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Identification of host blood-meal sources and *Borrelia* in field-collected *Ixodes ricinus* ticks in north-western Poland

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Abstract

Forest animals play fundamental roles in the maintenance of *Ixodes ricinus* and *Borrelia* species in the forest biotope. To identify the forest vertebrate species that are host for *I. ricinus* and for the recognition of the reservoirs of *Borrelia* species, the blood-meal of 325 *I. ricinus* ticks collected at two forest sites in north-western Poland were analysed. Nested PCR was used to detect polymorphisms in a fragment of the mitochondrial *12S rRNA* gene for the identification of the hosts species. The products were digested with the restriction enzymes, a combination that allows the identification of 60 vertebrate species, comprising 17 bird, 4 reptile and 39 mammalian species. Host DNA was detected in 244 (75%) *I. ricinus* individuals, with the species being detected and classified for 210 (86%) samples. The restriction patterns resulted in the identification of 14 vertebrate species, including 2 species of birds, lizard, badger, rabbit, deer; most of the samples contained DNA from wild boar (*Sus scrofa*), red fox (*Vulpes vulpes*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*). Identification of *Borrelia* species was based on the *flaB* gene using nested PCR coupled to RFLP. This method allows the identification of all *Borrelia* species transmitted by *I. ricinus* in Europe, including *B. miyamotoi* and 3 genetic variants of *B. garinii*. In the studied isolates, 2 species belonging to *B. burgdorferi* sensu lato were identified – *B. garinii* and *B. afzelii*, and *B. miyamotoi*, which are related to relapsing fever borreliae.

Key words

blood-meal, *Borrelia*, reservoir, *Ixodes ricinus*

INTRODUCTION

The main vector for the bacteria causing Lyme borreliosis in Europe is the *Ixodes ricinus* tick. The causative agents of Lyme borreliosis belonging to the *Borrelia burgdorferi* species complex (*B. burgdorferi* sensu lato (s.l.)) comprise of at least 19 species with a worldwide distribution [1]. As each species of these tick-transmitted pathogens shows preferences for specific hosts, and is also associated with different clinical manifestations, it is obvious that the eco-epidemiology of Lyme borreliosis agents is very complex [2]. The transmission of *B. burgdorferi* s.l. is affected by molecular processes at the tick-host interface, including mechanisms for the protection of spirochetes against the host's immune response. Molecular biology also increasingly provides important identification tools for the study of tick-borne disease agents and their hosts.

Many species of animals play an essential role in the maintenance of *I. ricinus* and *Borrelia* in nature, but a specific role is ascribed to forest animals which are the natural sources of infection. The lack of host specificity of *I. ricinus* has tremendous significance in the spread and maintenance of *Borrelia* spirochetes in a specific biotope. Infected ticks infect their host when sucking the host's blood, or the tick becomes infected when feeding on an infected animal. Every tick host is a potential reservoir of *B. burgdorferi* s.l., but the proper reservoir species is an animal that enables the persistence of the pathogenic factor, constituting a long-term source of infection for the ticks feeding on it [2]. It has been estimated that ticks can feed on 300 different species of vertebrates (mammals, birds and reptiles), and the capacity to distinguish among the blood meals of haematophagous arthropods has revolutionised our understanding of ectoparasite–host interactions and infectious disease ecology [1]. The methodology for the use of new protocols and molecular markers is under constant development. For the differentiation of reservoirs and *Borrelia* species, Kirsten and Gray [3] used the mitochondrially encoded *cytb* gene as the molecular marker and digested the PCR products using the restriction endonucleases HaeIII and DdeI, a protocol that allowed the authors to distinguish 11 animal species. Pichon et al. [4] identified vertebrate DNA in the tick gut using PCR amplification with universal primers targeting a portion of the 18S rRNA gene, followed by reverse line blot (RLB) hybridisation. In the study of Moran Cadenas et al. [5], a 12S rDNA gene fragment was used as a PCR marker, followed by RLB, with the identification of the host DNA from field-collected ticks being possible in 43.6% of the cases.

The presented work is a continuation of studies concerning the molecular identification of borrelial hosts and *Borrelia* species through an analysis of the blood meal in *I. ricinus* individuals in north-western Poland [6].

OBJECTIVES

The aim of the study was to improve the protocols of applied molecular methods. The protocol developed in this study is based on the polymorphism of a mitochondrial 12S rRNA gene fragment, allowing the identification of 60 vertebrate species, comprising 17 bird, 4 reptile and 39 mammalian

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species. The identification of *Borrelia* species was based on the PCR-RFLP protocol with the use of *flaB* gene as molecular target and 2 restriction enzymes [7], a method that allows the identification of all *Borrelia* species transmitted by *I. ricinus* in Europe.

MATERIALS AND METHOD

Study material and collection sites of *Ixodes ricinus*. The study included 325 specimens of *I. ricinus* ticks (25 males, 34 females, 266 nymphs) collected in 2 study forest areas in north-western Poland: Zielonczyn (N: 53°40'9"; E: 14°40'1") and Pobierowo (N: 54°2'44"; E: 14°52'54"). These sites are characterised as mixed forests with a prevalence of European beech (*Fagus sylvatica*), English oak (*Quercus robur*) and Scots pine (*Pinus sylvestris*), and are known as high-risk areas of *I. ricinus* ticks and *Borrelia*-infected ticks, and are often visited by hikers and mushroom pickers. The ticks were collected with a flannel flag, with which the vegetation was swept up to a height of 1 m, and the collected specimens stored at -20 °C until isolation of their DNA.

DNA extraction. DNA was extracted from the ticks according to the phenol-chloroform protocol. All of the tick individuals (nymph or adult) were crushed using a ceramic pestle and suspended in 500 µl of 2X buffer (0.19 M NH_4Cl, 0.011 M $KHCO_3$ and 0.024 M EDTA) plus 100 µl of Lysis buffer (0.017 M SDS, 0.01 M Tris and 0.01 M EDTA) and 1 µl of proteinase K (20 mg/ml) (BioShop, Canada). The tubes were placed in a 56 °C water bath for 3 hours. Following this incubation, 300 µl of phenol (BioShop) was added, and the tube vortexed for 30 sec and centrifuged for 10 min. at 9,000 rpm. The supernatant was extracted with 400 µl phenol-chloroform (1:1) and then twice with 300 µl chloroform (POCH, Poland), vortexing for 30 sec and centrifuging for 10 min. at 9,000 rpm between each extraction. The supernatant was then precipitated by adding 500 µl isopropanol (POCH). The pellet was rinsed with 70% ethanol and air-dried before suspension in TE buffer (pH 8.0). The samples were stored at -70 °C prior to the PCR analyses.

Detection of *I. ricinus* host DNA in blood meal remnants using nested PCR. The mitochondrially encoded *12S rRNA* gene was used as the molecular marker for the detection of the tick hosts by nested PCR. This gene has a mosaic structure with regions conserved for vertebrates that may serve as targets for PCR primers and variable regions that may be used as PCR-RFLP markers. The following 2 primer sets were used: outer, 532f12s (5'-CAAACTGGGATTAGATAC-3') and 1102r12s (5'-TGCTTACCTTGTTACGAC-3'), with a product length of approximately 520 bp; and inner, 539f12s (5'-GGATTAGATACCCCACTATGC-3') and 1015r12s (5'-TGAGGAGGGTGACGGGCGGT-3'), with product length of approximately 440 bp.

The first PCR was performed in a reaction volume of 10 µl containing 0.5 U Allegro Taq DNA polymerase (Novazym, Poland), 70 mM Tris-HCl (pH 8.6 at 25 °C), 16.6 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 0.75 µM each deoxyribonucleotide triphosphate (Novazym), 2 pmol each of the 2 outer primers (532f12s and 1102r12s) and 1 µl of the supernatant of the processed DNA sample in TB buffer. For the second PCR, 1 µl of a 10-fold dilution of the PCR product of the first reaction was added to 9 µl of reaction mixture prepared with inner primers 539f12s and 1015r12s. The PCRs were performed using a T-gradient thermal cycler (Biometra, Germany). The templates were subjected to an initial denaturation step of 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 sec, 60 °C for 45 sec and 72 °C for 1 min. The DNA of *Cervus elaphus* (hunted near Dobra Szczecińska, Zachodniopomorskie province) was used as a positive control. TE buffer was used as the negative control. The PCR products were analysed on 1.5% agarose gels (BioShop) with the addition of GelView DNA stain (Novazym, Poland) at 5 V/cm for 1 hour. The Nova 100 DNA Ladder (Novazym) was used for evaluation of the PCR product size. The results of the PCR were viewed under UV light and computer archived using VisionWorksLS Image Acquisition and Analysis Software (UVP, CA, USA).

Identification of *Ixodes ricinus* hosts by PCR-RFLP. The DNA amplified using the primer set 539f12s and 1015r12s was digested with enzymes AluI, Cfr13I and Tru1I. These enzymes were selected on the basis of computer analysis (DNAMAN) of the *12S rDNA* gene sequence downloaded from GenBank to obtain the RFLP patterns of 60 vertebrate species: 17 bird, 4 reptile and 39 mammalian species (including Muridae, hares, rabbits, bats, hedgehogs, minks, dormice, roe deer, wild boar, red deer and fallow deer) that might be present in the habitats studied.

Detection of *B. burgdorferi* s.l. DNA by nested PCR-RFLP. The nested PCR method with 2 primer sets (outer 132f and 905r and inner 220f and 823r) used to detect the *flaB* gene fragment of *B. burgdorferi* s.l. has been described previously [7, 8]. DNA isolated from a reference strain of *B. burgdorferi* s.s. IRS (German Collection of Microorganisms and Cell Cultures – DSMZ, Germany) was used as the positive control, and TE buffer as the negative control. The PCR products were separated on 1.5% agarose gels with the addition of GelView DNA stain at 5 V/cm for 1 hour. The Nova 100 DNA Ladder was used for evaluation of the product. The results of the PCR were viewed under UV light and archived as described above.

The *flaB* gene fragments amplified using the primer set 220f and 823r were digested with enzymes HpyF3I and Ecl136II (Fermentas, Lithuania) to obtain RFLP patterns of different *Borrelia* species, as described previously [7]. The digestion products were analysed on 3% agarose gels at 5 V/cm for 2 hours and archived as described above.

RESULTS

Host species determination. Host DNA was detected in 244 *I. ricinus* individuals; 15 types of restriction patterns consistent with those from GenBank based on the analysis of the *12S rRNA* sequence were obtained for 210 (86%) of the samples. A list of the observed restriction patterns characteristic for each species of vertebrate is presented in Table 1. The results from the remaining 34 samples (14%) did not allow identification of the host to the species level. The characteristic restriction patterns obtained in the digested nested PCR products for 4 selected vertebrate species are shown in Figure 1.

***Borrelia* species determination.** The DNA of *Borrelia* spirochetes was detected using nested PCR in 11 of the 244 *I. ricinus* isolates, which was 4.5% of the population showing

Table 1. Restriction patterns obtained after digestion of the mitochondrial *12S rRNA* gene sequence with enzymes AluI, Cfr13I and Tru1I for vertebrate selected species

Host species	Enzyme	Restriction pattern	No. of I. ricinus individuals with the host DNA (n/%)
Sus scrofa (wild boar)	AluI	167-274	39/18.57
Vulpes vulpes (red fox)	AluI	52-76-304	38/18.09
Cervus elaphus (red deer)	AluI Tru1I	83-354 28-57-95-257	38/18.09
Capreolus capreolus (roe deer)	AluI Tru1I	83-354 10-23-85-95-224	34/16.19
Accipiter gentilis (northern goshawk)	AluI	41-175-223	24/10.43
Myodes glareolus (bank vole)	AluI	42-89-300	16/7.62
Lacerta viridis (green lizard)	AluI	41-400	14/6.67
Meles meles (European badger)	AluI	52-175-209	1/0.48
Dama dama (fallow deer)	AluI Tru1I	83-354 85-352	1/0.48
Natrix natrix (grass snake)	AluI Cfr13I	206-231 436	1/0.48
Orytolagus cuniculus (European rabbit)	AluI Cfr13I	82-149-200 92-339	1/0.48
Plecotus austriacus (grey long-eared bat)	AluI Tru1I	98-158-173 28-88-147-170	1/0.48
Castor fiber (European beaver)	AluI Tru1I	83-354 85-131-222	1/0.48
Ciconia nigra (black stork)	AluI Cfr13I	206-234 39-398	1/0.48

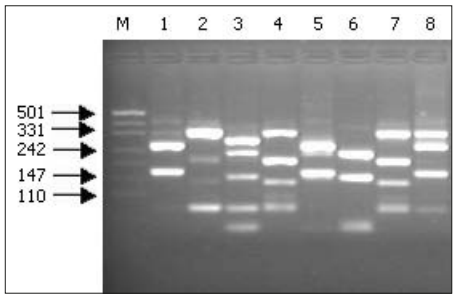

Figure 1. Restriction patterns obtained for the hosts of *I. ricinus* ticks (collected in Zielonczyn area) by the digestion of mitochondrial gene *12S rRNA* fragments and the products of nested-PCR reactions with AluI: M – DNA molecular weight marker MW 501.
1, 5, 8 – *Sus scrofa*; 3 – *Myodes glareolus*; 6 – *Accipiter gentilis*; 2, 4, 7 – *Cervus elaphus*

host DNA. Restriction analysis of the nested PCR product using the 220f and 823r primers and the HpyF3I enzyme generated 3 restriction patterns characteristic for 3 *Borrelia* species (Fig. 2): 2 species belonging to *B. burgdorferi* s.l., *B. garinii* and *B. afzelii*, and *B. miyamotoi,* which is related to relapsing fever borreliae. The results of the identification of the *Borrelia* species using the PCR–RFLP method is shown in Figure 2. *B. garinii* DNA was detected along with DNA from 2 host species (*Myodes glareolus* and *Accipter gentilis*) in nymphs, and DNA from *B. afzelii* and 2 hosts (*Sus scrofa* and *Oryctolagus cuniculus*) was also detected in nymphs. DNA from *B. miyamotoi* and 3 species of vertebrates (*Capreolus capreolus, Cervus elaphus* and *Sus scrofa*) was detected in 2 nymphs and 1 male.

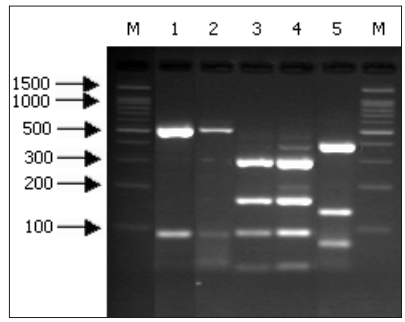

Figure 2. Restriction patterns obtained for *Borrelia* species after the digestion of *flaB* gene fragments with HpyF3I (identification results for the Zielonczyn area).
M – DNA molecular weight marker MW501; 1, 2 – *B. miyamotoi*; 3, 4 – *B. afzelii*; 5 – *B. garinii*.

DISCUSSION

The capacity to distinguish the blood meals of haematophagous arthropods has revolutionised our understanding of ectoparasite-host interactions and infectious disease ecology [1]. Such an indirect method of identification of *I. ricinus* hosts was applied, as based on unfed ticks, in the presented work. Each DNA isolate obtained from a single tick was useful for the identification of both host species and *Borrelia* species. The host species was determined with great precision based on host blood meal remnants in adult and nymphal *I. ricinus* ticks using species-specific restriction patterns with a PCR-RFLP protocol. This method may be very useful for the development of studies concerning the reservoir of *B. burgdorferi* s.l. spirochetes because the reservoirs for all of the *Borrelia* species have not been determined to date.

The protocol presented in this study improves on the method of Kirsten and Gray [3], allowing the identification of 60 vertebrate species, including 17 birds, 4 reptiles and 39 mammals; DNA of host species was detected in 210 *I. ricinus* individuals (86%). In similar studies, the detectability of host DNA in nymphal ticks was much lower. The low level of detected host DNA was obtained in a study conducted in Spain [9] in which host DNA was detected only in 37.7% of the ticks, and in Ireland with 49.4% of host DNA detectability [4], whereas the DNA of vertebrate hosts was detected in 53.5% of *Borrelia*-infected ticks in an earlier study in Poland by the authors of the current study [6]. It is difficult to compare DNA results among studies because of differences in the number of ticks, method of DNA isolation, and molecular target for host DNA detection. However, in studies in which the entire population of ticks were tested, the level of detection of host DNA appeared to depend on the DNA isolation method. The method described by Guy and Stanek [10] using ammonium hydroxide is the most frequent and simplest protocol for DNA isolation from hard ticks (*Ixodidae*), yet it is also the least effective method. Studies applying 2 isolation protocols, the first with ammonium hydroxide and the second with Chelex, showed higher levels of host DNA detection for ticks prepared with the Chelex protocol (72.8%) than with ammonium hydroxide (48.4%) [11]. The phenol-chloroform protocol used in presented study is the most time-consuming method, but it appears to be the most effective for host DNA detection. Further studies are required to compare this protocol with those using ammonium hydroxide.

The presented protocol allows for precise identification of individual host species in that 14 species of forest-dwelling animals were successfully detected and classified, and may be considered as possible or natural reservoirs of *B. burgdorferi* s.l. spirochetes in the selected area. The described protocol is a variation of an earlier method [6] developed to identify as much as 60 species of tick hosts. In contrast to the presented work, other protocols allow identification only at the genus level or larger group of organisms, such as songbirds, gamebirds, small mammals, squirrels and ruminants [4, 9, 11]. However, studies of the ticks collected from birds revealed that among songbirds only 2 species are important, blackbird (*Turdus merula*) and song thrush (*T. philomelos*) [12, 13], which suggests the necessity for a precise method for tick host identification.

The most common host species detected in the ticks were large mammals, i.e., red deer (*C. elaphus*), roe deer (*C. capreolus*), wild boar (*S. scrofa*) and red fox (*V. vulpes*), results which are in accordance with the results of previous studies. When the entire population of collected nymphs were tested for host DNA, ruminants predominated or were one of predominant groups of vertebrate hosts [4, 11]. The indicated species, particularly roe deer and red deer, are considered as the main hosts of the *I. ricinus* mature stage. However, a comparison of the 2 methods of tick collection from large mammals [14] revealed that, in collecting ticks from living animals (live method), most of the feeding larvae and nymphs are absent. In contrast, the method of collecting from the separated parts of hunted animals (destructive method) allows establishment of the actual proportion of tick stages, which fluctuated from 62.8% – 68% for larvae, from 25.9% – 31.8% for nymphs, and from 5.4% – 6.1% for adults. The mean number of ticks collected from an individual animal using the destructive sampling method was 40 – 60 times higher than for the live method. The prevalence of large-mammal DNA detected in non-engorged nymphs (acquired during the feeding process in the larval stage) found in the present and other studies is in accordance with the above-described findings.

Vertebrate tick hosts that are not associated with the life cycle of *Borrelia* may interrupt the cycle or serve as competent reservoirs and participate in the enzootic cycle of these bacteria [12, 15]. Animals that retain the pathogen and constitute a source of infection for a long time to allow ticks to feed on them are competent reservoirs of *B. burgdorferi* s.l. [1]. However, even incompetent hosts are able to participate in the circulation of *B. burgdorferi* s.l. because of the so-called co-feeding phenomenon [16]. Detection of the pathogen in the tissue of such animals reveals susceptibility of the host to the infection, but does not necessarily mean that the animal host is able to transfer *B. burgdorferi* through feeding ticks [2].

In Europe, animal reservoirs have been determined with regard to approximately 40 species of birds and mammals [2, 17]. Thus far, it has been found that large forest mammals, such as roe deer, red deer and wild boar, which are the basic hosts for adult *I. ricinus*, are not able to constitute reservoirs for the species belonging to the *B. burgdorferi* s.l. complex [17]. Although the DNA of the roe deer *C. capreolus* has been identified in 34 samples from ticks, although this species does not seem to have any relevance as a reservoir of *B. burgdorferi* s.l. in north-western Poland because the DNA of a *Borrelia* spirochete was present in only 1 nymph of *I. ricinus* (2.9%) – *B. miyamotoi* of the relapsing fever borreliae group. *C. capreolus* plays a short-term role as a *Borrelia* host, enabling the transmission to the vector organism during the process of feeding: when a tick acquires spirochetes from the subcutaneous tissue in the direct area of an earlier bite by another infected tick [16]. The studies of Telford et al. [18] in addition to earlier studies by the authors of the presented study [6, 19], have proved that roe deer is not an important reservoir of *B. burgdorferi* s.l.

There are data concerning the possibility of surviving spirochetes in the blood of red deer (*Cervus elaphus*), remaining in their skin and an absence of the possibility to survive [17, 18]. The studies of Wodecka [20] have provided negative results for the occurrence of *B. burgdorferi* s.l. spirochetes in the DNA isolated from red deer tissues and from the ticks found on them. In the presented work, the DNA of *C. elaphus* was identified in 38 individuals of *I. ricinus;* however, the DNA of *Borrelia* was detected only in a single male tick (2.6%), and the species was *B. miyamotoi*. These results confirm that *C. elaphus* does not function as a *B. burgdorferi* s.l. reservoir.

It has been reported that *Sus scrofa* is not a competent reservoir of *B. burgdorferi* s.l., but these animals are the main hosts of the adult stage of *I. ricinus* [6]. In the presented work, the DNA of *S. scrofa* was identified in 39 individuals of *I. ricinus*, and the DNA of *B. afzelii* and *B. miyamotoi* was detected in 2 nymphs (5.1%).

Reservoir competence for *B. burgdorferi* spirochetes has not yet been confirmed for *Vulpes vulpes*. However dogs, the domesticated relatives of red fox, often suffer from Lyme borreliosis [21, 22, 23, 24]. The studies of Heidrich et al. [25] have proven the possibility of *B. burgdorferi* s.l. spirochetes occurring in the skin of *V. vulpes*. In the present work, fox DNA was detected in 38 samples, but none of these samples contained *Borrelia* spirochetes.

According to Gern [2], *Myodes glareolus* (bank vole) is thought to be a natural reservoir of *B. burgdorferi* s.l. spirochetes. However, in the opinion of Talleklint and Jaenson [26], *M. glareolus* cannot serve as a reservoir of the spirochetes because the infected bank voles easily die during the winter. In the current study, the DNA of *Myodes glareolus* was found in 16 samples, and *B. garinii* DNA was detected in 2 (12.5%) samples.

Fallow deer (*Dama dama*) is not recognised as a competent reservoir of *B. burgdorferi* s.l. This assertion was confirmed by the studies of Talleklit and Jaenson [27] in south-central Sweden where the registered frequency of infection with *Borreli*a spirochetes was low (7–10%), and was explained by the occurrence of a large number of fallow deer in this region. In the presented study, the presence of *D. dama* DNA was identified only in 1 tick, and spirochete DNA was not detected in this sample.

The studies of Gern and Sell [28] conducted in Switzerland indicate that *Meles meles* may act as a reservoir of *B. afzelii*. However, there is lack of convincing data concerning the ecological relevance of badgers with regard to Lyme borreliosis in Europe. In the current study, the DNA of *M. meles* was detected only in one tick, whereas the DNA of *B. burgdorferi* s.l. was not present in this sample.

The other 7 identified vertebrate species with genetic material present in the blood found in the tick's alimentary tract were *Accipiter gentilis* (northern goshawk) in 24 samples and *Lacerta viridis* (lizard) in 16 samples. The DNA of the following species was found in only 1 sample: *Natrix natrix*

(grass snake), *Ciconia nigra* (black stork), *Castor fiber* (European beaver), *Plecotus austriacus* (grey long-eared bat) and *Oryctolagus cuniculus* (European rabbit). These are likely accidental hosts because they do not appear on the list of reservoir species [2]. Among these species, only the DNA of goshawk and rabbit occurred together with the genetic material of *B. afzelii and B. miyamotoi*, respectively.

The identified species of vertebrates play a large part in the ecology of Lyme borreliosis, but only *M. glareolus* constitutes a reservoir for *Borrelia* spirochetes. The other species do not exhibit reservoir competence with regard to *B. burgdorferi* s.l.; however, as significant hosts for *I. ricinus* ticks, these species are elements of the enzootic cycle of the bacterium, enabling the tick to survive and reproduce. The fact that these animals are short-lasting hosts for some *Borrelia* species is essential in enabling the bacteria to survive and transfer to the vector during the feeding process. Unfortunately, in the presented study it was not possible to determine which of these animals is the most important for spreading *Borrelia* infections because the degree of infection was small: the DNA of *B. burgdorferi* s.l. was detected only in a few individuals of *I. ricinus* that had fed on *M. glareolus*, *S. scrofa*, *C. capreolus*, *C. elaphus*, *O. cuniculus* and *A. gentilis*.

In summary, the modification of a previous protocol allowed the detection of host DNA with the highest efficiency and accuracy of the individual identified species. The method confirmed the finding that the main hosts of all of the stages of *I. ricinus* are large mammals, particularly deer, wild boars and foxes. Undoubtedly, all of the identified animals have essential roles in the circulation of *Borrelia* spirochetes, and they also enable the survival and reproduction of *I. ricinus* ticks. Moreover, these species also serve as short-lasting hosts for some *Borrelia* species, enabling their survival and transmission to feeding vectors.

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