A molecular survey of spotted fever group rickettsiae in introduced raccoons (Procyon lotor)

Joanna Hildebrand1*, Agnieszka Perec-Matysiak1, Marcin Popiołek1, Dorota Merta2, Izabella Myśliwy1 and Katarzyna Buńkowska-Gawlik1

Abstract

Background: The raccoon Procyon lotor (Linnaeus, 1758) (Carnivora; Procyonidae) is one of the most important and most intensively studied invasive mammal species in Europe. Within the last 30 years the raccoon has spread at an increasing rate, resulting in the establishment of local populations in various regions of Europe. In these newly colonised areas, gaps in knowledge of the raccoon's biology concern not only most aspects of its ecology in a broad sense, but also its pathogens and parasites. Most micropathogens recorded hitherto in the raccoons that have colonised Europe have documented epizootic and zoonotic potential. Thus, it is considered especially important to investigate the role played by the raccoon in the spread of pathogens through both animal-animal and animal-human pathways.

Methods: Tissue samples of raccoons from Poland and Germany were examined in this study. In total, 384 tissue samples from 220 raccoons (170 spleen samples, 82 liver biopsies, 132 ear biopsies) were examined using molecular methods. The presence of Rickettsia spp. DNA was screened through amplification of a fragment of the gltA gene. Samples that were PCR positive for gltA were tested for other rickettsial genes, ompB and a 17-kDa antigen. For taxonomic purposes, the obtained sequences were compared with corresponding sequences deposited in GenBank using the Basic Local Alignment Search Tool, and phylogenetic analyses were conducted using Bayesian inference implemented in MrBayes software.

Results: Rickettsia DNA was confirmed only in skin biopsies; no isolates from the spleen or liver were positive for Rickettsia DNA. With the exception of one sample from Germany, which was positive for Rickettsia helvetica DNA, all the samples positive for Rickettsia DNA derived from the Polish population of raccoons. DNA of Rickettsia spp. was detected in 25 samples, i.e. 11.4% of the tested raccoons, and R. helvetica was confirmed in 52% of the positive samples. Additionally, single cases of Rickettsia monacensis, Rickettsia raoultii, and Candidatus Rickettsia kotlani-like were found, and in 32% of all the positive samples similarity was shown to different Rickettsia endosymbionts. Out of the samples that tested positive for gltA, amplicons of ompB and 17 kDa were successfully sequenced from 14 and three samples, respectively.

Conclusions: To the best of our knowledge, this study provides, for the first time, evidence of the occurrence of Rickettsia pathogens and endosymbionts in the European population of raccoons. Further, broader research on different
Background

The raccoon Procyon lotor (Linnaeus, 1758) (Carnivora; Procyonidae) is one of the most important and most intensively studied invasive mammal species in Europe. Its natural distribution includes much of North America, from southern Canada to Panama. It was first introduced into Europe in the early twentieth century to meet the demands of the then rapidly developing fur industry. Local raccoon populations comprise escapees from fur farms and individuals that have been intentionally released into the wild. The geographical distribution of the species in Europe underwent widespread expansion ca. 20 years after its introduction, mainly from Germany and—to a lesser extent—from Belarus [1, 2]. During the last 30 years the raccoon has spread at an increasing rate, resulting in the establishment of local populations in various regions of Europe. In these newly colonised areas, gaps in knowledge of the raccoon’s biology concern not only most aspects of its ecology in a broad sense, but also its pathogens and parasitoids. While knowledge of parasitic helminths in European raccoon populations is slowly but steadily improving [3–14], knowledge of the parasitic protozoans and bacterial pathogens of this host is still insufficient and fragmentary [15–17]. The possibility that the raccoon may be able to transmit pathogens that are new to the Old Continent’s fauna, together with its phenomenal habitat plasticity, omnivorous diet, opportunism, synanthropy and synurbization, suggest that it may pose a risk as a new reservoir of pathogens for European mammals. Though the species composition of the parasites of the European raccoon populations is still being studied, the results of earlier studies indicate that the problem of introduced parasites pertains mostly to parasitic helminths, e.g. one of the most important zoonotic parasites of raccoons is the nematode Baylisascaris procyonis. The species compositions of parasitic protozoans and bacterial pathogens of this carnivore in North America and Europe differ somewhat. Leśniańska et al. [15] and Hildebrand et al. [16] showed that the micro-parasites and micropathogens recorded in the European raccoon populations are not the same as those recorded in North American populations. On the one hand, this suggests that an obstacle to the transcontinental transmission of these pathogens may be, for example, a lack of adequate vectors of these organisms in the environments newly colonised by the raccoons. On the other hand, raccoons colonising new areas of Europe may acquire new pathogens, and thus may play a role as a potential new environmental reservoir for them. Most micropathogens recorded hitherto in the raccoon colonists of Europe have documented epizoonotic and zoonotic potential. Raccoons co-occurring with native carnivores are increasingly encountered near human habitations. Thus it seems especially important to examine the part played by this species in the spread of pathogens through both animal-animal and animal-human pathways.

Rickettsiae are strictly intracellular vector-borne bacteria which are transmitted to vertebrates by a variety of arthropods, primarily fleas and ticks. The genus Rickettsia includes over 20 validated species, which have been classified into five groups: the spotted fever group (SFG) including Rickettsia helvetica; the transitional group including Rickettsia felis; the typhus group; the ancestral group Rickettsia bellii and Rickettsia canadensis [18, 19]; and an ever-growing number of unnamed and non-cultivated genotypes [20]. Some of these newly identified rickettsiae have been proven to be the causative agents of emerging human diseases; they are often first recognized through their associations with different animals and their ectoparasites and only later detected in clinical specimens and associated with specific diseases [21]. To date, 18 species of Rickettsia are recognized human pathogens [22]. In Europe, rickettsioses are well documented, and there are several circulating Rickettsia species and Candidatus Rickettsia species [23].

Molecular studies on the occurrence of Rickettsia spp. in populations of wild carnivores are scarce in comparison to the large number of studies that have been undertaken on Rickettsia spp. in vectors. The occurrence of Rickettsia spp. among raccoons has been reported in Hokkaido, Japan from a molecular study. To the best of our knowledge, no Rickettsia strain has been isolated from raccoons in Europe so far. Therefore, little is known about the role of raccoons in the epidemiological cycles of Rickettsia in Europe, or elsewhere in the world. Thus, the aim of the present study was to investigate, through polymerase chain reaction assays, the presence of potentially zoonotic agents, such as Rickettsia spp., in the tissues of invasive raccoons (P. lotor) in Poland and Germany. This survey is a first step in achieving a full understanding of the host–parasite relationship between invasive raccoons and Rickettsia spp. Knowledge of the genetic diversity of Rickettsia species and their reservoirs is useful for predicting the potential risk of infection.
posed by them and for making decisions regarding their effective management. The isolation of new *Rickettsia* from wild carnivores such as raccoons will contribute to an understanding of the complexity of these bacteria in wildlife.

**Methods**

**Sample collection**

Raccoons were sampled from Poland (Ruszów Forest District, Zgorzelecka Forest) and Germany (districts of Kassel and Dresden). Ruszów Forest District is located in the western part of the Lower Silesian Wilderness—the largest lowland forest complex in Europe. It is part of the large, compact forest complex of the Bory Dolnośląskie Forest, which has a low proportion of deciduous forest types and a high proportion of coniferous forest types [24]. We used tissues in the present study that were obtained through collaboration with other projects financed by different grants. The raccoon carcasses comprised those obtained from hunters undertaking raccoon culling as part of game management activities, road kills, and those collected during a predator control operation conducted as a part of a program to reintroduce the capercaillie (*Tetrao urogallus*) into the Lower Silesian Forest, which was co-financed by the European Commission. Ear, spleen and liver samples were obtained, if possible, during the autopsies, and stored at −20°C until analysis. In total, 384 tissue samples (170 spleen samples, 82 liver biopsies and 132 ear biopsies) from 220 raccoons were examined (Table 1).

**Molecular analysis**

DNA was extracted using the Bio-Trace DNA Purification Kit (EURx, Poland) in accordance with the manufacturer’s instructions, and stored at −20°C until further use. DNA concentrations were determined with a NanoDrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

The presence of *Rickettsia* spp. DNA was determined through the amplification of a 338-bp fragment of the *gltA* gene, which has conserved regions shared by all known *Rickettsia* species, in nested PCR using two primer sets, RpCS.877p-RpCS.1258n and RpCS.896p-RpCS.1233n [25]. All the *gltA*-positive samples were further examined using nested PCR assays amplifying parts of the other protein coding genes examined, namely *ompB* (primers Rc.rompB.4362p, Rc.rompB.4,836n, Rc.rompB.4,496p, Rc.rompB.4,762n) [26] and a 17-kDa antigen (primers 17-k5, 17 k-3, 17KDK1, 17KDK2) [27]; the resulting 355-bp and 434-bp products were considered to indicate positive samples. Each PCR reaction was performed with 2× PCR Mix Plus (A&A Biotechnology, Gdynia, Poland) in a total reaction volume of 25 µl containing 1 µl of each primer (10 µM) and 3 µl (first reaction) or 1 µl (second reaction) of the DNA sample. Negative controls with nuclease-free distilled water, in the absence of template DNA, were included for each PCR reaction. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, and stained with Midori Green stain (Nippon Genetics). In order to prevent contamination of the PCR, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separate rooms.

The selected amplicons were purified using Exo-BAP (EURx) and directly sequenced in both directions by Macrogen (Amsterdam, the Netherlands) with the primers used for DNA amplification. Finally, the nucleotide sequences obtained in this study were edited using DNA Baser Sequence Assembly software (Heracle BioSoft, Romania) and compared with each other and with corresponding sequences registered in GenBank using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program [http://blast.ncbi.nlm.nih.gov/Blast.cgi].

Phylogenetic analyses were conducted using Bayesian interface implemented in MrBayes v3.2.7 software [28]. The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR+G+I) model was identified as the best-fitting nucleotide substitution model for both *gltA* and *ompB* alignments using MEGA X software [29]. The generated consensus trees were visualized using FigTree ver. 1.4.4 software [30].

**Results**

A total of 220 raccoons collected from two localities in Germany and two localities in Poland were tested for the presence of rickettsial DNA. Due to limited access to a complete range of tissues from all the specimens from each location, only one type of tissue was examined for some of the individual raccoons (Table 1). However, due to a lack of data on the role of raccoons in *Rickettsia* sp. circulation in Europe, all the results are presented here. In total, 384 tissue isolates derived from the skin, spleen and liver were used in this study.

| Country | Ear biopsies | Spleen samples | Liver biopsies | Total |
|---------|--------------|----------------|----------------|-------|
| Poland  | 50           | 73             | 56             | 179   |
| Germany | 0            | 15             | 26             | 41    |
| Total   | 50           | 88             | 82             | 220   |
Using partial gltA gene as a marker, DNA of Rickettsia spp. was detected in 25 samples, i.e. 11.4% [95% confidence interval (CI) 8.7–14.7] of the tested individuals. Amplicons of the rickettsial gltA gene were generated for all positive PCR products, and DNA sequences obtained from 20 isolates along with homologous sequences deposited in GenBank were used for analysis. Rickettsia helvetica was confirmed in 13 isolates, i.e. 52% (95% CI 31.7–70.4) of positive samples. All these sequences were identical to each other and to R. helvetica (GenBank no. U59723). Additionally, single cases of Rickettsia monacensis, Rickettsia raoultii and Candidatus Rickettsia kotlani—like (100% similarity to all three available sequences of Candidatus Rickettsia kotlani for gltA deposited in GenBank) are reported. The remaining eight isolates [32% (95% CI 16.1–52.0) of all positive samples] showed varying degrees of similarity to different Rickettsia endosymbionts (Fig. 1). Out of the samples that tested positive for gltA, 14 amplicons of ompB were successfully sequenced, i.e. from R. monacensis (n = 1), R. raoultii (n = 1), R. helvetica (n = 6), and Rickettsia endosymbionts (n = 6) (Fig. 2), but only three sequences of 17 kDa were obtained, i.e. from R. helvetica (n = 2) and Rickettsia endosymbionts (n = 1).

Rickettsia DNA was confirmed only in skin biopsies; no isolates from the spleen or liver were positive. With the exception of evidence of R. helvetica in one raccoon specimen from Germany (Kassel), all samples positive for this bacterium derived from the Polish population of raccoons.

**Discussion**

The role of wild animals in the life cycles of different vector-borne pathogens, and primarily those of tick-borne pathogens (TBPs), has been indicated in recent years [31, 32]. Certain species of wildlife, including wild carnivores, are suitable hosts for ticks and other haematophagous arthropods, and are also the main reservoirs of some vector-borne pathogens of medical and veterinary concern. However, for some TBPs, the role of wildlife is related to the persistence of pathogens in the environment, or is still not completely understood. On the one hand, in recent years the spectrum of TBPs affecting domestic animals and humans has increased, whilst on the other hand, due to urbanization and changes in natural ecosystems, populations of many wild species have increased, and they have adapted to environments in close proximity to human populations [33]. Therefore, investigation
of the distribution of TBPs among wildlife and domestic animals is important and necessary in studies on their epidemiology and ecology [31, 33–35]. A group of wildlife of particularly interest in this context are invasive alien species, as they create new opportunities for pathogens present in the environment by increasing their abundance and their range, which may result in bidirectional pathogen transmission [36–38].

Raccoons are hosts to ticks and associated pathogens which can impact the health of humans, livestock, and indigenous wildlife. Although raccoons are widespread in Europe, only a limited number of studies on the prevalence of zoonotic agents in these animals have been undertaken to date, e.g. on Baylisascaris procyonis [10], Trichinella spp. [12], Toxoplasma gondii [17], Cryptosporidium spp. and Enterocytozoon bieneusi [15] and Anaplasma phagocytophilum [16].

Data on Rickettsia pathogens detected in both native and introduced raccoon populations are rather scarce. In areas endemic for Rickettsia pathogens in the USA, various serological investigations have shown a very high prevalence, i.e. 45.8%, of R. rickettsii, the causative agent of Rocky Mountain spotted fever, and 73.7% of Rickettsia parkeri, which is closely related to R. rickettsii [39, 40]. Interestingly, a very recent molecular study confirmed the presence of Rickettsia DNA in only a few raccoon tissue samples, i.e. out of the 39 tested raccoons from the city of New York, Rickettsia spp. was confirmed in three ear biopsy tissue samples and one blood sample [41]. Research carried out in Japan using molecular methods revealed low levels of Rickettsia infection in introduced populations of raccoons, i.e. 1.6% for Rickettsia helvetica, 1.5% for Rickettsia amblyommii and single samples for Rickettsia felis and Rickettsia heliongjiangensis. It should be noted that the tested material, i.e. blood or spleen tissue, derived from a large number of animals, i.e. 194 and 699 samples, respectively [42, 43]. There are no similar data, to the best of our knowledge, on European raccoon populations.

This study showed, to our knowledge for the first time, the presence of genetic material of members of the genus Rickettsia in tissue samples of free-living raccoons in Europe. The detected prevalence of Rickettsia spp., 11.4%, was calculated from all the tested individuals, regardless of the type of tissue examined; however, when we considered only the biopsy samples of the skin (ear) (Table 1),
which was the only tissue in which the presence of *Rickettsia* DNA was confirmed, the prevalence reached 18.9% (95% CI 13.4–26.0). These results suggest that raccoons are not competent (or not yet competent) definitive hosts for *Rickettsia* spp. in Europe because *Rickettsia* infection in the present study was limited to a skin reaction. The most frequently identified rickettsiae [64% (95% CI 43.9–80.4) of all the positive samples] were members of the SFG, i.e. *R. helvetica*, *R. monacensis*, *R. raoultii* and *Candidatus* Rickettsia kotlaniii-like. These results are not surprising as tick-borne rickettsiae have been reported from almost all European countries. *Ixodes ricinus* is the most widespread tick species in Europe, and is known to carry mainly *R. helvetica* and *R. monacensis*. The dominant *Rickettsia* species in Europe is *R. helvetica*, and numerous European studies have confirmed the presence of this bacterium in *I. ricinus*, although its prevalence varied greatly [44]. *Rickettsia raoultii* is the species most commonly connected with the tick *Dermacentor reticulatus*, which has undergone a rapid expansion in recent years in Europe [45, 46], and specifically in Poland [47–49].

Recording the presence of a pathogen in a vertebrate is not sufficient evidence for classifying that host species as a reservoir; it can only be classified as a candidate reservoir if its physiological and behavioural features support amplification and transmission of the pathogen to vectors, or it can be classified as a simple carrier host or a dead-end host [44]. However, it is currently considered that vertebrates can act as amplifying hosts of rickettsiae, thus contributing to their spread in ecosystems, even in the absence of systemic infection [32].

The identification of *Candidatus* Rickettsia kotlaniii-like in Polish raccoons is interesting for various reasons. The genotype of *Candidatus* Rickettsia kotlani was described in 2006 as new within the SFG in ixodid ticks from Hungary, but its potential for pathogenicity is still unknown [44]. Ours is the fourth report of this species for the entire world and the third for Europe, and specifically the first from outside Hungary. All previous reports are from studies on *Haemaphysalis* ticks, i.e. *Haemaphysalis concinna* was reported as a host for *Candidatus* Rickettsia in Europe and *Haemaphysalis megaspinosa* for *Candidatus* Rickettsia in Japan [50, 51]. Ipso facto, the identification of the DNA of *Candidatus* Rickettsia in the skin biopsy of a raccoon is the first report of this *Candidatus* bacterial species from a vertebrate. Additionally, our finding of *Candidatus* Rickettsia kotlaniii-like may be indirect evidence of the occurrence of *H. concinna* in southwestern Poland. A recent study confirmed the presence of *H. concinna* among collections of juvenile ticks from rodent hosts and questing ticks from vegetation in western Poland. Interestingly, the occurrence of this tick in Poland has been reported only once so far, in 1953, in northwestern Pomerania, close to the border with Germany [49].

Our research on the potential reservoir role of the raccoon—a relatively new member of the carnivore fauna of Poland and the rest of Europe—in the circulation of vector-borne pathogens showed the presence of the DNA of *Rickettsia* endosymbionts in the examined tissues at a comparatively high frequency (one-third of positive isolates). We identified seven different endosymbiont strains (five of these are presented on the phylogenetic tree; Fig. 1) with varied homology to sequences previously deposited in GenBank. These endosymbionts have been previously identified as *Coxiella*, *Wolbachia*, and *Rickettsia* spp., and the relationship between them and the pathogenic bacteria which are transmitted by ticks to animals and humans remains unclear, as discussed in the literature [32]. Recent studies have revealed that rickettsial endosymbionts have negative effects on pathogenic rickettsiae within tick vectors, and that they preclude secondary infection. Other studies have addressed the positive influence of rickettsial endosymbionts on tick hosts [32]. However, the ancestral origins of these endosymbionts have yet to be elucidated, although it is speculated that they were originally animal pathogens acquired by ticks through their feeding on bacteremic hosts. These ancestral species of microorganisms probably divided into two groups: the endosymbionts, which have become specialists and completely adapted to ticks; and pathogenic bacteria, which have become generalists and are able to infect and reproduce in ticks as well as in vertebrate hosts [52]. If we accept this theory, it is understandable that reports of endosymbionts detected in vertebrate tissues are so infrequent. Therefore, in the light of our results, Noda et al. [52] appear to be justified in suggesting that there is a need to determine the potential of tick endosymbionts to emerge or reemerge as pathogens under natural conditions.

**Conclusions**

To the best of our knowledge, this study provides the first evidence of the occurrence of *Rickettsia* pathogens and endosymbionts in the raccoon population of Europe. The results presented here thus indicate the need for further, broader research on different species of wild vertebrates, and ticks, as potential vectors and hosts of TBPs in natural as well as in peri-urban environments.

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Author contributions
JH conceived and designed the study, DM, IM and MP collected and prepared the tissues for further research. APM, IM and KBG carried out the molecular analyses. JH performed the phylogenetic analysis. JH, MP and KBG drafted the manuscript. All the authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in the present article, and all the sequences were deposited in GenBank (accession numbers ON157065-ON157075 for gltA and ON157076-ON157083 for ampB genes).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 Department of Parasitology, University of Wroclaw, Wroclaw, Poland. 2 Department of Ecology and Environmental Protection, Institute of Biology, Pedagogical University of Kraków, Kraków, Poland.

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References
1. Bartoszewicz M, Okarma H, Zalewski A, Szczęsna J. Ecology of the raccoon (Procyon lotor) from western Poland. Ann Zool Fenn. 2008;45:291–8.
2. Fischer ML, Hochkirch A, Heddergott M, Schulze C, Anheyer-Behmenburg HE, Lang J et al. Historical invasion records can be misleading: genetic evidence for multiple introductions of invasive raccoons (Procyon lotor) in Germany. PLoS ONE. 2015;10:e0125441
3. Priemer J, Lux E. Abnataenia incisa (Cestoda), a parasite of the badger, Meles meles, and the raccoon Procyon lotor, in Brandenburg, Germany. Can J Zool. 1994;72:1848–53.
4. Gey A. Synopsis der Parasitenfauna des Waschbären (Procyon lotor) unter Berücksichtigung von Befunden aus Hessen. Ph.D. Dissertation. Fachbereich Veterinärmedizin, Justus Liebig Universität. Gießen, Germany. 1998,201.
5. Popiolek M, Szczęsna-Stasiakiewicz J, Bartoszewicz M, Okarma H, Smalec B, Zalewski A. Helminth parasites of an introduced invasive carnivore species, the raccoon (Procyon lotor L.), from the Warta Mouth National Park (Poland). J Parasitol. 2011;97:357–60.
6. Davidson RK, Ólives Ó, Hamnes IS, Schluze J. Illegal wildlife imports more than just animals—Baylisascaris procyonis in raccoons (Procyon lotor) in Norway. J Wildl Dis. 2013;49:986–90.
7. Rentería-Solís ZM, Hamedy A, Michler FU, Michler BA, Lücke E, Steer N, et al. AluA alata mcsorecaniae in raccoons (Procyon lotor) in Germany. Parasitol Res. 2013;112:3595–600.
8. Rentería-Solís Z, Birka S, Schmäschke R, Krol N, Obiegała A. First detection of Baylisascaris procyonis in wild raccoons (Procyon lotor) from Leipzig, Saxony, eastern Germany. Parasitol Res. 2018;117:3289–92.
9. Karamon J, Kochanowski M, Cencek T, Bartoszewicz M, Kusyk P. Gastrointestinal helminths of raccoons (Procyon lotor) in western Poland (Lubuskie province)—with particular regard to Baylisascaris procyonis. Bull Vet Inst Pulawy. 2014;58:547–52.
10. Al-Sabi MNS, Chriel M, Hansen MS, Enemark HL. Baylisascaris procyonis in wild raccoons (Procyon lotor) in Denmark. Vet Parasitol. Reg Stud Rep. 2015;1:2–55–8.
11. Pińoś A, Kusmierek N, Popiolek M. The occurrence of avian acanthocephalans Polymorphus minutus (Goeze, 1782) in raccoons (Procyon lotor L.) introduced to Europe. Ann Parasitol. 2018;64:249–52.
12. Cybulska A, Skoplek R, Kornacka A, Popiolek M, Pińoś A, Laskowska Z, et al. First detection of Trichinella pseudospiralis infection in raccoon (Procyon lotor) in Central Europe. Vet Parasitol. 2018;254:114–9.
13. Heddergott M, Steinbach P, Schwarz S, Anheyer-Behmenburg HE, Sutor A, Schliephake A, et al. Geographic distribution of raccoon roundworm, Baylisascaris procyonis, Germany and Luxembourg. Emerg Infect Dis. 2020;26:821–3.
14. Biedrzycka A, Popiolek M, Zalewski A. Host-parasite interactions in non-native invasive species are dependent on the levels of standing genetic variation at the immune locus. BMC Evol Biol. 2020;20:43.
15. Leśniarska K, Perek-Matsiak A, Hildebrand J, Burkowska-Gawlik K, Pińoś A, Popiolek M. Cryptosporidium spp. and Enterocytozoon bieneusi in introduced raccoons (Procyon lotor)—first evidence from Poland and Germany. Parasitol Res. 2016;115:4355–41.
16. Hildebrand J, Burkowska-Gawlik K, Adamczyk M, Gajda E, Merta D, Popiolek M, et al. The occurrence of Anaplasma phagocytophilum in European populations of invasive carnivores. Ticks Tick Borne Dis. 2018;9:934–7.
17. Kornacka A, Cybulska A, Popiolek M, Kusmierek N, Moskwa B. Survey of Toxoplasma gondii and Neospora caninum in raccoons (Procyon lotor) from the Czech Republic, Germany and Poland. Vet Parasitol. 2018;262:47–50.
18. Merhej V, Raoult D. Rickettsial evolution in the light of comparative genomics. Biol Rev Camb Philos Soc. 2011;86:379–405.
19. Murray GG, Weinert LA, Ruhel EL, Welch JJ. The phylogeny of Rickettsia using different evolutionary signatures: how tree-like is bacterial evolution? Syst Biol. 2016;65:265–79.
20. Parola P, Paddock DC, Cordovoschi C, Labruna MB, Medianinov O, Kemf T, et al. Update on tick-borne rickettsiosis around the world: a geographic approach. Clin Microbiol Rev. 2013;26:657–702.
21. Eremeeva ME, Dasch GA. Challenges posed by tick-borne rickettsiae: eco-epidemiology and public health implications. Front Public Health. 2015;3:155.
22. Sekeyová Z, Danchenko M, Filipík P, Fournier PE. Rickettsial infections of the central nervous system. PLoS Negl Trop Dis. 2019;13:e0007469.
23. Portillo A, Santibáñez S, García-Alvarez L, Palomar AM, Oteo JA. Rickettsioses in Europe. Microbes Infect. 2015;17:834–8.
24. Bobek B, Furtok J, Merta D, Wojcich-Ploskonka M. The effect of fencing the forest regeneration stages upon the level of damage caused by deer in the lowland forests of south-western Poland. Preprints. 2018;2018070161.
25. Prakash JA, SohanLal T, Rosemol V, Verghese VP, Pulimood SA, Reller M, et al. Molecular detection and analysis of spotted fever group Rickettsia infections in patients with fever and rash at a tertiary care centre in Tamil Nadu. India Pathog Glob Health. 2012;106:40–5.
26. Choi J, Yang W, Kim J, Ryu J, Lee S, Park K, et al. Spotted fever group and typhus group rickettsioses in humans, South Korea. Emerg Infect Dis. 2005;11:237–44.
27. Heise SR, Elshahed MS, Little SE. Bacterial diversity in Amblyomma americanum (Acanthocheilocnema) with a focus on members of the genus Rickettsia. J Med Entomol. 2010;47:235–44.
28. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohen A, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61:539–42.
29. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1647–57.
30. Rambaut A. FigTree v1.4.4. Computer program and documentation distributed by the author. 2016. http://tree.bio.ed.ac.uk/software/figtree/
31. Otranto D, Cantacessi C, Dantas-Torres F, Brianti E, Pfeffer M, Genchi C, et al. The role of wild canids and felids in spreading parasites to dogs and cats in Europe. Part II. Helminths and arthropods. Vet Parasitol. 2015;213:24–37.
32. Tomassone L, Berriatua E, De Sousa R, Duscher GG, Mihalca AD, Silaghi C, et al. Neglected vector-borne zoonoses in Europe: into the wild. Vet Parasitol. 2018;251:17–26.
33. Alvarado-Rybak M, Solano-Gallego L, Millán J. A review of piroplasm infections in wild carnivores worldwide: importance for domestic animal health and wildlife conservation. Parasit Vectors. 2016;9:538.

34. Dantas-Torres F, Chomel BB, Ottonato D. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol. 2012;28:437–46.

35. Hodžič A, Mitković B, Modrý D, Juráňková J, Frigelecová L, Forejtek P, et al. A new case of the enigmatic Candidatus Neoehrlichia sp. (FU98) in a fox from the Czech Republic. Mol Cell Probes. 2017;31:59–60.

36. Daszak P, Cunningham AA, Hyatt AD. Emerging infectious diseases of wildlife—threats to biodiversity and human health. Science. 2000;287:443–9.

37. Sutor A, Schwarz S, Conraths FJ. The biological potential of the raccoon dog (Nyctereutes procyonoides, Gray 1834) as an invasive species in Europe—new risks for disease spread? Acta Theriol. 2014;59:49–59.

38. André MR. Diversity of Anaplasma and Ehrlichia/Neoehrlichia agents in terrestrial wild carnivores worldwide: implications for human and domestic animal health and wildlife conservation. Front Vet Sci. 2018;5:293.

39. Magnarelli LA, Anderson JF, Philip RN, Burgdorfer W, Chappell WA. Rickettsiae–infected ticks (Acari: Ixodidae) and seropositive mammals at a focus for Rocky Mountain spotted fever in Connecticut, USA. J Med Entomol. 1983;20:151–6.

40. Castellau AH, Chenney EF, Varela-Stokes AS. Tick-borne disease agents in various wildlife from Mississippi. Vector-Borne Zoonotic Dis. 2011;11:439–42.

41. Tufts DM, Goodman LB, Benedict MC, Davis AD, VanAcker MC, Diuk-Wasser M. Association of the invasive Haemaphysalis longicornis tick with vertebrate hosts, other native tick vectors, and tick-borne pathogens in New York City, USA. Int J Parasitol. 2021;51:149–57.

42. Sashika M, Abe G, Matsumoto K, Inokuma H. Molecular survey of rickettsial agents in feral raccoons (Procyon lotor) in Hokkaido, Japan. Jpn J Infect Dis. 2010;63:353–4.

43. Baba K, Kaneda T, Nishimura H, Sato H. Molecular detection of spotted fever group Rickettsia in feral raccoons (Procyon lotor) in the western part of Japan. J Vet Med Sci. 2013;75:195–7.

44. Rizzoli A, Silaghi C, ObiegalA A, Rudolf l, Hubálek Z, Foldvári G, et al. Ixodes ricinus and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health. Front Public Health. 2014;2:251.

45. Rubel F, Brugger K, Pfeffer M, Chitimia-Dobler L, Didyk YM, Leverenz S, et al. Geographical distribution of Dermacentor marginatus and Dermacentor reticulatus in Europe.Ticks Tick Borne Dis. 2016;7:224–33.

46. Foldvári G, Siroký P, Szekeres S, Majoros G, Spong H. Dermacentor reticulatus: a vector on the rise. Parasit Vectors. 2016;9:314.

47. Mierzejewska EJ, Estrada-Peña A, Alsaaraf M, Bajer A. Mapping of tick Dermacentor reticulatus expansion in Poland in 2012–2014. Ticks Tick Borne Dis. 2016;7:94–106.

48. Mierzejewska EJ, Estrada-Peña A, Bajer A. Spread of Dermacentor reticulatus is associated with the loss of forest area. Exp Appl Acarol. 2017;72:399–413.

49. Dwużnik D, Mierzejewska EJ, Alsaaraf M, Bajer A. A new focus of the tick Haemaphysalis concinna in western Poland. Exp Appl Acarol. 2019;78:93–112.

50. Hornok S, Meli ML, Perreten A, Farkas R, Willi B, Beugnet F, et al. Molecular investigation of hard ticks (Acari: Ixodidae) and fleas (Siphonaptera: Pulicidae) as potential vectors of rickettsial and mycoplasmal agents. Vet Microbiol. 2010;140:98–104.

51. Andoh M, Ogasa-wara Y, Sakata A, Ito T, Fujita H, Kawabata H, et al. Isolation of the rickettsial agent genetically similar to Candidatus Rickettsia kotani, from Haemaphysalis megaspinosa in Japan. Vector Borne Zoonotic Dis. 2014;14:681–4.

52. Noda H, Munderloh UG, Kortti TJ. Endosymbiotic of ticks and their relationship to Wolbachia spp. and tick-borne pathogens of humans and animals. Appl Environ Microbiol. 1997;63:3926–32.