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The occurrence of Anaplasmataceae in European populations of invasive carnivores

Joanna Hildebrand[a,*], Katarzyna Buńkowska-Gawlik[a], Maja Adamczyk[a], Ewa Gajda[a], Dorota Merta[b], Marcin Popiołek[a], Agnieszka Perec-Matysiak[a]

[a] Department of Parasitology, Institute of Genetics and Microbiology, University of Wrocław, Poland
[b] Department of Ecology and Environmental Protection, Institute of Biology, Pedagogical University of Kraków, Poland

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ABSTRACT

The raccoon (*Procyon lotor*) and the raccoon dog (*Nyctereutes procyonoides*) belong to a group of the invasive species. The introduced species as potential reservoirs for vector-borne pathogens have been the subject of recent research, though there are still no data with reference to the European population of the raccoon, and few studies concern only the raccoon dog. This study shows the occurrence of Anaplasmataceae representatives in raccoons and a sympatric population of the raccoon dogs obtained from the area of Poland and Germany. During the study, the occurrence of *Anaplasma phagocytophilum* ecotype I in the introduced raccoon in northwestern Poland was revealed. Additionally, *Candidatus* Neoehrlichia sp. (FU98) was identified for the first time in the raccoon dog in Central Europe and thereby the raccoon dog is a new host for this pathogen.

1. Introduction

The presence of invasive species, both on global and local scales, pose one of the greatest dangers to biological diversity. The raccoon (*Procyon lotor*) and the raccoon dog (*Nyctereutes procyonoides*) belong to this group of the invasive species. The expansion of the raccoon, originating in North America, its influence and the documented harmfulness to the native fauna have become an undisputable issue. Raccoons do not have any natural enemies and their growing number, and a fast pace of colonization forcibly make an effective control impossible (Biedrzycka et al., 2014; Hohmann et al., 2002). In this context, the presence of the raccoon dog introduced from the territories of Asia, an animal of slightly slower dynamics of population growth, is only seemingly less problematic (Kowalczyk, 2014).

Besides the negative influence of both species on native zoocenosis and their sympatric occurrence with other carnivorous mammals, the possibility of a transmission of new and foreign species of pathogens to the European fauna seems to be a serious problem (Beltrán-Beck et al., 2012; Duscher et al., 2017; Sutor et al., 2014). As the research into helminths of European populations of raccoons and raccoon dogs has already been discussed (Duscher et al., 2017; Lempp et al., 2017; Ostensacken et al., 2017; Popiołek et al., 2011), the knowledge of their micropathogens is largely fragmentary (Heddergott et al., 2017; Lempp et al., 2017; Leśniańska et al., 2016; Solarczyk et al., 2016). These introduced species as possible reservoirs for vector-borne pathogens has been the subject of recent research, though there are still limited data with reference to the European population of the raccoon, and few studies concern only the raccoon dog (Härtwig et al., 2014; Hodžić et al., 2017; Wodecka et al., 2016).

One of the arthropod-transmitted bacteria are intracellular representatives of the family Anaplasmataceae including *Anaplasma* spp. and *Candidatus* Neoehrlichia spp. that infect domestic and wild animals and humans (Welc-Falęciak et al., 2014). *Anaplasma phagocytophilum* and *Candidatus* Neoehrlichia lotoris (CNL) were molecularly confirmed in the raccoon's native area (Levin et al., 2002; Yabsley et al., 2008). Since the host spectrum and the genetic diversity of *A. phagocytophilum* have been already documented both in North America and Europe, *Candidatus* Neoehrlichia lotoris is a more enigmatic species detected so far only in raccoons in the USA (Yabsley et al., 2008). In the last decade in Europe, *Candidatus* Neoehrlichia mikurensis, transmitted by Ixodidae and appearing in different species of mammals, has been identified (Földvári et al., 2014; Silaghi et al., 2016). At the same time, *Candidatus* Neoehrlichia sp. (FU98) has been identified in the red fox and badger in Austria, the Czech Republic and Hungary, and has been determined to be molecularly similar to CNL (Hodžić et al., 2015, 2017; Hornok et al., 2017).

Perhaps because of research into only a few hosts, the occurrence of Anaplasmataceae in *P. lotor* introduced to Europe has not been confirmed so far. This study focuses on the wide-ranging research that includes the populations of the raccoon in Poland and Germany.

* Corresponding author.
E-mail address: joanna.hildebrand@uwr.edu.pl (J. Hildebrand).

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Table 1
Number of samples collected from the examined animals.

Country	raccoon no. of samples			raccoon dog no. of samples	
	spleen	blood	liver	spleen	liver
Poland	78	18	5	10	10
Germany	40	25	25	n.a.	n.a.
Total	**118**	**43**	**30**	**10**	**10**

n.a. – not available.

Additionally, samples from a sympatric population of the raccoon dog have been analysed.

2. Materials and methods

2.1. Study areas and sample collection

A total of 118 raccoons and 10 raccoon dogs were collected during 2016–2017 (Table 1). The animals were obtained from the area of western Poland (city of Kostrzyn on the Oder [1] and Warta Mouth National Park [2], both situated on the Polish-German border in the Lubuskie Voivodeship, and the Ruszów Forest District [3] – located in the North-West part of Dolnośląskie Voivodeship) as well as from Germany (city of Kassel in northern Hesse [4], and Müritz National Park [5] in the south of Mecklenburg-Vorpommern) (Fig. 1). Raccoons and raccoon dogs were either shot by hunters or found as roadkill. Frozen carcasses or individual tissue samples were delivered to the laboratory of the University of Wrocław Department of Parasitology and kept at −20 °C for further analysis.

2.2. DNA extraction and PCR amplification

Commercially available kits were used for DNA isolation from blood and other tissues such as liver and spleen i.e. Bio-Trace DNA Purification Kit and Quick Blood DNA Purification Kit (EURx, Poland), following the manufacturers' protocols. Isolates were stored at −20 °C for further use. All samples were screened for the representatives of the family Anaplasmataceae pathogens.

At first, PCR detecting a 420-bp fragment of 16S of Anaplasmataceae bacteria was performed under the cycling conditions elaborated by Stuen et al. (2003). Then, positive samples were further evaluated by amplifying a 334 bp fragment of *msp2* and a 573 bp portion of *groEL* of *A. phagocytophilum* genes (Alberti et al., 2005; Massung and Slater, 2003). Conventional PCR assays targeting 16S (1053 bp) and *groEL* (806 bp) gene fragments of *Candidatus* Neoehrlichia sp. were used according to the protocols of Hodžić et al. (2015). Negative controls were performed in the absence of template DNA. The amplification was carried out in T100 Thermal Cycler (Bio-Rad, USA). All PCR products were visualized in 1.0% agarose gel stained with Midori Green Advanced DNA stain (NIPPON Genetics EUROPE). The products of the expected size were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and stored at 4 °C until sequencing.

2.3. Nucleotide sequencing

The products were sequenced in both directions on Applied Biosystems ABI PRISM 3100-Avant Sequencer (Genomed, Poland). The nucleotide sequences obtained in this study were edited by the use of the DNA Baser Sequence Assembly software (Heracle BioSoft SRL Romania) and then aligned with sequences available in GenBank.

Phylogenetic analyses were performed using MEGA 6.0 software (Tamura et al., 2013). The trees were inferred by the maximum likelihood method (model GTR + G + I), and bootstrapping was performed by using 1000 replicates. The sequences of *groEL* of *A. phagocytophilum* and *Candidatus* Neoehrlichia sp. genes, and 16S rRNA of *Candidatus* Neoehrlichia sp. gene obtained in this study have been deposited in the GenBank® database under the accession numbers: MG670108, MG670109, MG670107 respectively.

3. Results and discussion

The amplification of part of the 16S rRNA, designed for the Anaplasmataceae detection, was successful for one isolate derived from the raccoon's spleen (1/118, 0.8%) and tissue samples (both isolates of spleen and liver) of three raccoon dogs (3/10, 30%). All Anaplasmataceae-positive samples originated from Western Poland. Sequence analysis (a 420-bp fragment) showed a 99–100% similarity to 16S rRNA of *A. phagocytophilum* (GenBank® accession no. CP006616, CP006617 and EU839849, respectively) in the case of the raccoon isolate (Fig. 2). The sequences of isolates derived from raccoon dogs showed 99% identity with 16S rRNA of *Candidatus* Neoehrlichia lotoris (GenBank® accession no. EF633744) reported from raccoons in North America (Yabsley et al., 2008) (Fig. 2) and 100% identity with *Candidatus* Neoehrlichia sp. (FU98) (GenBank® accession no. KT833357,

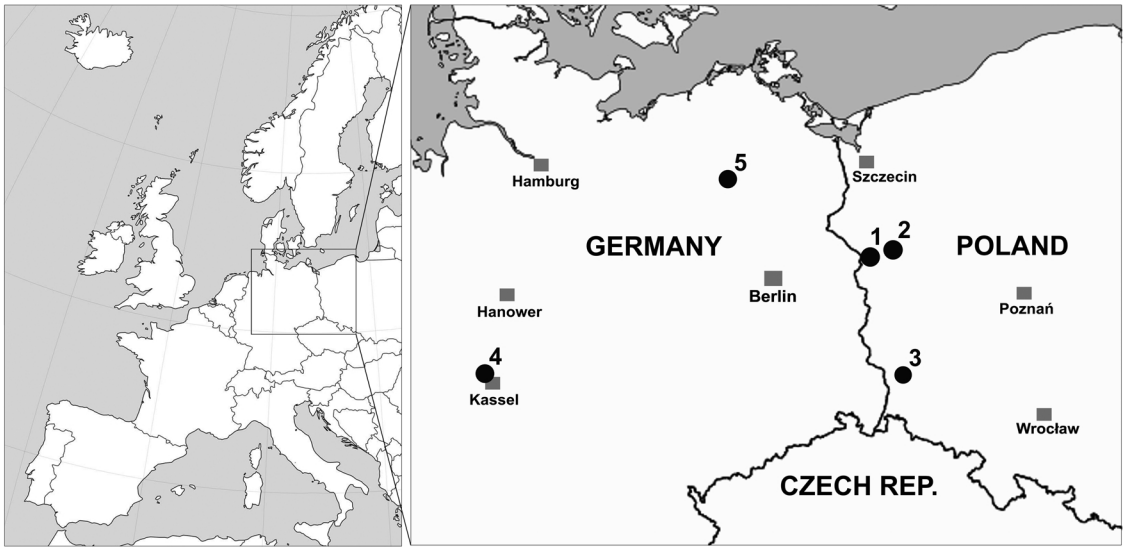

Fig. 1. Geographical origin (*black dots*) of raccoons and raccoon dogs obtained for this study.