Parasitology Research (2019) 118:3205–3216
https://doi.org/10.1007/s00436-019-06506-4

ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Ixodes inopinatus in northern Germany: occurrence and potential vector role for *Borrelia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum* in comparison with *Ixodes ricinus*

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Received: 5 April 2019 / Accepted: 11 October 2019 / Published online: 12 November 2019

Abstract

In 2014, a new tick species, *Ixodes inopinatus*, was described, which is closely related to *Ixodes ricinus.* So far, *I. inopinatus* has been found in Tunisia, Morocco, Spain, Portugal, Romania, Austria, and southern Germany. No data is yet available regarding occurrence of *I. inopinatus* in northern Germany and the potential role of *I. inopinatus* as a vector for tick-borne pathogens. Therefore, 3845 DNA samples from *Ixodes* ticks collected for prevalence studies on *Borrelia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum* during the years 2010–2015 in the northern German cities of Hamburg and Hanover were differentiated into *I. ricinus* or *I. inopinatus* by sequencing a part of the 16S rRNA gene. In total, 4% (137/3845) of the sequenced ticks were assigned to the species *I. inopinatus* and 96% (3708/3845) to *I. ricinus*. The prevalence of *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* DNA in *I. inopinatus* was 34% (46/137), 46% (63/137), and 3% (4/137), respectively, whereas the prevalence of these bacteria in *I. ricinus* was 25% (919/3708), 47% (1729/3708), and 4% (135/3708), respectively. Compared with *I. ricinus*, significantly more *I. inopinatus* ticks tested positive for *Borrelia*. To the best of our knowledge, this is the first report of *I. inopinatus* in northern Germany. Detection of the DNA of *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* in questing *I. inopinatus* indicates a potential role of this tick species as a vector of these pathogens, which needs to be confirmed by transmission experiments.

Keywords Ticks · Tick-borne diseases · *Borrelia burgdorferi* sensu lato · *Borrelia miyamotoi* · Rickettsiales · 16S rRNA

Introduction

The hard tick *Ixodes ricinus* is a widespread tick species in Europe. Its current distribution extends from approximately 10° W (Ireland) to 45° E (Ural Mountains, Russia) and from 65° N (Norway) to 30° N (Egypt) (Randolph et al. 2002; Soleng et al. 2018). In many parts of Europe, *I. ricinus* constitutes the main vector for a range of tick-borne bacterial and viral pathogens with relevance for human and animal health. Notable bacterial tick-borne pathogens transmitted by *I. ricinus* are spirochetes of *Borrelia burgdorferi* sensu lato (s.l.) complex including the causative agents of Lyme borreliosis (LB), as well as the relapsing-fever spirochete *B. miyamotoi*, which has been associated with febrile illness and, additionally, with meningoencephalitis in immunocompromised patients (Gugliotta et al. 2013; Hovius et al. 2013). Furthermore, several species of *Rickettsia* occur in Europe, which may cause spotted fever or lymphadenopathy in humans (Dobler and Wölfel 2009). In Germany, *R. helvetica*, *R. monacensis*, *R. massiliae*, and *R. felis* were detected in *I. ricinus* ticks, the most prevalent of which was *R. helvetica* (Dobler and Wölfel 2009; Schicht et al. 2012; Tappe and Strube 2013; May and Strube 2014; Blazejak et al. 2017). Another member of the order Rickettsiales,

Section Editor: Neil Bruce Chilton

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-019-06506-4) contains supplementary material, which is available to authorized users.

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Anaplasma phagocytophilum, may lead to granulocytic anaplasmosis in humans, dogs, horses, and cattle (Dumler et al. 2005).

In 2014, Estrada-Peña et al. described a new tick species, *I. inopinatus*, which is closely related to *I. ricinus*. As the life stages of both species can only be distinguished by subtle morphological characteristics, such as punctuations on the dorsal scutum and the number of conscutal setae in males, the shape of the anal groove, and the number of concentric rows of goblets in the spiracular plate in nymphs and females, many *I. inopinatus* specimens may have been misclassified as *I. ricinus* in the past (Chitimia-Dobler et al. 2018; Estrada-Peña et al. 2014). Genetically, *I. ricinus* can be differentiated from *I. inopinatus* based on differences in the 16S rDNA sequence (Estrada-Peña et al. 2014).

Ixodes inopinatus has been reported from parts of Spain, Portugal, Morocco, and Tunisia, and in the southwestern German federal state Rhineland-Palatinate (Estrada-Peña et al. 2014). Recently, *I. inopinatus* was found in sympatry with *I. ricinus* in Romania and western Austria, and at three sampling sites in the southern German federal state Bavaria (Chitimia-Dobler et al. 2018). So far, the reported natural hosts include foxes, sheep, and lizards (Chitimia-Dobler et al. 2018; Estrada-Peña et al. 2014). While the vector potential of *I. ricinus* is well described, and many studies have assessed pathogen prevalence in questing *I. ricinus* throughout Europe (e.g., Blazejak et al. 2017; Blazejak et al. 2018; Fingerle et al. 1999; Franke et al. 2010; Henningsson et al. 2015; May et al. 2015; May and Strube 2014; Schorn et al. 2011; Venczel et al. 2016), the role of *I. inopinatus* as a vector of bacterial pathogens is still unknown. In a recent study, three *I. inopinatus* specimens tested positive for *Rickettsia* spp. (Chitimia-Dobler et al. 2018), but reports of *I. inopinatus* as a carrier of other pathogens are lacking.

In the present study, we aimed to investigate the occurrence of *I. inopinatus* in the northern German cities of Hanover and Hamburg and its potential role as a vector for *Borrelia*, *Rickettsia*, and *A. phagocytophilum*. Furthermore, the local distribution, seasonal activity patterns, and the respective pathogen DNA detection rates were compared with those of *I. ricinus*.

Materials and methods

Tick collection and pathogen testing

Questing nymphal and adult ticks were collected by the flagging method at ten different sampling sites in the city of Hanover from April to October of 2010 and 2015, and at ten sites in the city of Hamburg from April to October 2011, in previous studies on pathogen prevalence in *Ixodes* spp. (detailed information on sampling sites can be found in Blazejak et al. 2017; May and Strube 2014; Tappe and Strube 2013). Twenty (Hamburg) or thirty (Hanover) ticks were flagged per sampling site and month. Morphological differentiation of *I. inopinatus* was not possible, as a majority of the ticks were collected before the description of this species (Estrada-Peña et al. 2014) and had been used in pathogen prevalence studies. Thus, only DNA samples were available for this study. Methods regarding qPCR testing for *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* as well as *Borrelia* (geno-)species determination by Reverse Line Blot (RLB) and *Rickettsia* spp. identification by real-time pyrosequencing have been published elsewhere (Blazejak et al. 2017; Blazejak et al. 2018; May et al. 2015; May and Strube 2014; Tappe et al. 2014; Tappe and Strube 2013). Detection of *B. miyamotoi* was implemented for the ticks collected in Hanover in 2015, after modification of the RLB protocol for additional detection of *B. miyamotoi* (Blazejak et al. 2018).

Molecular tick species identification

In total, 3845 DNA samples consisting of 2709 from the city of Hanover (758 from 2010, 1951 from 2015) and 1136 from the city of Hamburg (from the year 2011) were available for sequencing. Part (420 bp) of the 16S rRNA gene was amplified using primers described by Mangold et al. (1998). The reaction mixture was partially adapted from previous studies (Mangold et al. 1997; Mangold et al. 1998) and contained 2 μl of each primer (10 μM each), 1 μl dNTPs (10 mM, Roti®-Mix PCR 3, Carl Roth GmbH + Co.KG, Karlsruhe, Germany), 5 μl 10× buffer, 0.5 μl Taq polymerase (Dream Taq, Thermo Fisher Scientific, Epsom, UK), and 4 μl tick DNA template in 50 μl of reaction volume. Thermocycling conditions were adapted from Mangold et al. (1998). After an initial denaturation at 95 °C for 3 min the first 7 cycles were performed as described in Mangold et al. (1998), followed by 31 cycles at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 45 s. Amplicons were separated by electrophoresis on 1.5% agarose gels stained with GelRed® (Biotium Inc., Fremont, CA, USA) and visualized under UV light. In case of non-amplification in the first PCR run, the amount of template was increased to 6 μl and the number of PCR cycles to 41. Obtained PCR products were Sanger-sequenced at the Microsynth Seqlab Laboratories (Göttingen, Germany) and aligned with corresponding 16S rRNA sequences of *I. ricinus* and *I. inopinatus* published by Estrada-Peña et al. (2014) (GenBank accession nos. KM211785, KM211786, KM211787, KM211788 [*I. ricinus*]; KM211789, KM211790 [*I. inopinatus*]) and other selected sequences available in GenBank (accession nos. GU074592, GU074605 [*I. ricinus*]; KY569415, KY569416, KY569417, KY569418 [*I. inopinatus*]) using Clone Manager 9 Professional Edition (Scientific & Educational Software, Denver, USA). Based on two adjacent single nucleotide polymorphisms of the published sequences, all sequences that were at least 110 bp in length were assigned either to *I. ricinus* (CT at

Table 1 Occurrence of *Ixodes inopinatus* and *I. ricinus* in the cities of Hanover and Hamburg (*N* nymphs, *AF* adult females, *AM* adult males)

Sampling site	*I. inopinatus*					*I. ricinus*				
	No./total	%	N	AF	AM	No./total	%	N	AF	AM
Total ticks	137/3845	3.6	96	24	17	3708/3845	96.4	2911	404	393
Hanover 2015	75/1951	3.8	40	21	14	1876/1951	96.2	1355	257	264
Hanover 2010	26/758	3.4	23	1	2	732/758	96.6	581	94	57
Hamburg 2011	36/1136	3.2	33	2	1	1100/1136	96.8	975	53	72

position 184/185, KM211785 used as reference sequence) or *I. inopinatus* (AG at position 184/185, KM211789 used as reference sequence).

Statistical analyses

Statistical analyses were conducted in R v. 3.3.1 (R Core R Core Team 2018). The ratio of *I. inopinatus* to *I. ricinus* was compared between tick stages and between the two different sampling locations (Hanover vs. Hamburg), as well as between the three sampling years (2010, 2011, and 2015) using Chi-square tests. Monthly and sampling-site specific differences regarding the number of samples identified as *I. inopinatus* vs. *I. ricinus* were then tested separately for Hamburg and Hanover, using generalized linear models (GLMs) with binominal error structure and "sampling site" as well as "month" as predictors. The model for the Hanover dataset also included the factor "sampling year." Each model was compared with a null model including only the intercept in a likelihood ratio test (R function "anova," test = "chisq").

Potential factors influencing the probability of *Anaplasma*, *Rickettsia*, and *Borrelia* spp. infections were tested in generalized linear mixed effects models (GLMMs) with logit link function and binomial assumption, with the fixed factors "tick species" (*I. inopinatus*/*I. ricinus*), "month" (April to October), "developmental stage" (nymph/adult), and "year" (2010, 2011, 2015). To account for repeated measurements at the same sampling site, this factor was included as a random effects term. Final models were compared with null models containing only the random effects term in a likelihood ratio test (R function "anova", test = "chisq"). The predictive variables sampling month and sampling year were subjected to a post hoc analysis, computing all pairwise differences between months and years in a Tukey's test based on the parameters of the fitted GLMM.

Results

Occurrence of *I. inopinatus* and *I. ricinus*

In total, 3.6% (137/3845) of the sequenced ticks were assigned to the species *I. inopinatus*, including 4.2% (17/410) of male adult ticks, 5.6% (24/428) of female adult ticks, and 3.2% (96/3007) of analyzed nymphs. The remaining 96.4% (3708/3845) of ticks were assigned to the species *I. ricinus*, namely 95.9% (393/410) of adult males, 94.4% (404/428) of adult females, and 96.8% (2911/3007) of nymphs.

Concerning the two different cities, 3.7% (101/2709) of analyzed ticks from Hanover (3.4% [26/758] in 2010 and 3.8% [75/1951] in 2015) and 3.2% (36/1136) of ticks from Hamburg were assigned to the recently described tick species *I. inopinatus*, while 96.3% (2608/2709) of analyzed ticks from Hanover (96.6% [732/758] in 2010 and 96.2% [1876/1951] in 2015) and 96.8% (1100/1136) of ticks from Hamburg were assigned to *I. ricinus*. More detailed results are listed in Table 1. No statistically significant differences in the proportion of ticks identified as *I. inopinatus* were found between tick stages, between the two locations (Hamburg and Hanover), and between the two different sampling years in Hanover (Chi-square tests, $P > 0.05$).

Distribution of *I. inopinatus* and *I. ricinus* per sampling site is presented in Supplementary Table 1. Concerning the ten different sampling sites in the city of Hanover, statistically significant differences regarding the *I. inopinatus* to *I. ricinus* ratio were detected only between the sites "Ricklinger Teiche" (*I. inopinatus*: 7.1% [22/308], *I. ricinus*: 92.9% [286/308]) and "Bornumer Holz" (*I. inopinatus*: 1.3% [4/299], *I. ricinus*: 98.7% [295/299]; $P = 0.043$, Table 2). No significant differences between sampling months were found. For the city of Hamburg, no statistically significant differences between sampling sites or sampling months were found after correcting for multiple comparisons (Table 3).

There was a slight intraspecific genetic variation among *I. ricinus* and *I. inopinatus* specimens. The nucleotide identity among *I. inopinatus* sequences was 97–100% and 98–100% among *I. ricinus* sequences, comparing a 100-bp area around the two adjacent single nucleotide polymorphisms (position 134 to 234 with reference to KM211789 for *I. inopinatus* and KM211785 for *I. ricinus*).

Table 2 Results of the GLM testing differences in the percentage of samples assigned to *I. inopinatus* between different sampling sites and sampling months in the city of Hanover. The full model was significantly different from a null model containing only an intercept term (Chi-square = 35.4, Df = 16, $P = 0.007$). Significant *P*-values (≤ 0.05) are indicated by italics.

	Estimate	SE	*z*	*P*
Intercept	3.530	0.526	6.717	*< 0.001*
Year 2015 vs. 2010	− 0.108	0.244	− 0.442	0.659
Sampling months*				
May vs. April	− 0.342	0.512	− 0.668	0.994
June vs. April	− 0.212	0.474	− 0.447	0.999
July vs. April	0.192	0.442	0.436	0.999
August vs. April	0.405	0.434	0.935	0.966
September vs. April	0.549	0.437	1.255	0.869
October vs. April	0.128	0.453	0.282	1.000
June vs. May	0.130	0.466	0.279	1.000
July vs. May	0.534	0.433	1.234	0.878
August vs. May	0.747	0.426	1.755	0.573
September vs. May	0.891	0.436	2.044	0.381
October vs. May	0.470	0.447	1.051	0.940
July vs. June	0.404	0.376	1.074	0.934
August vs. June	0.617	0.370	1.667	0.632
September vs. June	0.760	0.388	1.962	0.433
October vs. June	0.340	0.395	0.859	0.978
August vs. July	0.213	0.327	0.652	0.995
September vs. July	0.356	0.347	1.026	0.947
October vs. July	− 0.065	0.355	− 0.182	1.000
September vs. August	0.143	0.337	0.425	1.000
October vs. August	− 0.278	0.348	− 0.799	0.985
October vs. September	− 0.421	0.363	− 1.158	0.907
Sampling sites*				
Bornumer Holz vs. Annateiche	1.000	0.599	1.670	0.803
Eilenriede vs. Annateiche	− 0.134	0.458	− 0.292	1.000
Georgengarten vs. Annateiche	0.889	0.599	1.484	0.892
Große Heide vs. Annateiche	− 0.098	0.447	− 0.220	1.000
Maschpark vs. Annateiche	− 0.303	0.439	− 0.689	1.000
Mecklenheide vs. Annateiche	− 0.526	0.420	− 1.254	0.961
Misburger Wald vs. Annateiche	0.592	0.556	1.064	0.987
Ricklinger Teiche vs. Annateiche	− 0.758	0.392	− 1.933	0.632
Seelhorster Wald vs. Annateiche	0.171	0.484	0.354	1.000
Eilenriede vs. Bornumer Holz	− 1.133	0.599	− 1.891	0.661
Georgengarten vs. Bornumer Holz	− 0.110	0.713	− 0.155	1.000
Große Heide vs. Bornumer Holz	− 1.098	0.591	− 1.859	0.683
Maschpark vs. Bornumer Holz	− 1.302	0.585	− 2.225	0.424
Mecklenheide vs. Bornumer Holz	− 1.526	0.570	− 2.675	0.174
Misburger Wald vs. Bornumer Holz	− 0.408	0.677	− 0.602	1.000
Ricklinger Teiche vs. Bornumer Holz	− 1.758	0.551	− 3.191	*0.043*
Seelhorster Wald vs. Bornumer Holz	− 0.828	0.619	− 1.338	0.941
Georgengarten vs. Eilenriede	1.023	0.599	1.707	0.781
Große Heide vs. Eilenriede	0.036	0.447	0.079	1.000
Maschpark vs. Eilenriede	− 0.169	0.439	− 0.385	1.000
Mecklenheide vs. Eilenriede	− 0.392	0.419	− 0.935	0.995
Misburger Wald vs. Eilenriede	0.726	0.557	1.304	0.950

Table 2 (continued)

	Estimate	SE	*z*	*P*
Ricklinger Teiche vs. Eilenriede	− 0.625	0.394	− 1.586	0.847
Seelhorster Wald vs. Eilenriede	0.305	0.484	0.631	1.000
Große Heide vs. Georgengarten	− 0.988	0.591	− 1.671	0.802
Maschpark vs. Georgengarten	− 1.192	0.586	− 2.036	0.559
Mecklenheide vs. Georgengarten	− 1.415	0.571	− 2.481	0.266
Misburger Wald vs. Georgengarten	− 0.297	0.678	− 0.439	1.000
Ricklinger Teiche vs. Georgengarten	− 1.648	0.551	− 2.988	0.078
Seelhorster Wald vs. Georgengarten	− 0.718	0.619	− 1.159	0.977
Maschpark vs. Große Heide	− 0.204	0.429	− 0.477	1.000
Mecklenheide vs. Große Heide	− 0.428	0.408	− 1.049	0.988
Misburger Wald vs. Große Heide	0.690	0.547	1.260	0.959
Ricklinger Teiche vs. Große Heide	− 0.660	0.380	− 1.737	0.763
Seelhorster Wald vs. Große Heide	0.270	0.474	0.569	1.000
Mecklenheide vs. Maschpark	− 0.224	0.399	− 0.560	1.000
Misburger Wald vs. Maschpark	0.894	0.541	1.652	0.812
Ricklinger Teiche vs. Maschpark	− 0.456	0.372	− 1.225	0.966
Seelhorster Wald vs. Maschpark	0.474	0.466	1.016	0.991
Misburger Wald vs. Mecklenheide	1.118	0.525	2.128	0.492
Ricklinger Teiche vs. Mecklenheide	− 0.232	0.349	− 0.666	1.000
Seelhorster Wald vs. Mecklenheide	0.698	0.448	1.558	0.860
Ricklinger Teiche vs. Misburger Wald	− 1.350	0.505	− 2.674	0.174
Seelhorster Wald vs. Misburger Wald	− 0.420	0.578	− 0.728	0.999
Seelhorster Wald vs. Ricklinger Teiche	0.930	0.423	2.197	0.443

SE standard error

*Multiple comparisons between the levels of month and sampling site were performed using Tukey contrasts with single-step *P* value adjustment

I. inopinatus as a potential vector of bacterial pathogens

In addition to *I. ricinus*, DNA of all three investigated bacterial pathogens (*Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum*) were detected in the recently described species *I. inopinatus*. The prevalence of each bacterium for each tick species is shown in Table 4. A coinfection rate of 21.2% (29/137) in *I. inopinatus* compared with 14.1% (524/3708) in *I. ricinus* was determined. Statistical analyses revealed a significantly higher prevalence of *Borrelia* spp. DNA in *I. inopinatus* compared with *I. ricinus* ($P = 0.020$; odds ratio = 1.56; Table 5, Fig. 1). In addition, the detection rate of *Borrelia* spp. DNA was significantly higher in adult ticks than that in nymphs ($P < 0.001$). Significantly less *Borrelia*-positive ticks were observed in April than there were in June, August, and October. Furthermore, significant differences were detected between study years (Table 5).

Borrelia (geno-)species determination by RLB was successful in 52.1% (503/965) of *Borrelia*-positive ticks; *Borrelia* (geno-)species determination revealed *B. afzelli* as the most frequent genotype in *I. inopinatus* (40.9%, 9/22) as well as *I. ricinus* (37.4%, 180/481), followed by *B. valaisiana* 22.7% (5/22) and *B. garinii*/*B. bavariensis* as well as *B. burgdorferi* sensu stricto (s.s.)/*B. carolinensis* (each 18.2%, 4/22) in *I. inopinatus* and *B. garinii*/*B. bavariensis* (24.3%, 117/481) and *B. valaisiana* (21.6%, 104/481) in *I. ricinus*. Further details on (geno-)species distribution in the different developmental stages of both tick species are provided in Table 6. Subsequent Sanger sequencing revealed the presence of *B. garinii* DNA in all four *B. garinii*/*B. bavariensis*-positive *I. inopinatus* samples. Among the 117 *B. garinii*/*B. bavariensis*-positive *I. ricinus* samples, *B. garinii* was identified in 57 cases, while *B. bavariensis* was successfully identified in 4 samples. Differentiation of *B. burgdorferi* (s.s.)/*B. carolinensis* (Blazejak et al. 2018) revealed *B. burgdorferi* (s.s.) in 2/2 *I. inopinatus* samples and 22/32 *I. ricinus* samples, while species differentiation by

Table 3 Results of the GLM testing differences in the percentage of samples assigned to *I. inopinatus* between sampling sites and sampling months in the city of Hamburg. The full model was significantly different from a null model containing only an intercept term (Chi-square = 51.3, Df = 15, $P < 0.001$). Significant *P*-values (≤ 0.05) are indicated by italics.

	Estimate	SE	*z*	*P*
Intercept	− 5.515	1.237	− 4.459	*< 0.001*
Sampling months*				
May vs. April	1.355	1.115	1.215	0.876
June vs. April	1.787	1.090	1.640	0.630
July vs. April	2.528	1.054	2.399	0.180
August vs. April	1.062	1.135	0.935	0.962
September vs. April	− 0.388	1.427	− 0.271	1.000
October vs. April	0.802	1.173	0.684	0.993
June vs. May	0.432	0.610	0.708	0.991
July vs. May	1.173	0.543	2.160	0.293
August vs. May	− 0.294	0.692	− 0.424	1.000
September vs. May	− 1.743	1.108	− 1.573	0.675
October vs. May	− 0.553	0.749	− 0.739	0.989
July vs. June	0.741	0.486	1.525	0.706
August vs. June	− 0.726	0.648	− 1.120	0.913
September vs. June	− 2.175	1.081	− 2.011	0.380
October vs. June	− 0.985	0.708	− 1.391	0.788
August vs. July	− 1.466	0.586	− 2.504	0.141
September vs. July	− 2.915	1.045	− 2.790	0.069
October vs. July	− 1.726	0.652	− 2.648	0.100
September vs. August	− 1.449	1.129	− 1.284	0.844
October vs. August	− 0.260	0.780	− 0.333	1.000
October vs. September	1.189	1.165	1.021	0.943
Sampling sites*				
Oejendorfer Park vs. Raakmoor	0.841	0.935	0.899	0.996
Volksdorfer Wald vs. Raakmoor	2.056	0.777	2.647	0.179
Neugrabener Heide vs. Raakmoor	0.040	1.015	0.039	1.000
Schwarzenberg vs. Raakmoor	1.096	0.835	1.313	0.944
Bergedorfer Gehoelz vs. Raakmoor	0.533	0.931	0.573	1.000
Gosslers Park vs. Raakmoor	− 0.754	1.237	− 0.609	1.000
Altonaer Volkspark vs. Raakmoor	− 0.526	1.238	− 0.425	1.000
Alster vs. Raakmoor	− 0.565	1.239	− 0.456	1.000
Stadtpark Winterhude vs. Raakmoor	0.457	0.930	0.492	1.000
Volksdorfer Wald vs. Oejendorfer Park	1.215	0.668	1.819	0.698
Neugrabener Heide vs. Oejendorfer Park	− 0.801	0.936	− 0.856	0.997
Schwarzenberg vs. Oejendorfer Park	0.255	0.736	0.347	1.000
Bergedorfer Gehoelz vs. Oejendorfer Park	− 0.308	0.844	− 0.365	1.000
Gosslers Park vs. Oejendorfer Park	− 1.594	1.172	− 1.360	0.930
Altonaer Volkspark vs. Oejendorfer Park	− 1.367	1.172	− 1.166	0.974
Alster vs. Oejendorfer Park	− 1.406	1.174	− 1.198	0.968
Stadtpark Winterhude vs. Oejendorfer Park	− 0.384	0.842	− 0.456	1.000
Neugrabener Heide vs. Volksdorfer Wald	− 2.016	0.777	− 2.596	0.201
Schwarzenberg vs. Volksdorfer Wald	− 0.960	0.520	− 1.846	0.680
Bergedorfer Gehoelz vs. Volksdorfer Wald	− 1.523	0.664	− 2.294	0.366
Gosslers Park vs. Volksdorfer Wald	− 2.810	1.050	− 2.675	0.168
Altonaer Volkspark vs. Volksdorfer Wald	− 2.582	1.051	− 2.457	0.269
Alster vs. Volksdorfer Wald	− 2.622	1.052	− 2.492	0.250

Table 3 (continued)

	Estimate	SE	*z*	*P*
Stadtpark Winterhude vs. Volksdorfer Wald	− 1.599	0.661	− 2.421	0.289
Schwarzenberg vs. Neugrabener Heide	1.057	0.835	1.265	0.955
Bergedorfer Gehoelz vs. Neugrabener Heide	0.494	0.931	0.530	1.000
Gosslers Park vs. Neugrabener Heide	− 0.793	1.237	− 0.641	1.000
Altonaer Volkspark vs. Neugrabener Heide	− 0.566	1.238	− 0.457	1.000
Alster vs. Neugrabener Heide	− 0.605	1.239	− 0.488	1.000
Stadtpark Winterhude vs. Neugrabener Heide	0.418	0.930	0.449	1.000
Bergedorfer Gehoelz vs. Schwarzenberg	− 0.563	0.730	− 0.771	0.999
Gosslers Park vs. Schwarzenberg	− 1.850	1.094	− 1.691	0.780
Altonaer Volkspark vs. Schwarzenberg	− 1.623	1.096	− 1.481	0.887
Alster vs. Schwarzenberg	− 1.662	1.096	− 1.516	0.872
Stadtpark Winterhude vs. Schwarzenberg	− 0.639	0.729	− 0.877	0.997
Gosslers Park vs. Bergedorfer Gehoelz	− 1.287	1.169	− 1.101	0.982
Altonaer Volkspark vs. Bergedorfer Gehoelz	− 1.059	1.171	− 0.905	0.996
Alster vs. Bergedorfer Gehoelz	− 1.099	1.172	− 0.937	0.994
Stadtpark Winterhude vs. Bergedorfer Gehoelz	− 0.076	0.837	− 0.091	1.000
Altonaer Volkspark vs. Gosslers Park	0.227	1.426	0.159	1.000
Alster vs. Gosslers Park	0.188	1.426	0.132	1.000
Stadtpark Winterhude vs. Gosslers Park	1.211	1.168	1.037	0.988
Alster vs. Altonaer Volkspark	− 0.039	1.427	− 0.027	1.000
Stadtpark Winterhude vs. Altonaer Volkspark	0.983	1.169	0.841	0.998
Stadtpark Winterhude vs. Alster	1.023	1.170	0.874	0.997

SE standard error

*Multiple comparisons between the levels of month and sampling site were performed using Tukey contrasts with single-step *P* value adjustment

sequencing was not successful for the remaining samples. Due to the low sample size of *Borrelia*-positive *I. inopinatus* specimens, a satisfactory statistical analysis of differences in *Borrelia* (geno-)species distribution between *I. inopinatus* and *I. ricinus* could not be performed.

Regarding *Rickettsia* spp., 46.0% (63/137) of *I. inopinatus* and 46.6% (1729/3708) of *I. ricinus* samples were PCR positive. Due to the high prevalence of *R. helvetica* (Tappe and Strube 2013; May and Strube 2014), only a random sample of *Rickettsia*-positive ticks collected in Hanover in 2015 was subjected to pyrosequencing (Blazejak et al. 2017), in addition to all *Rickettsia*-positive samples from the previous studies. Thus, species differentiation of *Rickettsia*-positive ticks by pyrosequencing was conducted for a subset of 34 positive *I. inopinatus* and 997 positive *I. ricinus*, respectively. *Rickettsia helvetica* was identified in 47.1% (16/34) of *I. inopinatus* and in 38.6% (385/997) of *I. ricinus*, while one *I. ricinus* sample contained *R. monacensis*. Species discrimination of the remaining *Rickettsia*-positive samples was not successful. Regarding *Rickettsia* spp. and *A. phagocytophilum* prevalence, no significant difference between tick species was found, but significant differences between tick developmental stages, sampling months, and sampling years were noted (Table 5).

Discussion

The present study demonstrates occurrence of *I. inopinatus* in northern Germany, namely in the cities of Hamburg and Hanover, although in low abundance as compared with *I. ricinus*. Nevertheless, the fact that *I. inopinatus* was detected at all sampling sites within the cities and during all three study years between 2010 and 2015 leads to the conclusion that there are established northern German *I. inopinatus* populations. Data from southern Germany show that *I. inopinatus* and *I. ricinus* can occur in sympatry (Chitimia-Dobler et al. 2018; Petney et al. 2015), contradicting the original assumption that *I. inopinatus* occurs in allopatry with *I. ricinus* (Estrada-Peña et al. 2014). In northern Germany, *I. inopinatus* was also found in sympatry with *I. ricinus*, confirming the observations from southern Germany. However, it is still unclear whether any hybridization

Table 4 Prevalence of *Borrelia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum* in *I. inopinatus* and *I. ricinus* (*N* nymphs, *AF* adult females, *AM* adult males)

	Borrelia spp.				*Rickettsia* spp.				*Anaplasma phagocytophilum*			
	Total	N	AF	AM	Total	N	AF	AM	Total	N	AF	AM
I. inopinatus	33.6% (46/137)	25.0% (24/96)	50.0% (12/24)	58.8% (10/17)	46.0% (63/137)	39.6% (38/96)	58.3% (11/24)	64.7% (11/17)	2.9% (4/137)	2.1.% (2/96)	4.2% (1/24)	5.9% (1/17)
I. ricinus	24.8% (919/3708)	22.9% (666/2911)	35.4% (143/404)	28.0% (110/393)	46.6% (1729/3708)	45.7% (1330/2911)	48.3% (195/404)	46.8% (184/393)	3.6% (135/3708)	3.0% (87/2911)	7.7% (31/404)	4.3% (17/393)

occurs between *I. inopinatus* and *I. ricinus*, as in sympatric populations of *I. ricinus* and *I. persulcatus*, for example (Kovalev et al. 2016). Since only one genetic locus, situated in mitochondrial DNA, was known for differentiation of the two tick species and was consequently analyzed at the time this study was conducted, the presence of hybrids within the study material cannot be excluded. To detect hybrids, nuclear loci would be needed (Kovalev et al. 2016); however, no nuclear *I. inopinatus* reference sequence is yet available.

For *I. ricinus*, two activity peaks were described in Central Europe: one in spring and another in autumn (Kurtenbach et al. 2006). As *I. inopinatus* generally occurred at low numbers compared with *I. ricinus* in the present study, no monthly differences in the *I. inopinatus* to *I. ricinus* ratio could be observed, i.e., no peak in *I. inopinatus* activity could be discerned. Similarly, no difference concerning the abundance of *I. inopinatus* in the two sampling regions, the cities Hanover and Hamburg, was detected. Among sampling sites within these two cities, which consisted of urban parks as well as urban forested areas (May and Strube 2014; Tappe and Strube 2013), the proportion of *I. inopinatus* ranged from 1 to 9%. However, a significant difference in the *I. inopinatus* to *I. ricinus* ratio was only detected between two of the ten locations in Hanover, whereas no significant geographical differences were detected in Hamburg. Thus, the analyzed data suggest that *I. inopinatus* shows the same habitat preferences as *I. ricinus* among the studied urban sites.

The importance of *I. ricinus* as a vector of bacterial pathogens is well described, and many studies have investigated pathogen prevalence in questing *I. ricinus* throughout Europe. In the *Ixodes* ticks from Hanover included in the present study, the detection rates for *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* were 23%, 26%, and 3% in 2010, and 24%, 51%, and 4% in 2015 (Blazejak et al. 2017; Blazejak et al. 2018; Tappe et al. 2014; Tappe et al. 2016; Tappe and Strube 2013; Tappe and Strube 2016), while detection rates in *Ixodes* ticks from Hamburg were 34%, 53%, and 4% in 2011 (May et al. 2015; May and Strube 2014). Due to the close phylogenetic relationship of *I. inopinatus* and *I. ricinus* (Estrada-Peña et al. 2014), *I. inopinatus* has been discussed as a potential vector of these pathogens (Petney et al. 2015). The present study shows that DNA of *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* occurs in *I. inopinatus*. Moreover, there was a significantly higher rate of detection of *Borrelia* spp. DNA in *I. inopinatus* (34%) than in *I. ricinus* (25%). In addition, DNA of causative agents of Lyme borreliosis, namely *B. afzelii*, *B. burgdorferi* (s.s.), *B. garinii*, *B. valaisiana*,

Table 5 Results of GLMMs testing the influence of different predictor variables on the infection probability with *Borrelia* spp. (model A), *Rickettsia* spp. (model B), and *Anaplasma phagocytophilum* (model C). Full models were significantly different from null models containing only the random factor "sampling location" (Chi-square = 86.2, Df = 10, *P* < 0.001 [model A]; Chi-square = 280.6, Df = 10, *P* < 0.001 [model B]; Chi-square = 51.8, Df = 10, *P* < 0.001 [model C]). Significant *P*-values (≤ 0.05) are indicated by italics.

	Model A: *Borrelia* spp.				Model B: *Rickettsia* spp.				Model C: *Anaplasma phagocytophilum*			
	Estimate	SE	*z*	*P*	Estimate	SE	*z*	*P*	Estimate	SE	*z*	*P*
Intercept	− 1.518	0.200	− 7.579	< *0.001*	− 1.506	0.165	− 9.121	< *0.001*	− 4.422	0.570	− 7.757	< *0.001*
Tick species (ref.: *I. inopinatus*)	0.446	0.191	2.336	*0.020*	− 0.122	0.183	− 0.668	0.504	− 0.230	0.517	− 0.444	0.657
Developmental stage (ref.: nymph)	− 0.625	0.091	− 6.874	< *0.001*	− 0.327	0.085	− 3.874	< *0.001*	− 0.907	0.193	− 4.695	< *0.001*
Year*												
2011 vs. 2010	0.820	0.166	4.938	< *0.001*	1.404	0.132	10.647	< *0.001*	0.629	0.445	1.416	0.322
2015 vs. 2010	0.307	0.112	2.750	*0.016*	1.260	0.100	12.618	< *0.001*	0.522	0.253	2.065	*0.092*
2015 vs. 2011	− 0.513	0.145	− 3.530	*0.001*	− 0.144	0.108	− 1.329	0.373	− 0.107	0.400	− 0.268	0.960
Sampling month*												
May vs. April	0.362	0.175	2.075	0.364	− 0.013	0.146	− 0.091	1.000	0.981	0.509	1.927	0.447
June vs. April	0.599	0.168	3.575	*0.006*	0.305	0.141	2.163	0.315	1.211	0.494	2.453	0.167
July vs. April	0.367	0.170	2.160	0.314	0.856	0.140	6.123	< *0.001*	1.866	0.476	3.920	*0.002*
August vs. April	0.476	0.168	2.833	0.068	0.858	0.140	6.127	< *0.001*	0.775	0.514	1.508	0.728
September vs. April	0.463	0.172	2.695	0.098	0.730	0.143	5.096	< *0.001*	0.468	0.539	0.870	0.975
October vs. April	0.666	0.167	3.976	*0.001*	0.812	0.141	5.763	< *0.001*	1.112	0.500	2.223	0.270
June vs. May	0.237	0.142	1.667	0.635	0.318	0.130	2.451	0.176	0.231	0.324	0.712	0.991
July vs. May	0.005	0.145	0.031	1.000	0.870	0.128	6.778	< *0.001*	0.885	0.295	2.998	*0.040*
August vs. May	0.114	0.143	0.796	0.985	0.872	0.129	6.776	< *0.001*	− 0.206	0.354	− 0.582	0.997
September vs. May	0.101	0.149	0.677	0.994	0.743	0.133	5.585	< *0.001*	− 0.512	0.391	− 1.310	0.839
October vs. May	0.304	0.142	2.134	0.329	0.825	0.130	6.367	< *0.001*	0.131	0.333	0.394	1.000
July vs. June	− 0.233	0.133	− 1.750	0.579	0.552	0.119	4.640	< *0.001*	0.655	0.260	2.514	0.145
August vs. June	− 0.123	0.131	− 0.939	0.966	0.554	0.120	4.624	< *0.001*	− 0.437	0.327	− 1.337	0.825
September vs. June	− 0.136	0.140	− 0.973	0.959	0.425	0.126	3.365	*0.014*	− 0.743	0.371	− 2.003	0.398
October vs. June	0.066	0.131	0.508	0.999	0.507	0.121	4.188	< *0.001*	− 0.100	0.304	− 0.327	1.000
August vs. July	0.109	0.134	0.818	0.983	0.002	0.117	0.016	1.000	− 1.091	0.297	− 3.679	*0.004*
September vs. July	0.096	0.143	0.676	0.994	− 0.127	0.125	− 1.017	0.950	− 1.397	0.346	− 4.035	*0.001*
October vs. July	0.299	0.133	2.245	0.268	− 0.044	0.118	− 0.375	1.000	− 0.754	0.272	− 2.774	0.076
September vs. August	− 0.013	0.141	− 0.092	1.000	− 0.129	0.125	− 1.029	0.947	− 0.306	0.397	− 0.771	0.987
October vs. August	0.190	0.132	1.442	0.776	− 0.046	0.119	− 0.388	1.000	0.337	0.336	1.004	0.950
October vs. September	0.203	0.140	1.449	0.772	0.082	0.126	0.654	0.995	0.643	0.379	1.699	0.602

SE standard error

*Multiple comparisons between the levels of year and month were performed using Tukey contrasts with single-step *P* value adjustment

and *B. bissettiae* were identified in *I. inopinatus*. Detection of these *B. burgdorferi* s.l. genospecies suggests that the natural host spectrum of *I. inopinatus* includes rodents which constitute the main reservoir for *B. afzelii*, as well as birds which are the main reservoir hosts for *B. garinii* and *B. valaisiana* (Gern and Humair 2002). Besides agents of Lyme borreliosis, *B. miyamotoi*, the causative agent of tick-borne relapsing fever (Krause et al. 2016), was detected in one *I. inopinatus* specimen.

In total, 46% (63/137) of *I. inopinatus* specimens were *Rickettsia* positive. Sixteen of the thirty-four *Rickettsia*-positive ticks selected for species differentiation by pyrosequencing were found to contain *R. helvetica*, which is part of the spotted fever group rickettsiae. In Germany, *R. helvetica* is the most frequent *Rickettsia* spp. in *I. ricinus* ticks (Dobler and Wölfel 2009; Schicht et al. 2012; Tappe and Strube 2013; May and Strube 2014; Blazejak et al. 2017). This also seems to apply to

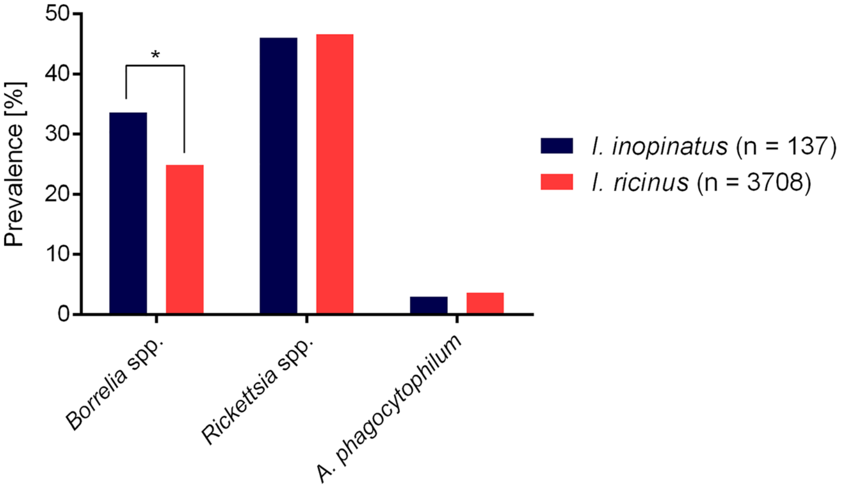

Fig. 1 Prevalence of *Borrelia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum* in questing *I. inopinatus* and *I. ricinus* collected in northern Germany (cities of Hanover and Hamburg). The asterisk indicates a statistical significant difference ($P < 0.05$) determined by generalized linear mixed model analysis, controlling for the influence of tick life stage as well as month, year, and location of sampling

I. inopinatus. The prevalence of *A. phagocytophilum* DNA in *I. inopinatus* (i.e., 3%) was comparable to that previously reported for *I. ricinus* (i.e., 0–10%) (Blazejak et al. 2017; Franke et al. 2010; Schorn et al. 2011).

The previous studies representing the basis for the study presented here showed that adult ticks were significantly more often infected with *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* than nymphs (Blazejak et al. 2017; Blazejak et al. 2018; May et al. 2015; May and Strube 2014; Silaghi et al. 2008; Tappe et al. 2014; Tappe et al. 2016; Tappe and Strube 2013; Tappe and Strube 2016). This higher prevalence in adult ticks can be explained by efficient transstadial transmission and an increased probability to acquire bacteria from infected hosts during the blood meal of the nymphal stage (Humair and Gern 2000). Unfortunately, it was not possible to separately assess differences in prevalence rates between *I. inopinatus* adults and nymphs due to the overall low number of *I. inopinatus* ticks and the unequal distribution of the identified adult and nymphal *I. inopinatus* ticks. Further investigations based on a larger number of *I. inopinatus* are necessary to confirm the same assumption for *I. inopinatus*. Similarly, differences in pathogen prevalence between sampling months as well as between sampling years could not be assessed for *I. inopinatus* separately. Significant differences in pathogen prevalence between sampling locations, months, and years calculated by GLMMs confirmed the results

Table 6 Prevalence of *Borrelia* (geno-)species in *I. inopinatus* and *I. ricinus* ticks determined by Reverse Line Blot (*N* nymphs, *AF* adult females, *AM* adult males)

	I. inopinatus				*I. ricinus*			
	Total	N	AF	AM	Total	N	AF	AM
B. afzelii	40.9% (9/22)	77.8% (7/9)	12.5% (1/8)	20.0% (1/5)	37.4% (180/481)	40.4% (132/327)	33.0% (30/91)	28.6% (18/63)
B. garinii / *B. bavariensis*	18.2% (4/22)	0.0% (0/9)	37.5% (3/8)	20.0% (1/5)	24.3% (117/481)	25.7% (84/327)	19.8% (18/91)	23.8% (15/63)
B. valaisiana	22.7% (5/22)	11.1% (1/9)	25.0% (2/8)	40.0% (2/5)	21.6% (104/481)	20.5% (67/327)	26.4% (24/91)	20.6% (13/63)
B. burgdorferi (s.s.) / *B. carolinensis*	18.2% (4/22)	22.2% (2/9)	25.0% (2/8)	0.0% (0/5)	14.4% (69/481)	12.5% (41/327)	15.4% (14/91)	22.2% (14/63)
B. spielmanii	0.0% (0/22)	0.0% (0/9)	0.0% (0/8)	0.0% (0/5)	8.7% (42/481)	8.0% (26/327)	11.0% (10/91)	9.5% (6/63)
B. bissettiae	4.6% (1/22)	0.0% (0/9)	12.5% (1/8)	0.0% (0/5)	1.7% (8/481)	1.8% (6/327)	1.10% (1/91)	1.6% (1/63)
B. lusitaniae	0.0% (0/22)	0.0% (0/9)	0.0% (0/8)	0.0% (0/5)	0.2% (1/481)	0.3% (1/327)	0.0% (0/91)	0.0% (0/63)
*B. miyamotoi**	8.3% (1/12)	0.0% (0/2)	0.0% (0/7)	33.3% (1/3)	18.2% (42/231)	20.0% (26/130)	14.0% (8/57)	18.2% (8/44)

*Detection of *B. miyamotoi* was implemented for the ticks collected in Hanover in 2015, after establishment of a modified RLB protocol for detection of *B. miyamotoi* by Blazejak et al. (2018)

obtained by Chi-square tests in previous studies (Blazejak et al. 2017; Blazejak et al. 2018; May et al. 2015; May and Strube 2014; Tappe et al. 2014; Tappe et al. 2016; Tappe and Strube 2013; Tappe and Strube 2016). Since only a low number of the differentiated ticks were assigned to *I. inopinatus*, the pathogen prevalence published in these previous studies can still be regarded as representative for *I. ricinus*.

In conclusion, detection of pathogen DNA in questing *I. inopinatus* indicates a potential role of this tick species as a vector for *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum*. Thus, *I. inopinatus* may be of medical and veterinary significance. To confirm this hypothesis, the vector competence of *I. inopinatus* should be investigated using transmission experiments.

Funding information The study was supported by funding from Bayer Vital GmbH and Bayer Animal Health GmbH.

Compliance with ethical standards

Disclaimer Study data collection and interpretation is completely independent from the company's opinion.

Conflict of interest SP is an employee of Bayer Vital GmbH, BS is an employee of Bayer Animal Health GmbH. The authors declare that there is no conflict with commercial interests.

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