red numbers. N refers to the number of *B. garinii* isolates used in the analyses. * cluster of STs from Norway.

Sup. Mat. 7. Figure caption - *Borrelia garinii* STs distribution by isolation source: questing ticks/ human isolates are indicated in rose, while ticks collected from birds are colour coded according to migratory status of birds (resident: dark grey, mixed populations - resident and short distance migrants: light grey, or migrant: blue). GoeBURST analysis included 137 STs, using TLV as parameter. Sixteen major clonal complexes and 4 minor clonal complexes (consisting of only two associated STs) were found. Thirty-three isolates formed singletons. A clonal founder was identified in five out of the 16 major clonal complexes (ST86, ST88, ST184, ST244 and ST251), these are indicated in red numbers. N refers to the number of *B. garinii* isolates used in the analyses.

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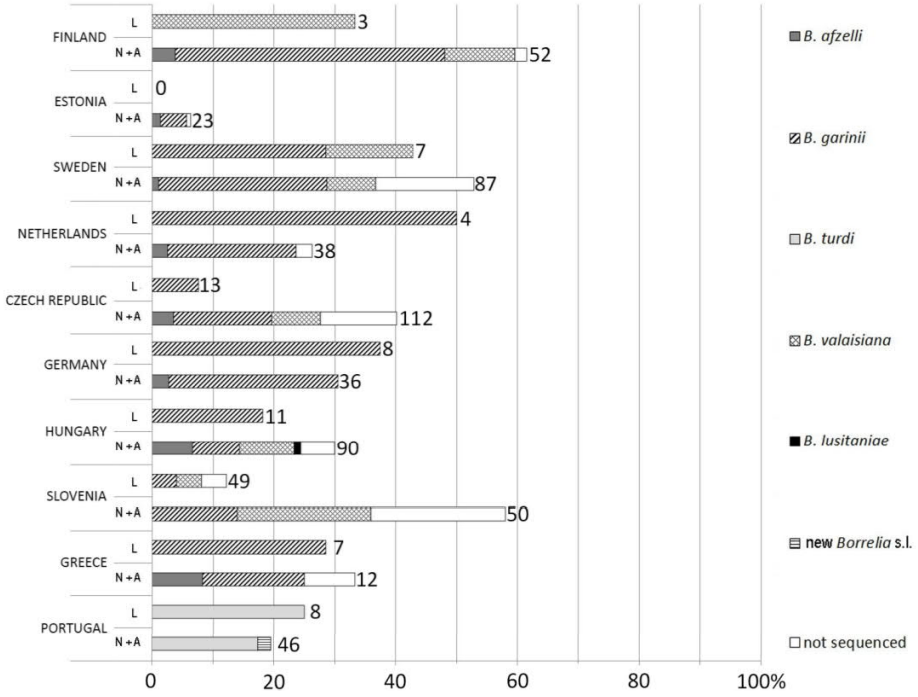

Fig. 2. Borrelia burgdorferi s.l. prevalence (%) and genospecies in Ixodes spp. ticks collected from different bird species. Bird species from which less than 10 ticks were tested were included in the category "other bird spp.". Numbers at the top of the bars represent the number of ticks tested. L – larva; N + A – nymph and adult.

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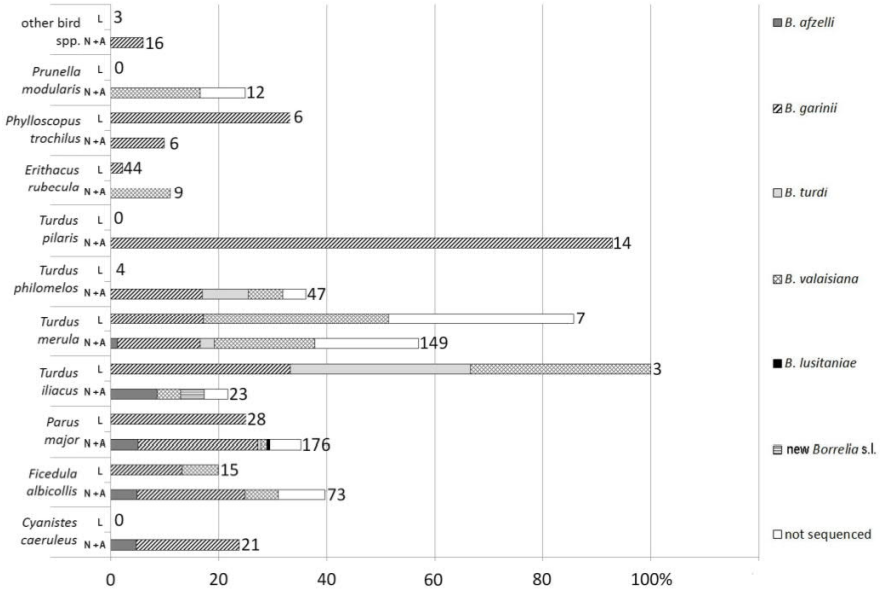

Fig. 3. Borrelia burgdorferi s.l. prevalence (%) and genospecies in Ixodes spp. ticks collected feeding on birds per country of collection. Numbers at the top of the bars represent the number of ticks tested. L – larva; N + A – nymph and adult.

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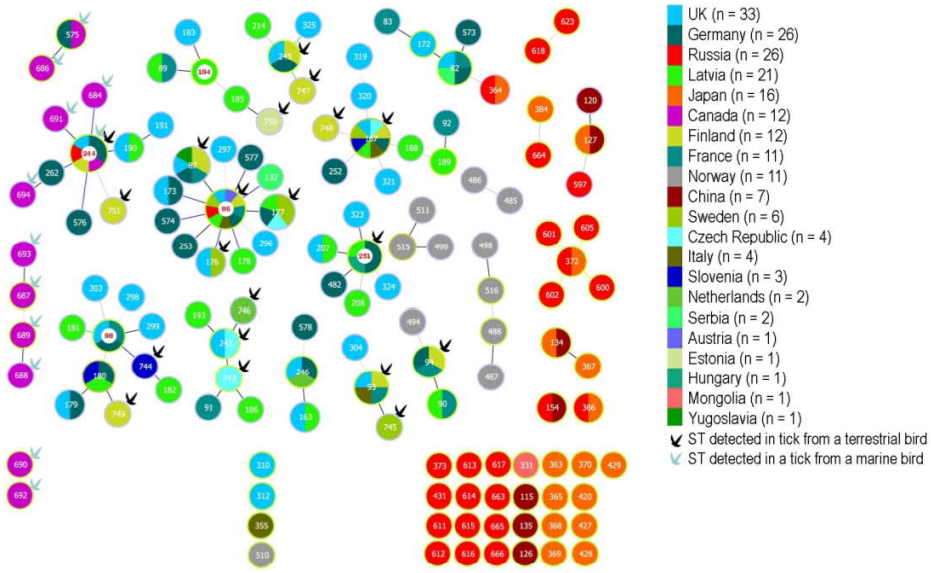

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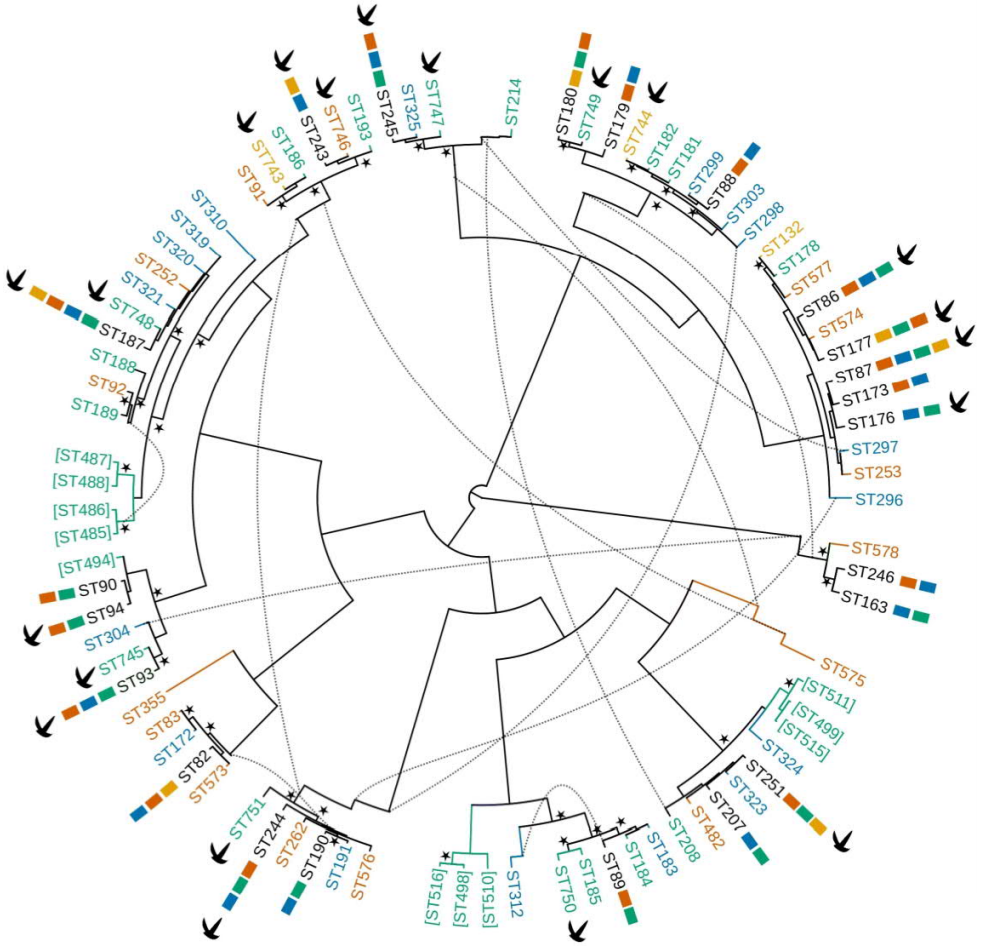

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189x184mm (300 x 300 DPI)