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Wide spectrum of tick-borne pathogens in juvenile *Ixodes ricinus* collected from autumn-migrating birds in the Vistula River Valley, Poland

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Abstract

Background Migratory birds serve as potential hosts for ticks and can be reservoirs of tick-borne pathogens (TBPs). The aim of our study was to investigate the prevalence of TBPs in juvenile *Ixodes ricinus* collected from *Erithacus rubecula*, *Turdus merula*, and *Turdus philomelos* passing through the Vistula River Valley, Poland — one of the most important European north-south routes for migratory birds.

Methods To detect TBPs in collected ticks we used a high-throughput microfluidic real-time PCR method. In addition, we performed a phylogenetic analysis of *Borreliella garinii flaB* and *Rickettsia helvetica ompB* sequences, considering haplotype diversity through a Median Joining Network.

Results Our results showed a high prevalence and wide spectrum of TBPs in both larvae and nymphs of *I. ricinus*. Overall, including co-infections, 47.41% of the tested tick specimens were infected with at least one TBP. Borreliaceae spirochetes were detected in ticks collected from all examined bird species. Ticks (larvae and nymphs) collected from *T. merula* showed the highest prevalence of *Bo. garinii* (33.33%), *Bo. burgdorferi* s.s. (7.69%) and *Borrelia miyamotoi* (2.56%), while the highest number of ticks infected with *Bo. valaisiana* were collected from *T. philomelos* (8.11%). In turn, the highest prevalence of *R. helvetica* (20.00%) was observed in ticks collected from *E. rubecula*. Additionally, infections with *A. phagocytophilum* (5.00%), *Ehrlichia* spp. (2.50%), *Ba. divergens* (2.50%) and *Ba. venatorum* (2.50%) were only confirmed in ticks collected from this bird species. The phylogenetic analysis of *Bo. garinii* revealed that the detected haplotype circulates widely across various hosts and is geographically widespread, while the haplotype of *R. helvetica* is mainly detected in ticks in Central Europe.

Conclusions Ticks carried by *T. merula*, *T. philomelos*, and *E. rubecula* migrating along the Vistula River Valley, Poland are characterized by a high prevalence and a wide spectrum of detected TBPs. Tested ticks carry widespread strains

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of *Bo. garinii*, in contrast to *R. helvetica*, which is mainly found in Central Europe. Therefore, further research on the possible role of birds as reservoirs of TBPs is needed.

Keywords Ticks, *Ixodes ricinus*, Tick-borne pathogens, Birds, Pathogen-host interactions

Background

Birds belong to a wide host spectrum of both argasid and ixodid ticks, including species of major medical and veterinary importance. Depending on latitude, the most commonly identified argasid tick species collected from birds and/or in their habitat/nests belong to *Argas* and *Ornithodoros* genera [1–4]. In turn, the most common ixodid ticks feeding on birds include representatives of *Ixodes* spp., *Hyalomma* spp., and *Haemaphysalis* spp [4–6].

Birds are considered to be reservoirs of tick-borne pathogens (TBPs) in the environment, contributing to the spread of emerging and re-emerging diseases [7–9]. By inhabiting tick-occupied habitats, birds facilitate the transmission of ticks, becoming a key link in the circulation of TBPs. Particularly, birds play a significant role in the dissemination of Borreliaceae representatives, whereas their role as reservoirs of *Anaplasma phagocytophilum* seems to be limited. Previous studies have confirmed that only ecotype IV of *A. phagocytophilum* is associated with birds [10, 11]. Thus, birds indirectly influence the epidemiological risk of tick-borne diseases (TBDs) transmission to humans [5, 12, 13]. Previously published studies reported a large number of TBPs detected in ticks collected from birds with *Borreliella* spirochetes, the causative agent of Lyme disease (LD), being the most frequently confirmed [14, 15]. Other commonly found TBPs in bird-carried ticks include *Rickettsia* (mainly *R. helvetica*), *Coxiella burnetii*, *Neoehrlichia mikurensis*, *Babesia* spp. and viruses, e.g. *Orthoflavivirus encephalitidis*, an etiological agent of tick-borne encephalitis (TBE) and Crimean-Congo hemorrhagic fever virus (CCHFV) [16–19]. Additionally, considering the role of birds in the circulation of TBPs in the environment it should be mentioned that the majority of pathogen species detected in ticks collected from them, depending on the pathogen species, can be transmitted transstadially, transovarially (e.g. *B. burgdorferi* s.l., *Rickettsia* spp.) and during co-feeding [20–22].

Studies on the role of birds in the spread of ticks and associated TBPs are particularly interesting in areas recognized as major corridors of avian migration, within global networks, such as the Palearctic-African migration system. The birds' migration routes in the southern part of the Western Palearctic cover the Iberian Peninsula, Tunisia, Turkey, Italy, and the Middle East [4, 5, 23–26], while Poland lies on their migratory pathway from Africa and the Middle East to Northern and Central Europe [27–29]. In this region, numerous birds' stopover sites are situated along the Vistula ecological corridor [30–32].

This study aimed to investigate the prevalence and diversity of TBPs in juvenile *Ixodes ricinus* collected during our previous fieldwork [33]. In the current study, we examined ticks collected from the most frequently captured and tick-infested bird species, i.e., *Turdus merula*, *Turdus philomelos*, and *Erithacus rubecula*, passing the Vistula Valley. Additionally, considering the migratory behavior of birds, a phylogenetic analysis of the detected TBPs was also performed to assess the potential introduction of pathogen strains in the study area.

Methods

All processes were carried out in conformity with applicable rules and legislation. The study, including birds capture and tick collection procedure, was approved by the General Directorate of Nature Conservation in Warsaw (DZP-WG.6401.102.2020.TŁ), the Regional Directorate of Nature Conservation in Lublin (WPN.6401.108.2021.KC) and was conducted in accordance with the ARRIVE principles (Animal Research: Reporting of in Vivo Experiments). The study was designed using the 3R concept (Replacement, Reduction, and Refinement) to keep the number of animals utilized to a minimum required for the reliability of the results and to ensure adequate amounts of data for statistical analysis.

Study area

The field study was conducted in cooperation with the Kaliszany Ornithological Station, a member of the National Bird Ringing Stations Network, and the SE European Bird Migration Network (SEEN). The station is located on a river island, within the Vistula River Gorge of Lesser Poland (Fig. 1) [34]. The valley is a natural bird migration corridor between northern and northeastern Europe and the southern and southwestern parts of the continent [35], while the island is their stopover site. A detailed description of the study area can be found in our previously published paper [33].

Tick collection

Fieldwork was conducted during the autumn bird migration season in 2021. Ticks were collected from birds captured in ornithological nets (Ecotone, Gdynia, Poland) extending over a total length of 500 m. The nets were placed along a transect spanning 1.5 km, passing through riverside vegetation dominated by riparian flora transitioning to a meadow community. Nets were inspected

Fig. 1 Location of the study area. The study area is displayed against the background of Europe and Poland (A) and the localization of the river island – a birds' stopover on the route of their seasonal migrations – is shown (B). Based on Google Maps and Wikimedia (By Alexrk2 - Own workData from/ Données issues de http://naturalearthdata.com/, Scale: 1:10 Mio, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=9701652) with own modifications

hourly, with the inspection periods adjusted to half an hour in the presence of unfavorable weather conditions such as rainfall or extreme air temperatures (either low or very high).

Upon capturing, each bird was carefully released from the net and identified to the species level, with the use of an identification key and ringed [36, 37]. Next, all captured birds were examined for the presence of attached ticks and immediately released once the procedure was completed. A detailed description of this procedure can be found elsewhere [33]. In the current study, however, we only tested for TBPs ticks collected from the three most frequently captured and tick-infested bird species, i.e., *T. merula*, *T. philomelos*, and *E. rubecula* [33].

The collected ticks were carefully stored in Eppendorf tubes containing 70% ethanol. Next, ticks were identified at species level and developmental stage as reported in our previous study [33]. Then, the specimens were kept frozen at -80˚C (Arctico, Esbjerg, Denmark) until DNA extraction.

Molecular analyses

Study material

We used juvenile *I. ricinus* ticks collected during our earlier study [33] for the molecular analysis. The sample size analyzed for TBPs was set to include a comparable number of ticks regardless of the bird species from which they were collected. From 266 collected *I. ricinus* ticks [33], we randomly selected partially engorged 54 larvae and 62 nymphs (adult specimens were not recorded) for molecular analyses to assess the presence of TBPs. Tested ticks were collected from 76 bird individuals, including

30 tick-carrying *E. rubecula* (19 larvae, 21 nymphs), 29 *T. merula* (19 larvae, 20 nymphs), and 17 *T. philomelos* (16 larvae and 21 nymphs).

DNA extraction

In preparation for molecular analysis ticks were washed with distilled water, dried, and cut into smaller fragments using a sterile scalpel. The isolation of DNA was carried out using the Genomic Mini AX Tissue kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The concentration of extracted DNA was measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) at a 260/280 nm wavelength. We obtained isolates with DNA concentrations ranging from 10 to 80 ng/µL, which were stored at −20 °C until further processing.

DNA pre-amplification

The DNA pre-amplification was performed using the PreAmp Master Mix kit (Standard Biotools, San Francisco, USA) following the manufacturer's protocol [38]. All primer pairs targeting searched microorganisms species were pooled, combining equal volumes with a final concentration of 0.2 µM each in a final volume of 5 µL containing 1 µL Perfecta Preamp 5×1.25 µL pooled primer mix, 1.5 µL distilled water, and 1.25 µL DNA. Prepared samples were thermocycled as follows: one cycle at 95 °C for 2 min, 14 cycles at 95 °C for 15 s, and 4 min at 60 °C. Next, the products were diluted 1:10 in Milli-Q ultrapure water (45 µL) and stored at −20 °C until further analysis.

Microfluidic real-time PCR for high-throughput detection of microorganisms

The BioMark™ real-time PCR system (Standard Biotools, San Francisco, USA) was run for pathogen detection. Real-time PCR reactions were performed using 6-carboxyfluorescein (FAM)-labeled and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene expression master mix according to the manufacturer's protocol (Applied Biosystems, France), as followed: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of two-step amplification of 15 s at 95 °C and 1 min at 60 °C.

In the real-time PCR reaction, we used primers listed in Table S1. High-throughput microfluidic real-time PCR protocol allowed us to perform screening for 43 pathogen taxa in one tick sample simultaneously. In addition, primers targeting *I. ricinus* were used to confirm tick species (previously described based on morphological features). *Escherichia coli* DNA and *Escherichia*-specific primers were used as positive controls (Table S1) [38, 39]. Ultra-pure water was used for a negative control. The obtained results were analyzed using Fluidigm Real-time PCR Analysis Software v.2.1.

Validation of microfluidic real-time PCR assay and sequencing

To validate the obtained results, *Anaplasma*, *Babesia*, *Rickettsia*, and *Borreliella*-positive samples were randomly selected to undergo additional conventional and nested PCR assays using primers different than those used in high-throughput microfluidic real-time PCR listed in Table S2 [40].

Next, amplicons were sequenced by Eurofins MWG Operon (Ebersberg, Germany), trimmed using BioEdit v.7.2.5 software (Ibis Biosciences, Carlsbad), and analyzed to identify microorganism/tick species using the GenBank database through the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) and Basic Local Alignment Sequence Tool (BLAST) search engine (http://www.ncbi.nlm.nih.gov/blast, accessed on 5 November 2023). Nucleotide sequences data obtained in the current study were submitted to GenBank under the following accession numbers: OR544364 for *Bo. garinii* and OR654151 for *R. helvetica*.

Phylogenetic analysis

We searched a GenBank database to analyze relationships of obtained sequences (targeted at *flaB* gene of *Bo. garinii* and *ompB* gene of *R. helvetica*) with different host species and geographical locations. To this end, sequences of both analyzed species were uploaded to BLAST. This allowed us to select previously identified sequences reported from different host species and different regions. Selected sequences were then aligned using the MUSCLE algorithm in MEGA 11 software and initial phylograms were constructed using Maximum Parsimony (MP), Neighbour-Joining (NJ), and Maximum Likelihood (ML) methods. Due to their similar topologies, the ML method was used in the final analysis. According to the lowest Bayesian Information Criterion (BIC) and Corrected Akaike Information Criterion (AICc), the Jukes-Cantor model (JC) and Tamura 3-parameter model (T92), were used to build the trees of *Bo. garinii* and *R. helvetica*, respectively. The reliability of internal branches was assessed using the bootstrapping method with 1000 replicates [41].

Moreover, to determine the genetic diversity of *Bo. garinii flaB* and *R. helvetica ompB*, sequences showed in the phylograms were grouped into haplotypes (genotypes) using the DnaSP software (Universitat de Barcelona, Spain, http://www.ub.edu/dnasp.; accessed on 5 November 2023). The relationships of specific haplotypes with hosts (taking into account their geographic origin), were illustrated using the Median Joining Network method available in POPArt software (University of Otago Popart, https://popart.maths.otago.ac.nz).

Statistical analysis

To analyze whether the prevalence of pathogen species in ticks was dependent on the bird host species and on the developmental stage of the ticks we used binomial Generalized Linear Mixed Models (GLMMs) with a logit link. In the first model, the prevalence of any pathogen (pooling all pathogen genera) was treated as a binomial response, while the identity of the host species and the developmental stage of ticks were entered as categorical independent factors. To get better insight into the prevalence of the most common pathogen taxa we constructed two additional binomial GLMMs with the same fixed and random factors as in the overall model but treating the prevalence of Borreliaceae in one sub-model and that of *R. helvetica* in another sub-model as binomial response variables. Non-significant interactions ($P<0.1$) between fixed factors were dropped. On the assumption that transovarial transmission of Borreliaceae is relatively rare compared to other common TBPs including Rickettsiales, *Borreliella* and *Borrelia* infection of tick larvae would point to bird hosts acting as reservoir hosts [20]. To account for sampling multiple ticks from the same individual, bird identity was fitted as a random factor. Additionally, to assess potential differences between bird host species in the role as reservoirs of TBPs, i.e., in the richness of pathogens, the number of pathogen species carried by ticks collected from individual birds was related to bird species identity using a Poisson GLMM (log link) with a random term for number of examined ticks per individual bird. The predicted means and 95% confidence intervals were back-transformed for presentation. The computations were performed using Genstat 15.1 (VSN International Ltd).

A significance level of 0.05 was adopted. We calculated 95% confidence interval for the overall prevalence of TBPs in ticks; given the samples sizes, we did not calculate confidence limits for very low prevalence (p) estimates, when $np \geq 5$ and $n(1-p) \geq 5$ [42].

Results

Prevalence of TBPs in juvenile *Ixodes ricinus* ticks

Molecular analysis confirmed the presence of genetic material of ten TBPs in the tested samples. A high prevalence and wide spectrum of TBPs were observed in both larvae and nymphs of *I. ricinus* (Fig. 2; Table 1). Overall, including co-infections, 47.41% (95% CI: 38.19-56.63%) of collected ticks were infected with at least one TBP, i.e., 45.00% (95% CI: 28.89-61.11%) of ticks collected from *E. rubecula*, 40.54% (95% CI: 23.94-57.14%) from *T. philomelos*, and 56.41% (95% CI: 40.13-72.69%) from *T. merula* (Fig. 3). The differences between bird species in the overall pathogen prevalence were not statistically significant (Wald $\chi^2=1.69$, $df=2$, $P=0.430$) (Table 2).

Taking into account all examined bird species, the most frequently detected pathogens in juvenile *I. ricinus* were Borreliaceae spirochetes (35.34%; 95% CI: 26.51-44.17%), i.e., *Borreliella* spp. (4.31%; 95% CI: 0.56-8.06%), *Bo. burgdorferi* s.s. (5.17%; 95% CI: 1.08-9.26%), *Bo. garinii* (18.10%; 95% CI: 10.99-25.22%), *Bo. valaisiana* (6.90%; 95% CI: 2.22-11.58%) and *Borrelia miyamotoi* (0.86%) — a member of relapsing fever group *Borrelia* (RFGB). In addition, we confirmed the presence of the genetic

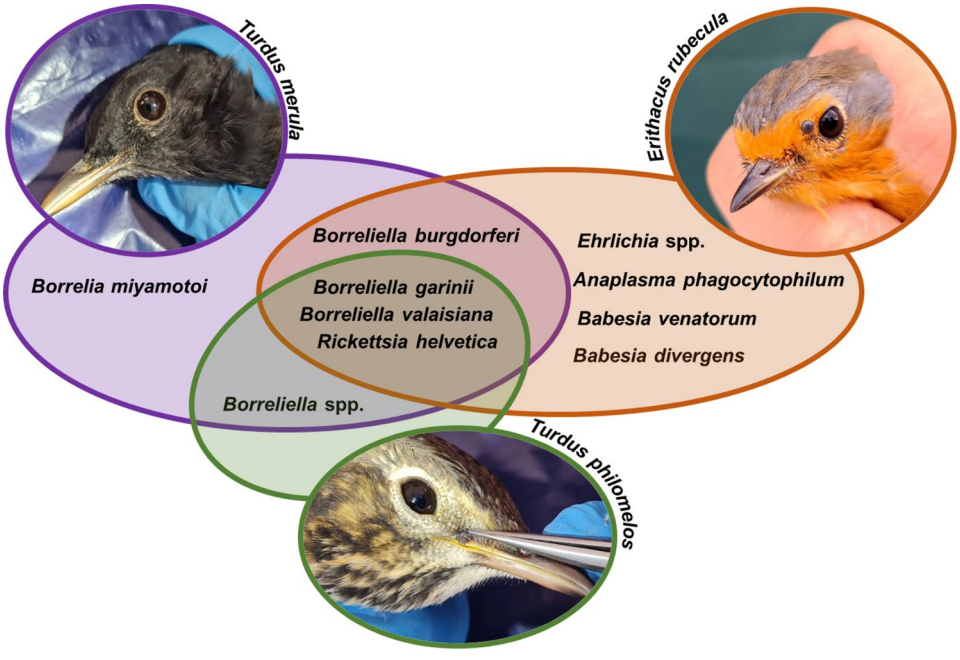

Fig. 2 Spectrum of tick-borne pathogens detected in *Ixodes ricinus* larvae and nymphs depending on bird host species

Table 1 Prevalence of tick-borne pathogens in juvenile *Ixodes ricinus* specimens depending on bird host species

Bird species	Tick stage	Tick-borne pathogens/number of positive samples and percentage rate [%]										
		Borreliella burgdorferi s.s.	**Borreliella garinii**	**Borreliella valaisiana**	**Borreliella spp.**	**Borrelia miyamotoi**	**Rickettsia helvetica**	**Anaplasma phagocytophilum**	**Ehrlichia spp.**	**Babesia divergens**	**Babesia venatorum**	**Overall prevalence of pathogens in ticks***
Erithacus rubecula	L	2	2	1	0	0	5	1	0	0	0	11
(*n* = 30)	(*n* = 19)	(10.53)	(10.53)	(5.26)	(0.00)	(0.00)	(26.32)	(5.26)	(0.00)	(0.00)	(0.00)	(57.89)
	N	1	0	1	0	0	3	1	1	1	1	7
	(*n* = 21)	(4.76)	(0.00)	(4.76)	(0.00)	(0.00)	(14.29)	(4.76)	(4.76)	(4.76)	(4.76)	(33.33)
	L + N	3	2	2	0	0	8	2	1	1	1	18
	(*n* = 40)	(7.50)	(5.00)	(5.00)	(0.00)	(0.00)	(20.00)	(5.00)	(2.50)	(2.50)	(2.50)	(45.00)
Turdus merula	L	2	5	1	1	1	2	0	0	0	0	10
(*n* = 29)	(*n* = 19)	(10.53)	(26.32)	(5.26)	(5.26)	(5.26)	(10.53)	(0.00)	(0.00)	(0.00)	(0.00)	(52.63)
	N	1	8	2	0	0	2	0	0	0	0	12
	(*n* = 20)	(5.00)	(40.00)	(10.00)	(0.00)	(0.00)	(10.00)	(0.00)	(0.00)	(0.00)	(0.00)	(60.00)
	L + N	3	13	3	1	1	4	0	0	0	0	22
	(*n* = 39)	(7.69)	(33.33)	(7.69)	(2.56)	(2.56)	(10.26)	(0.00)	(0.00)	(0.00)	(0.00)	(56.41)
Turdus philomelos	L	0	2	1	2	0	3	0	0	0	0	7
(*n* = 17)	(*n* = 16)	(0.00)	(12.50)	(6.25)	(12.50)	(0.00)	(18.75)	(0.00)	(0.00)	(0.00)	(0.00)	(43.75)
	N	0	4	2	2	0	0	0	0	0	0	8
	(*n* = 21)	(0.00)	(19.05)	(9.52)	(9.52)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(38.10)
	L + N	0	6	3	4	0	3	0	0	0	0	15
	(*n* = 37)	(0.00)	(16.22)	(8.11)	(10.81)	(0.00)	(8.11)	(0.00)	(0.00)	(0.00)	(0.00)	(40.54)
All examined bird species (*n* = 3)	L	4	9	3	3	1	10	1	0	0	0	28
	(*n* = 54)	(7.40)	(16.67)	(5.56)	(5.56)	(1.85)	(18.52)	(1.85)	(0.00)	(0.00)	(0.00)	(51.85)
	N	2	12	5	2	2	5	1	1	1	1	27
	(*n* = 62)	(3.23)	(19.35)	(8.06)	(3.23)	(3.23)	(8.06)	(1.61)	(1.61)	(1.61)	(1.61)	(43.55)
	L + N	6	21	8	5	3	15	2	1	1	1	55
	(*n* = 116)	(5.17)	(18.10)	(6.90)	(4.31)	(2.59)	(12.93)	(1.72)	(0.86)	(0.86)	(0.86)	(47.41)

s.s. - sensu stricto, *n* - number of tested specimens, L - larvae, N – nymphs; *- overall prevalence of pathogens including co-infecions

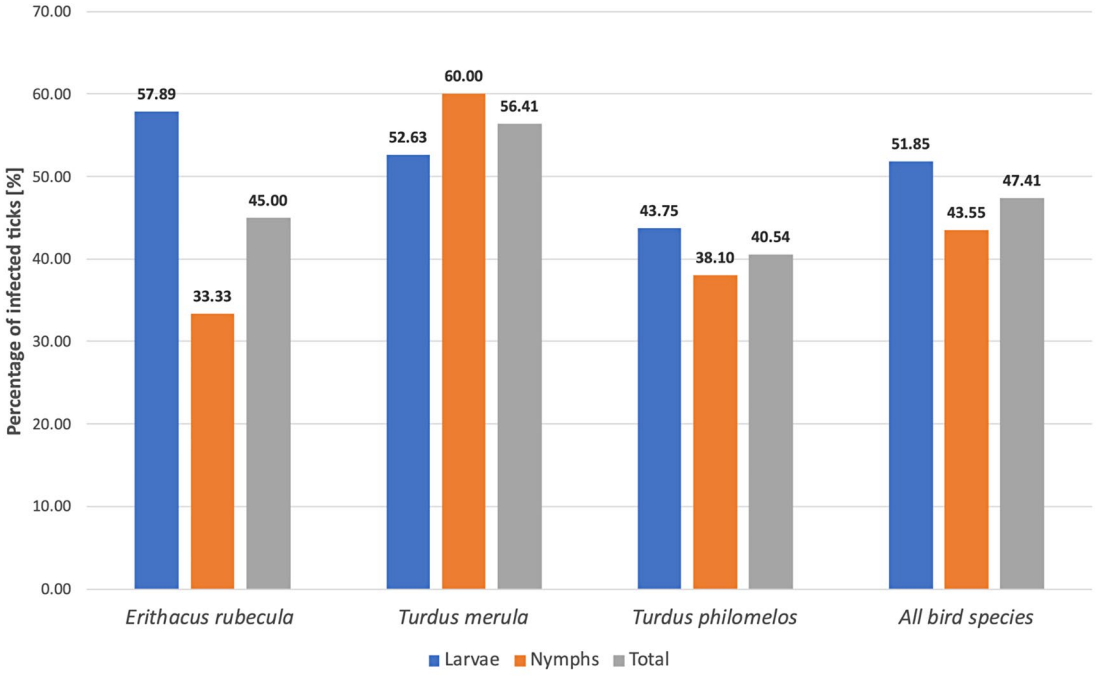

Fig. 3 Prevalence of infection with minimum one tick-borne pathogen in *Ixodes ricinus* larvae and nymphs, depending on bird host species

Table 2 Results of three binomial GLMMs relating the overall prevalence of pathogens (present or absent), the prevalence of Borreliaceae, and the prevalence of *Rickettsia helvetica* in ticks to the tick developmental stage (larvae, nymphs) and to the identity of bird host species. The effects were estimated using the larval stage and *Erithacus rubecula* as the reference levels. Mean effect size with standard error of differences (SED; for bird species the average standard error of differences) is reported. Means and confidence intervals were back-transformed. Individual bird identity was fitted as a random term

Dependent variable	Predictor	Wald	df	*P*	Mean	95% CI	Effect	SED
Overall prevalence of pathogens in ticks	**Stage**							
	Larvae	0.70	1	0.401	0.52	0.37–0.66	–0.339	0.404
	Nymphs				0.43	0.31–0.51		
	Bird species							
	E. rubecula	1.69	2	0.430	0.44	0.27–0.63	0.000	0.522
	T. philomelos				0.57	0.39–0.73	–0.120	
	T. merula				0.41	0.26–0.59	0.515	
Prevalence of Borreliaceae in ticks	**Stage**							
	Larvae	0.16	1	0.690	0.35	0.21–0.52	–0.189	0.475
	Nymphs				0.30	0.18–0.46		
	Bird species							
	E. rubecula	9.50	2	0.009*	0.14	0.07–0.27	0.000	0.649
	T. philomelos				0.35	0.17–0.59	1.158	
	T. merula				0.55	0.33–0.75	1.981	
Prevalence of *Rickettsia helvetica* in ticks	**Stage**							
	Larvae	2.62	1	0.108	0.17	0.07–0.35	–0.956	0.591
	Nymphs				0.07	0.04–0.14		
	Bird species							
	E. rubecula	2.62	2	0.274	0.19	0.10–0.35	0.000	0.735
	T. philomelos				0.10	0.04–0.23	-1.027	
	T. merula				0.08	0.02–0.22	-0.816	

df - degree of freedom, *P* - level of significance, CI - confidence intervals of means, SED - standard error of differences, * - statistically significant

material of Rickettsiales, i.e., *R. helvetica* (12.93%; 95% CI: 6.73-19.13%), *A. phagocytophilum* (1.72%), and *Ehrlichia* spp. (0.86%) as well as apicomplexan parasites *Ba. divergens* (0.86%) and *Ba. venatorum* (0.86%) (Table 1).

Juvenile *I. ricinus* specimens collected from *T. merula* exhibited a high prevalence of Borreliaceae spirochetes, with *Bo. garinii* being the most frequently detected genospecies (up to 40.00% of nymphs), while *B. miyamotoi* infection was confirmed in one *I. ricinus* larva collected from this bird species (Table 1). The prevalence of Borreliaceae spirochetes differed depending on the bird host species from which they were collected (Table 2). However, based on standard errors of differences of means, significant differences were observed only between ticks collected from *E. rubecula* and those from *T. merula* (Wald $\chi^2=9.50$, $df=2$, $P=0.009$) (Table 2).

Rickettsia helvetica infection was confirmed in ticks collected from all examined bird species (Fig. 2; Table 1). The genetic material of *A. phagocytophilum*, *Ehrlichia* spp., *Ba. divergens* and *Ba. venatorum* was confirmed only in ticks collected from *E. rubecula*. Thus, the widest spectrum of TBPs was detected in ticks collected from this bird species (Fig. 2; Table 1). However, the Poisson GLMM did not reveal any significant differences between avian host species in the species richness of pathogens in ticks per individual bird, with back-transformed means ranging from 0.54 (95% CI 0.35–0.82) in *E. rubecula* to 0.79 (0.48–1.28) in *T. merula* (Wald $\chi^2=1.31$, $df=2$, $P=0.520$).

Coinfections of TBPs in juvenile *Ixodes ricinus* ticks

Co-infections of two TBPs, i.e., representatives of Borreliaceae and *R. helvetica* were confirmed in ticks collected from *T. merula* (up to 5.13%), and *T. philomelos* (2.70%). We also confirmed co-infections of *A. phagocytophilum* and *R. helvetica* (2.50%), as well as *Bo. valaisiana* and *Ba. divergens* (2.50%) in ticks collected from *E. rubecula* (Fig. S1; Table 3).

Table 3 Co-infections of tick-borne pathogens detected in juvenile *Ixodes ricinus* ticks

Bird species	Number of tested ticks	Vector-borne pathogens	Number of infected ticks/tick stage	Coinfection rate [%]
Erithacus rubecula *n* = 30	40	*Borreliella valaisiana* and *Babesia divergens*	1/N	2.50
		Anaplasma phagocytophilum and *Rickettsia helvetica*	1/N	2.50
Turdus merula *n* = 29	39	*Borreliella burgdorferi* s.s. and *Rickettsia helvetica*	2/L	5.13
		Borreliella garinii and *Rickettsia helvetica*	1/N	2.56
Turdus philomelos *n* = 17	37	*Borreliella garinii* and *Rickettsia helvetica*	1/L	2.70

n - number of tested individuals, L - larvae, N - nymphs

Genetic diversity of TBPs in juvenile *Ixodes ricinus* and tick-host-pathogen associations

The phylogenetic analysis of the *Bo. garinii flaB* sequence confirmed the species identity. The genetic diversity of the examined sequence confirmed its assignment to the H1 haplotype, commonly found in Poland and other European countries, as well as in Eastern Asia. This haplotype was shown to be frequently detected in a wide spectrum of hosts, i.e., in ticks, rodents, insects, canids, and humans (Fig. 4). Phylogenetic analysis of the *R. helvetica ompB* sequence allowed us to conclude that the analyzed sequence shows similarity to the H3 haplotype circulating among ticks, mainly *I. ricinus*, while geographically the range of this haplotype is limited to Central Europe (Fig. 5).

Discussion

In the current study we found a high prevalence of TBPs in juvenile *I. ricinus* collected from all examined bird species. Additionally, we observed the widest spectrum of TBPs in ticks collected from *E. rubecula.* In our opinion, high infection with TBPs is supported by the ground-foraging behavior of *E. rubecula*, *T. merula*, and *T. philomelos* which may favor tick infestation. Other studies have also confirmed such relationships [43].

Our results highlight spirochetes Borreliaceae as the most frequently detected pathogens in ticks collected from birds, followed by *R. helvetica* and *Babesia* spp. Similar observations regarding the high prevalence of *Borreliella* spp. in ticks collected from birds have been confirmed several times in other studies [44–47]. Notably, the prevalence of *Borreliella* spp. in *I. ricinus* larvae, particularly *Bo. garinii* and *Bo. valaisiana*, considered to be bird-associated Borreliaceae species, may suggest the significant role of birds in the circulation of this pathogen in the environment [48, 49]. Taking into account only sporadic transovarial transmission of *Borreliella* spirochetes (or during ticks co-feeding) [20, 50], infected *I. ricinus* larvae are considered informative indicators of TBPs prevalence in birds, as their microbial communities often reflect the composition of the host's blood or skin microbiome [51, 52]. Also, previous studies have suggested the importance of *T. merula* and *T. philomelos* as a reservoir of *Bo. garinii* [53, 54]. Our results confirmed the presence of *Borreliella* spirochetes also in larvae removed from *E. rubecula*. In addition, we found one *I. ricinus* larva collected from *T. merula* infected with *B. miyamotoi*. This may suggest a role for *T. merula* as a competent reservoir of this pathogen as reported in previous studies [55, 56]. The differences between bird

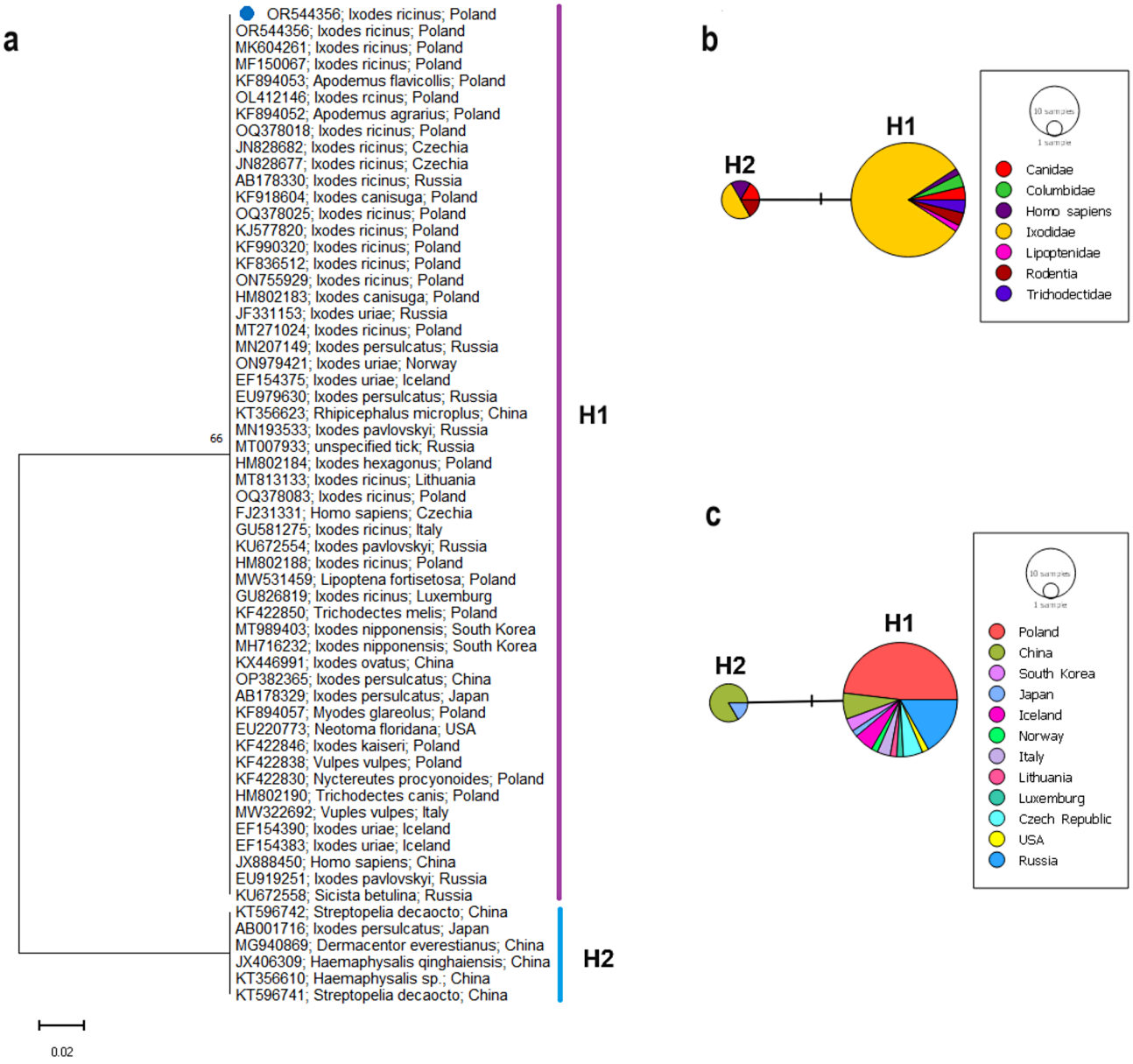

Fig. 4 Phylogeny and genotype analysis of *Borreliella garinii* inferred from sequences depending on host reservoir species and country of origin. Phylogram (**a**) shows the position of the sequence obtained in the current study targeted *flaB* gene (marked blue) and other previously reported sequences available in GenBank (accession numbers and country of origin are displayed). The phylogenetic tree was inferred using the Maximum Likelihood method and the Jukes-Cantor model (JC). The tree is drawn to scale with branch lengths measured in the number of substitutions per site. This analysis involved 60 nucleotide sequences with a total of 6 positions in the final dataset. Analysis was conducted with the use of the complete deletion option. Bootstrap values are represented as percent of internal branches (1000 replicates). Only values ≥ 60% are shown. Sequences are grouped into haplotypes (H1 and H2). Panels (**b**) and (**c**) present networks of haplotype divergence analysis of the same *Borreliella garinii* sequences as in panel (**a**) with the use of the Median Joining Network method regarding the host types (**b**) and country of origin (**c**). The proportional size of nodes indicates the frequency of haplotypes. Small black dots represent inferred haplotypes. The diagonal lines indicate the number of mutations between the haplotypes

species (here between *E. rubecula* and *T. merula*) in the prevalence of Borreliaceae in the carried ticks require further research. A possible explanation can be the high overall tick infestation in *T. merula* [33], which increases the probability of birds' infection and transfer of pathogens to ticks. *T. merula* as a reservoir/amplifying host of Borreliaceae spirochetes, is all the more important from the epidemiological perspective, as the species has been urbanized since the 19th century [57]. It is worth noting that in our study, some larvae collected from the same bird differed in terms of detected TBPs (Fig. S1), which could be explained by many factors, including duration of feeding time (increased length of feeding promotes the probability of TBPs transmission from the host),

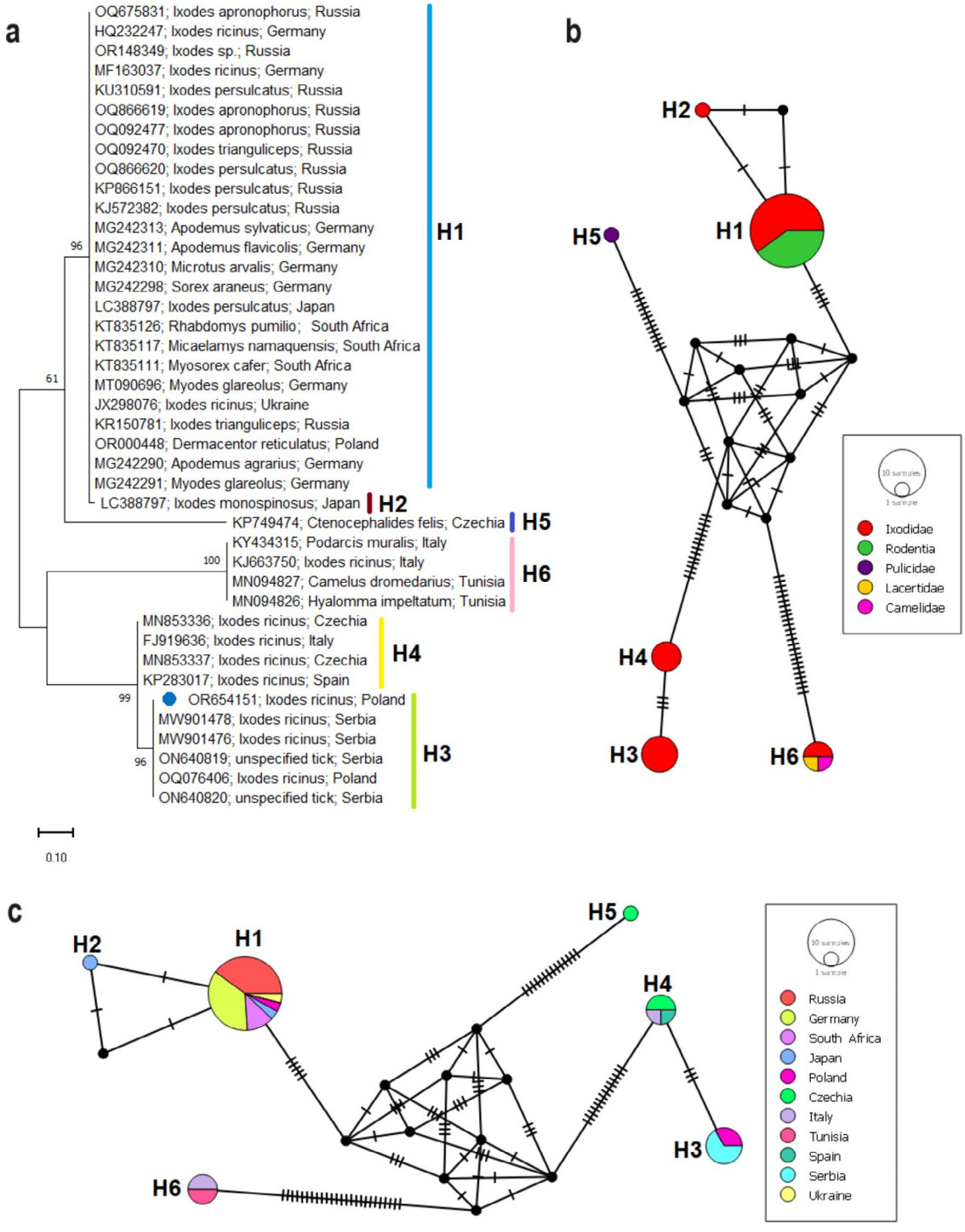

Fig. 5 Phylogeny and genotype analysis of *Rickettsia helvetica* inferred from sequences depending on host reservoir species and country of origin. Phylogram (**a**) shows the position of the sequence obtained in the current study targeted *ompB* gene (marked blue) and other previously reported sequences available in GenBank (accession numbers and country of origin are displayed). The phylogenetic tree was inferred using the Maximum Likelihood method and the Tamura 3-parameter model (T92). The tree is drawn to scale with branch lengths measured in the number of substitutions per site. This analysis involved 41 nucleotide sequences with a total of 62 positions in the final dataset. Analysis was conducted with the use of the complete deletion option. Bootstrap values are represented as percent of internal branches (1000 replicates). Only values ≥ 60% are shown. Sequences are grouped into haplotypes (H1 to H6). Panels (**b**) and (**c**) present networks of haplotype divergence analysis of the same *Rickettsia helvetica* sequences as in panel (**a**) with the use of the Median Joining Network method regarding the host types (**b**) and country of origin (**c**). The proportional size of nodes indicates the frequency of haplotypes. Small black dots represent inferred haplotypes. The diagonal lines indicate the number of mutations between the haplotypes

interference of compounds found in tick saliva and host immune response [58].

Rickettsiales were the second most frequently detected TBPs in juvenile *I. ricinus* ticks, with a high prevalence of *R. helvetica* infections. Other studies have shown that *Rickettsia* spp. is one of the most frequently detected TBPs in ticks feeding on birds [59, 60]. Despite frequent detection of this pathogen in ticks feeding on birds, the role of birds as a competent reservoir host for *R. helvetica* remains uncertain [59], mainly due to the potential of transovarial transmission [61, 62], and demands further research using samples taken directly from birds.

Anaplasma phagocytophilum is relatively rarely detected in ticks collected from birds and the prevalence of infection with this pathogen varies, e.g. up to 9.00% as previously reported [11]. We found *A. phagocytophilum* infection only in two ticks (one larva and one nymph) removed from one *E. rubecula* with a prevalence of up to 5.26% (Table 1).

Similarly, *Ehrlichia* spp. is relatively rare in bird-feeding ticks [63, 64], however, a large sample survey conducted in Sweden confirmed the importance of migratory birds in the spread of ticks infected with this pathogen [65]. As in the mentioned study, we confirmed *Ehrlichia* spp. infection in nymph (Table 1). The role of birds in the circulation of *Ehrlichia* spp. was also indicated in earlier studies, including Brazil [66] and Hungary where the pathogen was detected in the blood of *T. philomelos* [67].

Our results also confirmed the infection of *I. ricinus* larvae with the protozoan *Babesia* spp., with a low prevalence comparable to that found in ticks collected from migratory birds in other countries [14, 68, 69]. Although large domestic and wild ruminants, including cattle *Bos taurus* and roe deer *Capreolus capreolus*, are recognized as the main reservoir of *Babesia* spp., it is believed that birds can feature in the circulation of the pathogen in the environment [70–72].

Phylogenetic analyses of the *Bo. garinii flaB* and *R. helvetica ompB* sequences showed the complexity of the TBPs circulation phenomenon in the environment. We revealed that detected haplotype of *Bo. garinii* is strongly associated with ticks but is also characterized by a wide range of potential hosts, including pigeons, rodents, insects, canids, and humans (Fig. 4). Similarly, the results of our study on the genetic diversity of *R. helvetica* indicate that its main vector remains *I. ricinus* tick (Fig. 5). It is worth noting that *R. helvetica* is among the most frequently detected species of the *Rickettsia* genus in *I. ricinus* ticks [73, 74].

Conclusions

Our results confirmed the high prevalence and wide spectrum of TBPs in juvenile *Ixodes ricinus* ticks collected from *Turdus merula*, *Turdus philomelos*, and *Erithacus rubecula* migrating along the Vistula Valley, Poland. We found that Borreliaceae spirochetes and *Rickettsia helvetica* were the most frequently detected pathogens. In addition, tested ticks carry widespread strains of *Borreliella garinii*, in contrast to *R. helvetica*, which is mainly found in Central Europe. Therefore, further research on the possible role of birds as reservoirs of TBPs is needed.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04415-y.

Supplementary Material 1

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Author contributions
JK – conceptualization, methodology, fieldwork, molecular analysis, data analysis, phylogenetic analysis, visualization, writing original draft, writing – review and editing; ZZ – conceptualization, methodology, fieldwork, molecular analysis, data analysis, visualization, writing original draft, writing – review and editing; AFS – molecular analysis, data analysis, writing – review and editing; AW – fieldwork, molecular analysis, writing – review and editing; MF – fieldwork, statistical analysis, writing original draft, writing – review and editing; JKl – statistical analysis, writing – review and editing; RR – fieldwork, writing – review and editing; AC – phylogenetic analysis, writing – review and editing; KB – molecular analysis, writing – review and editing; SM – writing – review and editing; ACC – writing original draft, writing – review and editing.

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Data availability
All data supporting the findings of this study are available within the paper.

Declarations

Ethics approval and consent to participate
The study, including bird capture and tick collection procedure was approved by the General Directorate of Nature Conservation in Warsaw (DZP-WG.6401.102.2020.TŁ) and the Regional Directorate of Nature Conservation in Lublin (WPN.6401.108.2021.KC) and in accordance with the ARRIVE principles (Animal Research: Reporting of in Vivo Experiments). In light of current Polish legislation (Act on the Protection of Animals Used for Scientific or Educational Purposes. Dz. U. 2015 poz. 266) no further permissions, including ethics committee approval were required. Informed consent was not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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