Two New Haplotypes of Bartonella sp. Isolated from Lipoptena fortisetosa (Diptera: Hippoboscidae) in SE Poland

Katarzyna Bartosik 1,*, Weronika Maślaniko 1,*, Alicja Buczek 1, Marek Asman 3, Joanna Witecka 3, Ewelina Szwaj 1, Paweł Szczepan Błaszkiewicz 1 and Magdalena Świsłocka 4,*

1 Chair and Department of Biology and Parasitology, Faculty of Health Sciences, Medical University of Lublin, Radziwiłłowska 11 St., 20-080 Lublin, Poland; masman@sum.edu.pl (M.A.); jwitecka@sum.edu.pl (J.W.)
2 Department of Animal Ethology and Wildlife Management, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Radziwiłłowska 11 St., 20-950 Lublin, Poland
3 Department of Parasitology, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia, Jędrzejów 8 St., 41-200 Sosnowiec, Poland; masman@sum.edu.pl (M.A.); jwitecka@sum.edu.pl (J.W.)
4 Department of Zoology and Genetics, Faculty of Biology, University of Białystok, Ciołkowskiego 1f St., 15-245 Białystok, Poland
* Correspondence: katarzyna.bartosik@umlub.pl (K.B.); weronika.maslanko@up.lublin.pl (W.M.); magdaswi@uwb.edu.pl (M.Ś.)

Abstract: Insects of the genus Lipoptena are parasitic arthropods with a broad host range. Due to the type of parasitism (hematophagy), their potential role as vectors of pathogens, i.e., Bartonella sp., Anaplasma phagocytophilum, Rickettsia spp., and Borrelia burgdorferi is considered. As the range of their occurrence has been changing dynamically in recent years and infestations of humans have increasingly been reported, these organisms are now the subject of numerous studies. Our research aimed to present the molecular characteristics of Bartonella sp. detected in Lipoptena fortisetosa parasitizing wild cervids in south-eastern Poland. Adults of Lipoptena spp. were collected from carcasses of roe deer and red deer between spring and autumn in 2013. The PCR method was used to detect Bartonella sp. in the insects. We report two new haplotypes of the rpoB gene of Bartonella sp. isolated from L. fortisetosa feeding on wild cervids in south-eastern Poland and the presence of this invasive ectoparasitic species in the studied area since 2013. Phylogenetic analyses of newly obtained Bartonella sp. haplotypes confirmed their unique position on the constructed tree and network topology. The rpoB gene sequences found belonging to lineage B support the view that this phylogenetic lineage represents a novel Bartonella species.

Keywords: Lipoptena sp.; deer keds; invasive species; Cervus elaphus; ectoparasites; wild cervids

1. Introduction

Five of the 32 species of Lipoptena deer keds (Diptera: Hippoboscidae) widespread in the world fauna occur in Europe [1,2]. Two species, i.e., Lipoptena cervi (Linnaeus, 1758) and Lipoptena fortisetosa Maa, 1965, inhabit the central and northern parts of the continent. In recent years, progressive expansion of L. cervi [3–7] and L. fortisetosa [8–15] has been observed. As shown by literature data, the spread of L. fortisetosa species in Europe was most likely caused by its natural dispersal outside Asia, overlapping the ranges of Siberian
and European roe deer during periodic climate changes, or by introduction with alien mammal species, e.g., sika deer (*Cervus nippon* Temminck, 1838) [16,17].

In Poland, *L. fortisetosa* was first found in Lower Silesia at the end of the 1980s [18]. This deer ked species was found again in 2007–2014 on red deer (*Cervus elaphus* Linnaeus, 1758) and roe deer (*Capreolus capreolus* Linnaeus, 1758) in the north [19,20], in environments in north-eastern and southern Poland, including the Polish part of the Tatra Mountains [21,22], and recently in northern and western Poland [23].

Depending on the geographical region as well as climate and ecological conditions, the level of prevalence and severity of invasion of specific ectoparasites varies significantly, e.g., in the case of *L. cervi*, it mainly depends on the host species [4,24] and exhibits seasonal differences: the highest prevalence is most often noted in autumn and winter [25]. *L. cervi* parasitize domestic and wild animals, primarily representatives of Cervidae—red deer, roe deer, and moose (*Alces alces* Linnaeus, 1758) [15,26], own observations. The *L. fortisetosa* host species have not been clearly defined, but they are probably the same animals as the hosts of *L. cervi* [13,16]. Human infestations by *Lipoptena* adults in their habitats are increasingly being reported. Their bites cause dermatitis in humans [3,27–29]. In animals, the parasitism of these flies induces clinical symptoms related to anemia and skin mechanical damage [30,31].

In *Lipoptena* spp., microorganisms causing human and animal diseases have been detected. These include, e.g., *Anaplasma ovis* [32], *Anaplasma phagocytophilum* [23,33], *Bartonella* sp. [23,34–40], *Borrelia burgdorferi* [33], *Rickettsia* spp. [23,32], *Trypanosoma* spp. [41,42], Coxielia-like bacteria, *Theileria luwenshuni*, and *Theileria ovis* endosymbionts [23,43]. This fact contributed to the increased interest in the potential involvement of these arthropods in maintenance of foci of zoonotic diseases. In north-eastern Poland, researchers detected in *L. cervi* sequences of *Bartonella* sp. with 99% similarity with *B. schoenbuchensis* [44]. The presence of *Bartonella* sp. was also noted in *L. fortisetosa* sampled from cervids and from the environment in the northern and western parts of the country [23].

Our study presents the molecular characteristics of *Bartonella* sp. detected in *L. fortisetosa* parasitizing wild cervids in south-eastern Poland.

## 2. Materials and Methods

### 2.1. Sampling

Specimens of *Lipoptena* were collected in spring and autumn 2013 from carcasses of *C. capreolus* and *C. elaphus* harvested by hunters in accordance with the Act of 13 October 1995 (Hunting Law, Journal of Laws 2018, item 2033 as amended) near Polesie National Park (51°23′39″ N 23°11′4″ E) (Figure 1). These animals were culled in accordance with the Annual Hunting Plans in selected hunting circles operating in the studied macroregion, during hunting periods indicated in the Regulation of the Minister of the Environment of 16 March 2005 on the determination of hunting periods for game animals (Journal of Laws 2005, No. 48, item 459). Ectoparasites collected from the animals were placed in sterile plastic test tubes with 70% ethanol.
2.2. Species Identification

Identification of the species and sex of the adult insects was carried out in the laboratory using an OLYMPUS SZX16 (Olympus, Tokyo, Japan) stereoscopic microscope and the key for identification of arthropod species compiled by Borowiec [45].

2.3. Molecular Analysis

2.3.1. DNA Extraction and Polymerase Chain Reaction

The molecular analysis included 24 specimens of *Lipoptena* spp., each blood-fed adult from a different animal host. The DNA from 15 *L. cervi* (6 females and 9 males) and 9 *L. fortiseta* (7 females and 2 males) randomly selected for the pilot study was isolated with the ammonia method [46]. Next, its concentration was measured spectrophotometrically using a nanospectrophotometer Pearl (Implen, Germany) at a 260/280 wavelength. Then, the samples were frozen at −20 °C and stored until further analysis. The PCR method and a pair of primers (1400F and 2300R) specific to the *rpoB* gene were used to detect *Bartonella* sp. in the insects [47]. The amplification product was separated electrophoretically in 2% ethidium bromide-stained agarose gel. Then, the gel was visualized in ultraviolet light in an Omega 10 device (Ultra Lum, Claremont, CA, USA). Next, the samples were analyzed with the use of Total Lab software (TotalLab, Newcastle-Upon-Tyne, UK). The presence of an 825 base pair PCR product was treated as positive. Next, this product was isolated from the agarose gel with the use of an Agarose Out kit (EURx, Gdansk, Poland) according to the manufacture’s protocol and sequenced (Genomed, Warsaw, Poland).

2.3.2. Sequencing and Phylogenetic Analysis

The resulting sequences of the *rpoB* gene for RNA polymerase beta subunit were aligned and revised manually in BioEdit v7.0.4 [48]. The obtained sequences were submitted to GenBank. To test the phylogenetic relationships among our newly obtained *rpoB* gene haplotypes and sequences downloaded from GenBank, we constructed a phylogenetic tree using a maximum-likelihood (ML) algorithm in Mega v5.05 [49] with 1000 bootstrap
replicates. The GTR+I+G model of substitution was selected as the best-fitting model by the AIC test (Akaike Information Criterion) with jModelTest [50] for the ML tree. We also calculated and visualized the relationships among founders in our study and downloaded rpoB gene haplotypes from GenBank by constructing a haplotype network using the median-joining method available in Network version 10.2.0.0 (http://www.fluxus-engineering.com (accessed on 10 February 2021).

3. Results

Two species, i.e., *L. cervi* and *L. fortisetosa*, were identified among the Lipoptena adults collected from *C. elaphus* and *C. capreolus*. The preliminary analyses of the presence of Bartonella sp. in the deer keds involved 15 *L. cervi* specimens (3 females and 6 males from *C. elaphus* and 3 females and 3 males from *C. capreolus* and 9 *L. fortisetosa* specimens (3 females and 2 males from *C. elaphus* and 4 females from *C. capreolus*). In total, Bartonella sp. were detected in 3/24 (12.5%) of the studied insects. The presence of the bacteria was shown in only 3/9 (33.3%) *L. fortisetosa* adults (2/7 of the studied females and 1/2 of studied males). No Bartonella sp. were detected in the *L. cervi* adults. The derived sequences of Bartonella sp. were submitted to the GenBank database under the accession numbers: MZ061868, MZ061869. The sequences obtained in this study share from 96.6 to 98.3% similarity with Bartonella sp. Honshu isolated from sika deer blood in Japan (GenBank accession no. AB703145).

The analysis of a rpoB gene fragment yielded two new haplotypes of Bartonella sp.: haplotype H1 (MZ061868) and haplotype H2 (MZ061869), as defined by three polymorphic sites, all being transitions. The maximum-likelihood phylogenetic reconstructions produced a strong topology (Figure 2). The ML tree revealed that our two rpoB haplotypes belong to lineage B described by Sato et al. [51]. The median-joining network based on sequences from this study and haplotypes representing different species of Bartonella obtained from GenBank (Table 1) suggested the presence of a distinct phylogenetic branch created by the discovered haplotypes inside lineage B. It also showed that they are grouped closely with haplotypes H4 (AB703145) and H7 (AB703147) described for new species of Bartonella obtained from Japanese sika deer in Japan (Figure 3).

**Table 1.** List of species and GenBank accession numbers of their RNA polymerase beta subunit (rpoB) gene sequences used in the network phylogenetic analysis (Figure 3).

| Symbol of Haplotype | Scientific Name | GenBank Accession Number | Sequence Source |
|---------------------|----------------|-------------------------|-----------------|
| H1                  | Bartonella sp. | MZ061868                | This study      |
| H2                  | Bartonella sp. | MZ061869                | This study      |
| H3                  | Bartonella sp. | AB703144                | Sato et al. [51]|
| H4                  | Bartonella sp. | AB703145                | Sato et al. [51]|
| H5                  | Bartonella sp. | AB703146                | Sato et al. [51]|
| H6                  | Bartonella sp. | MF580655                | Szewczyk et al. [44] |
| H7                  | Bartonella sp. | AB703147                | Sato et al. [51]|
| H8                  | Bartonella sp. | AB703148                | Sato et al. [51]|
| H9                  | Bartonella sp. | AB703149                | Sato et al. [51]|
| H10                 | Bartonella capreoli | AB290188 | Inoue et al. [52] |
| H11                 | *Bartonella* schoenbuchensis | AY167409 | Unpublished |
| H12                 | Bartonella bovis | DQ356077 | Unpublished |
| H13                 | Bartonella bovis | EF432062 | Maillard et al. [53] |
| H14                 | Bartonella bovis | KF218216 | Bai et al. [54] |
| H15                 | Bartonella chomelii | JN646664 | Mediannikov et al. [55] |
| H16                 | Bartonella sp. | JQ765388 | Unpublished |
| H17                 | Bartonella bovis | KF218217 | Bai et al. [54] |
| H18                 | Bartonella bovis | KF218220 | Bai et al. [54] |
| H19                 | Bartonella bovis | KF218224 | Bai et al. [54] |
| H20                 | Bartonella bovis | KJ909808 | Rudoler et al. [56] |
Table 1. Cont.

| Symbol of Haplotype | Scientific Name        | GenBank Accession Number | Sequence Source                        |
|---------------------|------------------------|--------------------------|----------------------------------------|
| H21                 | Bartonella chomelii    | KM215709                 | Antequera-Gomez et al. [57]            |
| H22                 | Bartonella chomelii    | KM215710                 | Antequera-Gomez et al. [57]            |
| H23                 | Bartonella bovis       | KR733194                 | Kho et al. [58]                        |
| H24                 | Bartonella bovis       | KR733195                 | Kho et al. [58]                        |
| H25                 | Bartonella sp.         | LC485118                 | Sato et al. [51]                       |
| H26                 | Bartonella sp.         | MF80656                  | Szewczyk et al. [44]                   |
| H27                 | Bartonella sp.         | MF80657                  | Szewczyk et al. [44]                   |
| H28                 | Bartonella sp.         | MF80662                  | Szewczyk et al. [44]                   |

Figure 2. Maximum-likelihood topology computed with the GTR+I+G model of substitution evolution, representing the phylogenetic relationships among the sequences of the *rpoB* gene for RNA polymerase beta-subunit found in *Bartonella* sp. Numbers listed at the nodes represent the percent support for the node from 1000 bootstrap replicates. The ML tree has been rooted with sequences of *Brucella melitensis*, a microorganism closely related with *Bartonella* sp., as they together belong to the same order, Hyphomicrobiales. The haplotypes of *Bartonella* sp. found in this study are marked in blue. Lineage B, according to Sato et al. [51].
Figure 3. Median-joining network of rpoB haplotypes from Poland (H1 and H2, marked with a blue background) and haplotypes of different Bartonella species obtained from GenBank (H3–H28, symbols according to Table 1). Missing haplotypes are indicated by a grey dot.

4. Discussion

The zoonotic pathogen Bartonella sp. is a Gram-negative hemotropic bacterium, which is an etiological agent of bartonellosis. The disease usually manifests as an acute or sub-acute febrile illness in humans and animals [59]; however, a long-term symptomless infection with bacteremia in mammalian reservoir hosts (e.g., dogs and cats) was also noted [55,60,61]. The role of this bacterium as a causative agent or cofactor in endocarditis has been reported [62,63]. Lipoptena spp. may serve as a potential vector of this bacterium [34,35,39,40].

The prevalence of Bartonella sp. in Lipoptena is high. For instance, Bartonella DNA was detected in 85% of wingless adults of L. cervi collected from free-ranging cervids in Norway [64], and even in 94% of these deer keds collected from roe deer in France [35]. In Mazury forests (northern part of Poland), Szewczyk et al. showed the prevalence of Bartonella sp. in these insects at the level of 75.12% [44]. The latest data from northern and western Poland indicate the presence of Bartonella sp. in 49.4% of L. fortisetosa adults [23]. In turn, the Bartonella sp. infection rate in L. fortisetosa collected in Japan was estimated at 87.9% by real-time PCR and 51.5% in culture [40].
In this study, this bacterium was not detected in *L. cervi*. However, the absence of *Bartonella* sp. in the studied deer keds may be related to the smaller number of analyzed samples. Five sequences of *Bartonella* sp. obtained by Szewczyk et al. showed 94.4% similarity with *Bartonella* sp. from Japanese sika deer (GenBank accession no. AB703149) [44]. In turn, the two other sequences showed 99.7% similarity with *Bartonella* sp. isolated from Japanese sika deer in Wakayama Prefecture, Japan (GenBank accession no. AB703149) and with *Bartonella* sp. isolated from Japanese sika deer in Nara Prefecture, Japan (GenBank accession no. AB703146). The sequences obtained in this study showed high similarity with *Bartonella* sp. Honshu isolated from sika deer blood in Japan (GenBank accession no. AB703145) but did not show similarity with the sequences obtained by Szewczyk et al. from *L. cervi* [44].

In the maximum-likelihood (ML) algorithm based on the *rpoB* gene sequences, our two haplotypes formed a distinct branch with high bootstrap support within lineage B described by Sato et al. [51]. The ML phylogenetic analyses corroborated the result obtained from the nucleotide network and confirmed that the two haplotypes obtained in this study created a separate branch within the different species of *Bartonella*. Our newly discovered haplotypes differed by at least nine substitutions from haplotype 4 (GenBank accession no. AB703145, HonshuWD-9.3) and by at least 10 mutations from haplotype 7 (GenBank accession no. AB703147, *Bartonella* sp. HonshuWD-18.5), both described by Sato et al. [51]. Interestingly, these two GenBank haplotypes of *Bartonella* were isolated from Japanese sika deer in Japan. As shown by the network analysis, our two haplotypes and haplotypes 4 and 7 created a distinct group together, which additionally supports the view proposed by Sato et al. that lineage B represents a novel *Bartonella* species [51]. The presence of these two new *Bartonella* sp. haplotypes in *L. forsitetosa* and the haplotypes obtained by Szewczyk et al. in *L. cervi* may suggest that red deer in Poland seem to harbor the novel *Bartonella* species discovered in Japanese sika deer [44,51]. Moreover, it seems to be necessary to obtain and analyze more sequences of *Bartonella* directly from red deer blood to resolve the relationships of *Bartonella* species in deer from Japan and Poland. In turn, the role of this deer ked species as a potential vector of this bacterium needs further study.

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