



# Article Diversity of Flea Species (Siphonaptera) and Their Vector-Borne Pathogens from Bats (Chiroptera) in Lithuania

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Abstract: Bat fleas (Insecta: Siphonaptera: Ischnopsyllidae) are highly host-specific ectoparasites distributed worldwide (except Antarctica). The identification of flea species and the detection of fleaborne pathogens plays a significant role not only in veterinary and public health, but also in providing insights into the ecology, behaviour, and geographical distribution of fleas and flea-associated pathogens. The present study aimed to conduct a morphological and molecular identification of fleas collected from Lithuanian bats, as well as to screen for the presence of vector-borne pathogens. A total of 58 flea specimens were collected from bats in Lithuania. Based on morphological analysis, seven flea species were identified (Ischnopsyllus variabilis, I. octactenus, I. simplex, I. elongatus, I. hexactenus, Nycteridopsylla pentactena, and N. eusarca) and screened for the presence of Anaplasma spp., Borrelia spp., Babesia spp., Bartonella spp., Rickettsia spp., and Mycoplasma spp. Molecular analysis of fleas' COI and 18S rRNA genes showed that the flea species collected from bats are closely genetically related. Bartonella DNA was detected in bat fleas (41.4%). No DNA of Anaplasma, Borrelia, Babesia, Rickettsia, and Mycoplasma was amplified in the tested samples. The Wolbachia sp. was accidentally found in I. elongatus. The phylogenetic analysis of four Bartonella genes (16S-23S rRNA ITS, gltA, rpoB, and groEL) revealed specificity to bats or bats' ectoparasites. This is the first report about bat flea species and the pathogens in bat fleas in Lithuania. New host records for bat fleas were also reported. This study extends the knowledge about bat fleas and their role as vectors of different pathogens.

**Keywords:** *Ischnopsyllus; Nycteridopsylla;* phylogenetic analysis; *Anaplasma; Borrelia; Babesia; Bartonella; Rickettsia; Mycoplasma; Wolbachia* 

# 1. Introduction

Fleas (Siphonaptera) are globally distributed blood-sucking ectoparasites of mammals and birds that can play an important role in transmitting various pathogens. Currently, more than 2500 species in 16 families of fleas are known [1]. Fleas of the Ischnopsyllidae family, consisting of 20 genera and 125 species, are mainly parasites of bats [2]. The fleas of this family are highly host-specific but occasionally can be found on other bat species [3].

Bats (Chiroptera) are known as the second largest group of mammals, following rodents [4,5]. They form dense and large colonies and are characterized by a long lifespan [6]. These traits create appropriate conditions for bats to be reservoir hosts for different pathogens [7]. During the COVID-19 pandemic period, bats triggered a great interest [8]. Many research studies have focused on bat-associated viruses. Meanwhile, information about the bacterial pathogens in bats and their ectoparasites is limited [7,9].

In Lithuania, 15 bat species belonging to the Vespertilionidae family were recorded [10,11]. Only a few studies have focused on the distribution of Lithuanian bats [10,12]. Meanwhile, the fauna of bat ectoparasites has not been investigated and the species composition of fleas on Lithuanian bats is not known. Currently, no information about the pathogens circulating



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**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). in Lithuanian bats and their ectoparasites is available. To date, only a small number of studies have been published about the diversity of bat flea species [13–16] and the pathogens they might carry in Europe [17,18]. This highlights a need for studies to obtain knowledge about bat fleas and their role as vectors of different pathogens. Accordingly, the aims of this study were (i) to perform a morphological and molecular identification of the fleas collected from Lithuanian bats and (ii) to screen the presence of vector-borne pathogens (*Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Borrelia* spp., *Rickettsia* spp., and *Mycoplasma* spp.) in bat fleas.

#### 2. Materials and Methods

## 2.1. Flea Collection and Species Identification

Fleas were collected from both live and deceased bats by brushing the fur with tweezers, between 2019 and 2021. Live bats were either observed during ringing activities at the Vente's Ragas Ornithological Station or incidentally found in buildings. The carcasses of collected bats were stored at -40 °C and inspected for fleas before dissections. All aspects of this study were performed according to guidelines established for the ethical treatment of animals and complied with current Lithuanian regulations.

The fleas collected from each bat were placed in separate 1.5 mL tubes with 70% ethanol and were kept at +4 °C until investigation. Fleas were morphologically identified at the species level with taxonomic keys [19]. DNA was extracted from each specimen individually, using 2.5% ammonium hydroxide, while retaining flea exoskeletons [20]. The flea exoskeletons were mounted on permanent slides in Canada balsam [21]. The subsequent identification of the species was carried out by analysing the 18S ribosomal RNA (*18S rRNA*) and cytochrome c oxidase subunit I (*COI*) genes.

Conventional PCR was performed to amplify a ~1000 bp fragment of flea 18S rRNA using one primer set 18ai 5'-CCT GAG AAA CGG CTA CCA CAT C-3' and 18bi 5'-GAG TCT CGT TCG TTA TCG GA-3' [22]. Each 20 µL reaction contained 10 µL of 2x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania), 5 µL of nuclease-free water, 1 µL (10 mM) of each primer, and 3 µL of DNA. The PCR reaction conditions were as follows: initial denaturation at  $94^\circ$  for 2 min; followed by 35 cycles of denaturation at  $94^\circ$ for 1 min, annealing at  $58^{\circ}$  for 1 min, and extension at  $72^{\circ}$  for 1 min; with a final extension at  $72^{\circ}$  for 5 min, after the completion of all cycles. To amplify a 709 bp fragment of the COI gene, conventional PCR with primers LCO1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' was conducted [23]. The PCR reaction was performed in a 20 µL mixture containing 10 µL of 2x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania), 2.6 µL of nuclease-free water, 1.7  $\mu$ L (10 mM) of each primer, and 4  $\mu$ L of DNA. The PCR reaction conditions were as follows: initial denaturation at  $94^\circ$  for 1 min; followed by 5 cycles at  $94^\circ$ for 1 min, at 45° for 1.5 min, and at 72° for 1.5 min; followed by 35 cycles at 94° for 1 min, at  $50^{\circ}$  for 1.5 min, and at  $72^{\circ}$  for 1 min; with a final extension at  $72^{\circ}$  for 5 min after the completion of all cycles. All purified PCR products were prepared for DNA sequencing.

### 2.2. Pathogen DNA Amplification

Screening for the presence of six pathogens, *Anaplasma* spp., *Borrelia* spp., *Babesia* spp., *Bartonella* spp., *Rickettsia* spp., and *Mycoplasma* spp., was conducted by using different PCR assays with specific primers, as previously described (Supplementary Table S1) [24–41].

Multiplex-tandem (MT) real-time PCR for vector-borne pathogens was used to screen the samples. MT real-time PCR for *Anaplasma*, *Borrelia*, and *Babesia* species targeting *msp2*, 23S rRNA, and 18S rRNA genes, respectively, was run, as previously described [24,36]. To further screen MT real-time PCR for *Bartonella* and *Rickettsia* species targeting *ssrA* and *gltA* genes, respectively, was used, as previously described [31,36]. Results were considered positive when repeats yielded cycle threshold (Ct) values <38.00. All flea samples were further identified via conventional and nested PCRs targeting the specific genes of different pathogens. All PCR reactions were performed as previously described (Supplementary Tables S1 and S2). Positive (DNA of target pathogen, confirmed by sequencing) and negative (sterile, double-distilled water) controls were included in each PCR run. PCR products were visualized under UV light after electrophoresis on 1.5% agarose gel with ethidium bromide.

## 2.3. Sequencing

PCR products of all positive samples were extracted from agarose gel and purified using the GeneJET<sup>TM</sup> Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania) and sent to a sequencing service (Macrogen, Amsterdam, The Netherlands). The obtained DNA sequences were edited using the MegaX software package v10.2.6 [42] and aligned with each other, as well as with the previously published sequences in GenBank using the ClustalW multiple alignment option and BLASTn. The most appropriate model of nucleotide substitution for each alignment data set was determined according to the Bayesian information criterion (BIC). The phylogenetic tree was conducted using the Maximum Likelihood method and Tamura 3-parameter model. Outgroup taxa were obtained from GenBank. Bootstrap support was calculated by means of 1000 replicates.

Sequences for representative samples obtained in this study were submitted to the Gen-Bank database under the accession numbers PP057939–PP057943 and PP407542–PP407543 for the flea 18S rRNA gene, PP425999–PP426007 for the flea COI gene, PP430158–PP430160 for the Wolbachia, PP296528–PP296548 for the Bartonella 16S-23S rRNA ITS region, PP333876– PP333895 for the Bartonella groEL gene, PP333854–PP333875 for the Bartonella rpoB gene, and PP333838–PP333853 for the Bartonella gltA gene. Detailed information about the uploaded Bartonella sequences to GenBank, with accession numbers, is provided in Supplementary Table S3.

## 3. Results

#### 3.1. Identification of Flea Species

A total of 58 fleas were collected from 41 bats, representing 6 species, *Pipistrellus nathusii* (Keyserling and Blasius, 1839) (n = 32), *Pipistrellus pygmaeus* (Leach, 1825) (n = 2), *Nyctalus noctula* (Schreber, 1774) (n = 4), *Eptesicus nilssonii* (Keyserling and Blasius, 1839) (n = 1), *Myotis daubentonii* (Kuhl, 1817) (n = 1), and *Barbastella barbastellus* (Schreber, 1774) (n = 1). Seven species of fleas were identified morphologically, *Ischnopsyllus variabilis* (Wagner, 1898) (n = 24), *Ischnopsyllus octactenus* (Kolenati, 1856) (n = 16), *Ischnopsyllus simplex* (Rothschild, 1906) (n = 6), *Ischnopsyllus elongatus* (Curtis, 1832) (n = 4), *Nycteridopsylla pentactena* (Kolenati, 1856) (n = 5), *Ischnopsyllus hexactenus* (Kolenati, 1856) (n = 1), and *Nycteridopsylla eusarca* (Dampf, 1908) (n = 1) (Supplementary Figures S1–S7). One flea specimen collected from *N. noctula* was unidentified to species level (Table 1).

Analysis of the *18S rRNA* data revealed low variability, showing only one nucleotide difference between the *Ischnopsyllus* and *Nycteridopsylla* genus (G and C, respectively). At the genus level, all obtained sequences (*n* = 23) were 100% identical to each other and to sequences deposited in GenBank (Figure 1). Meanwhile, the partial sequences of the *COI* gene showed divergences on an individual level among flea specimens. A total of six variable positions were found between *I. variabilis* and four between *I. simplex* specimens (Supplementary Table S4). However, different bat flea species did not form separate clusters (Figure 2). All sequences from Lithuanian bat fleas had a maximum similarity to fleas from GenBank, varying between 93.8 and 100%. Moreover, the presence of *Wolbachia* endosymbionts was detected in three *I. elongatus* specimens using COI gene fragments. The NCBI BLAST analysis revealed that these sequences were 96.9–99.7% similar to the *Wolbachia* endosymbiont found in other insect species.

Bat Species	Flea Number, Sex, and Species				
	3 ♂, 10 ♀ <i>I. octactenus</i>				
P. nathusii	$3 \circ$ , $17 \circ I$ . variabilis				
	$2 \circ$ , $1 \circ I$ . simplex				
P. pygmaeus	0 ♂ , 3 ♀ I. octactenus				
	1  , 0				
N. noctula	$1 \circ$ , $3 \circ I$ . elongatus				
	1 ♂, 2 ♀ I. variabilis				
	$0 \circ, 1 \circ Ischnopsyllus sp.$				
E. nilssonii	2 ♂, 3 ♀ N. pentactena				
M. daubentonii	2 ♂, 1 ♀ <i>I. simplex</i>				
	1 ♂, 0 ♀ <i>I. hexactenus</i>				
B. barbastellus	$0 \circ$ , $1 \circ$ I. variabilis				
Total number (female/male ratio)	$16 \circ, 42 \circ (2.625, \chi^2 = 58.34)$				

Table 1. Sex and number of flea species collected from bats in Lithuania.



**Figure 1.** Phylogenetic tree of the Ischnopsyllidae flea species, based on the *18S rRNA* gene sequences constructed using the Maximum Likelihood method and Tamura 3-parameter model. Numbers on the tree indicate bootstrap support (values <50 % not shown). Samples sequenced in the present study are marked •. The number of samples represented by the sequence is given in parentheses (n = x).



0.02

**Figure 2.** Phylogenetic tree of the Ischnopsyllidae flea species based on the *COI* gene sequences constructed using the Maximum Likelihood method and Tamura 3-parameter model. Numbers on the tree indicate bootstrap support (values <50% not shown). Samples sequenced in the present study are marked •. The number of samples represented by the sequence is given in parentheses (n = x).

## 3.2. Detection of Pathogens

Results of real-time PCR revealed the presence of *Anaplasma* (3.4%, 2/58), *Borrelia* (5.2%, 3/58), *Babesia* (8.6%, 5/58), and *Bartonella* (41.4%, 24/58) DNA in bat fleas (Table 2). No DNA of *Rickettsia* was detected in the tested samples. DNA from fleas was further tested using a conventional and nested PCR for additional *Anaplasma* spp., *Borrelia* spp., *Babesia* spp., *Bartonella* spp., *Rickettsia* spp., and *Mycoplasma* spp. identification. Only *Bartonella* DNA was successfully detected in bat fleas (39.7%, 23/58) using nested PCR (Table 2). All *Bartonella*-positive samples were subjected to sequence analysis.

**Table 2.** Summary of vector-borne pathogen detection results from fleas collected from bats in Lithuania.

Pathogen	Gene	I.v. * n/N	I.o. * n/N	I.s. * n/N	I.e. * n/N	I.h. * n/N	N.p. * n/N	N.e. * n/N	I.sp. * n/N	Total n/N
Augulaana	RT-PCR msp2	0/24	1/16	0/6	0/4	0/1	1/5	0/1	0/1	2/58
	msp4	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
лпирияти	16S rRNA	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
	groESL	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
Borrelia	RT-PCR 23S rRNA	1/24	0/16	0/6	0/4	0/1	1/5	0/1	0/1	2/58
	ospA	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
	16S-23S rrs-rrlA ITS	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
	flA	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
Babesia	RT-PCR 18S rRNA	3/24	0/16	1/6	0/4	0/1	0/5	0/1	0/1	4/58
	18S rRNA	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58

Pathogen	Gene	I.v. * n/N	I.o. * n/N	I.s. * n/N	I.e. * n/N	I.h. * n/N	N.p. * n/N	N.e. * n/N	I.sp. * n/N	Total n/N
Bartonella	RT-PCR ssrA	14/24	6/16	1/6	3/4	0/1	0/5	0/1	0/1	24/58
	16S-23S rRNA ITS	14/24	6/16	1/6	2/4	0/1	0/5	0/1	0/1	23/58
	gltA	13/24	5/16	0/6	2/4	0/1	0/5	0/1	0/1	20/58
	rpoB	14/24	6/16	0/6	3/4	0/1	0/5	0/1	0/1	23/58
	groEL	14/24	6/16	0/6	3/4	0/1	0/5	0/1	0/1	23/58
Di Luci	RT-PCR gltA	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
Rickettsia	gltA	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58
Mycoplasma	16S rRNA	0/24	0/16	0/6	0/4	0/1	0/5	0/1	0/1	0/58

Table 2. Cont.

\* I.v.—I. variabilis; I.o.—I. octactenus; I.s.—I. simplex; I.e.—I. elongatus; I.h.—I. hexactenus; N.p.—N. pentactena; N.e.—N. eusarca; I.sp.—Ischnopsyllus sp.; N—number of tested fleas, n—number of PCR-positive fleas.

## 3.3. Phylogenetic Analysis of Bartonella spp. in Fleas

The phylogenetic analysis of *Bartonella* spp. detected from bat fleas of three species (*I. variabilis, I. octactenus,* and *I. elongatus*) collected from three different bat species (*P. nathusii, P. pygmaeus,* and *N. noctula*) was conducted through the examination of partial *gltA, groEL, rpoB,* and 16S-23S rRNA ITS sequences.

A total of 16 partial *gltA* gene (334 bp) sequences from fleas were obtained in this study. The derived sequences were clustered in three separate groups and had 23 variable sites between each other. The BLAST analysis showed a 97–100% similarity with *Bartonella* sp. sequences from the GenBank database. The sequences belonging to the first cluster were 100% identical to the *Bartonella* sp. sequences from the *I. variabilis* species flea collected from *P. nathusii* from the Netherlands. The second cluster sequences were 100% identical to sequences derived from two bat species (*Myotis emarginatus* (Geoffroy Saint-Hilaire, 1806) from Georgia and *Pipistrellus* sp. from United Kingdon). The sequences belonging to the third cluster had a 97% similarity with the sequences derived from the *N. eusarca* species of fleas from Ukraine (Figure 3).

A total of 20 partial *groEL* gene (290 bp) sequences were obtained in this study. Derived sequences were clustered in 2 separate clades and had 21 variable sites between each other. The BLAST analysis of sequences from the first cluster showed 97–99% similarity with *Bartonella* sp. sequences from *M. emarginatus* and *Eptesicus serotinus* (Schreber, 1774) bat species from Georgia. The rest of the sequences obtained in this study formed the second cluster, which is unique and exhibited only a 94–95% similarity with sequences from *Rhinolophus ferrumequinum* (Schreber, 1774) and *Myotis blythii* (Tomes, 1857) from Georgia (Figure 4).

A total of 22 partial *rpoB* gene (819–831 bp) sequences were obtained in this study. Derived sequences were clustered in 2 separate clades and had 100 variable sites between each other. The BLAST analysis of sequences from the first cluster showed a 97–98% similarity with sequences from the *Clethrionomys rutilus* (Pallas, 1779) vole from the USA and the *Myotis davidii* (Peters, 1869) bat from China. The sequence from the second cluster had a 93–96% similarity with sequences from the bat fly from China and the *h. ferrumequinum* bat from Georgia and formed a unique cluster (Figure 5).





**Figure 3.** Phylogenetic tree (Maximum Likelihood, Tamura 3-parameter model, 1000 bootstrap replicates) based on 285 bp alignment of *Bartonella* sp. *gltA* gene sequences. Samples sequenced in the present study are marked •. The blue colour indicates sequences derived from bat hosts, while the red colour sequences indicates those from ectoparasites associated with bats. The Roman numerals (I, II, and III) indicate clusters.



**Figure 4.** Phylogenetic tree (Maximum Likelihood, Tamura 3-parameter model, 1000 bootstrap replicates) based on 290 bp alignment of *Bartonella* sp. *groEL* gene sequences. Samples sequenced in the present study are marked •. The blue colour indicates sequences derived from bat hosts, while the red colour indicates sequences from ectoparasites associated with bats. The Roman numerals (I, II) indicate clusters.



**Figure 5.** Phylogenetic tree (Maximum Likelihood, Tamura 3-parameter model, 1000 bootstrap replicates) based on 757 bp alignment of *Bartonella* sp. *rpoB* gene sequences. Samples sequenced in the present study are marked •. The blue colour indicates sequences derived from bat hosts, while the red colour indicates sequences from ectoparasites associated with bats. The Roman numerals (I, II) indicate clusters.

A total of 21 partial (717–836 bp) *16S-23S rRNA ITS* region sequences were obtained in this study. Derived sequences were clustered in 3 separate clades and had 290 variable sites between each other. The first clade sequences clustered near the *Bartonella* sp. sequence from *M. emarginatus* from Georgia and had an 89% similarity. The second clade compounded five unique sequences, obtained in our study, and exhibited the highest similarity (90%) to the *Bartonella* sequences from the *Cimex adjunctus* (Barber, 1939) bat bug from the USA. The third clade contains only one sequence derived from our study which has a 99.8% similarity to the *Bartonella* sp. from *M. blythii* from Georgia (Figure 6).



**Figure 6.** Phylogenetic tree (Maximum Likelihood, Tamura 3-parameter model, 1000 bootstrap replicates) based on 574 bp alignment of *Bartonella* sp. *16S-23S rRNA ITS* sequences. Samples sequenced in the present study are marked •. The blue colour indicates sequences derived from bat hosts, while the red colour indicates sequences from ectoparasites associated with bats. The Roman numerals (I, II, and III) indicate clusters.

# 4. Discussion

The present study is the first report on fleas infesting bats in Lithuania. This finding presents seven flea species (*I. variabilis, I. octactenus, I. simplex, I. elongatus, I. hexactenus, N. pentactena,* and *N. eusarca*), extending the knowledge about the Lithuanian flea fauna. Also, it presents new data about the distribution of fleas belonging to the Ischnopsyllidae family in Europe.

*Ischnopsyllus octactenus* and *I. variabilis* were the main flea species collected from bats in this study. They are distributed in Europe, North Africa, and Central Asia [21]. In this study, *I. octactenus* was collected from both *P. nathusii* and *P. pygmaeus*, while *I. variabilis* was collected from *P. nathusii*, *N. noctula* and *B. barbastellus*. As far as we know, this is the first report of the *I. variabilis* flea species being collected from *B. barbastellus*. *Pipistrellus* spp. Bats are the principal host of these fleas [3]. However, there are records of findings on other bat species [3,15,43].

Six specimens of the flea *I. simplex* were collected from the *M. daubentonii* and *P. nathusii* bat species. To the best of our knowledge, this is the first report of the *I. simplex* flea species parasitizing *P. nathusii*. Previously, it was recorded on bats of *Myotis* spp., *Rhinolophus* spp., *B. barbastellus*, *Plecotus auritus*, *Eptesicus nilssonii*, *Vespertilio murinus*, and *P. pipistrellus* from European countries [3,15,43].

*Ischnopsyllus elongatus* was found on the *N. noctula* bat species in this study. *Nyctalus noctula* is the principal host of this flea species. It is distributed in Europe and Asia and was also previously recorded on other bat species [3,15,21,43].

Bats of *Plecotus auritus* and *Myotis* sp. are the principal hosts for fleas of the *I. hexactenus* species [3,21]. In this study, one specimen of *I. hexactenus* was found on *B. barbastellus*. This is not surprising as it has also been detected in other European countries [13,15,21]. Moreover, this flea species was reported from bats of the *Pipistrellus*, *Plecotus*, and *Eptesicus* genera [3,13,15].

According to Table 1, two winter flea species were found, as follows: *N. eusarca* and *N. pentactena*. One specimen of *N. eusarca* has been observed from *N. noctula*, which is the true host of these rare fleas [3,15,18,21]. Meanwhile, five specimens of *N. pentactena* were found on *E. nilssonii*. These flea species are distributed in European countries. Previously, they were recorded from bats of *B. barbastellus*, *Plecotus* spp., *Myotis* spp., *Pipistrellus* spp., *Eptesicus serotinus*, *Miniopterus schreibersii*, and *Rhinolophus ferrumequinum* [3,15,18,21].

The identification of flea species based on morphological characteristics can sometimes lead to misidentifications. Furthermore, recent studies have shown the presence of cryptic species between Siphonaptera [44,45]. For this reason, molecular data are needed to confirm the species of fleas. According to Figures 1 and 2, two of the most frequently used genes (COI and 18S rRNA) did not reveal intraspecific variation. The phylogenetic analysis of the 18S rRNA gene allowed us to separate fleas only to the genus level. This analysis was complicated by the fact that currently, only three 18S rRNA gene sequences of Ischnopsyllus spp. and five of Nycteridopsylla spp. are deposited in the GenBank. Moreover, previous studies conducted with other flea species also demonstrated that the phylogenetic analysis of 18S rRNA shows differences only above the species level [46]. Despite a larger number of COI gene sequences in the GenBank (n = 18), a detailed phylogenetic analysis was not feasible due to the fact that most of the sequences are from the I. variabilis flea species. Unlike the 18S rRNA gene, this gene revealed that all analysed bat flea species belonged to a single cluster. According to other authors, bat fleas belong to a monophyletic family [2,47]. Possibly, flea species of the Ischnopsyllidae family may be closely genetically related to each other; therefore, further research with additional genetic markers is needed in the future.

*Wolbachia* endosymbionts were accidentally detected in *I. elongatus* flea species. Previous studies indicated that the LCO1490 and HCO2198 primers targeting the *COI* gene may lead to the amplification of *Wolbachia* DNA rather than the host's [48,49]. Also, bat fleas as hosts for *Wolbachia* spp. have been detected in recent studies [18,50,51]. However, the detection of *Wolbachia* endosymbionts in bat fleas is not unexpected, considering their widespread occurrence in many insect species.

In this study, we screened the fleas collected from bats for pathogens, including Anaplasma, Borrelia, Babesia, Bartonella, Rickettsia, and Mycoplasma, which have been previously detected in bats or their ectoparasites [5,7,9,17,18,52]. Only the detection of Borrelia spp. (3/58) and Anaplasma sp. (2/58) were exclusively achieved through real-time PCR (Table 2). However, efforts to amplify those samples for sequence analysis were unsuccessful, leaving the samples without further identification. Furthermore, real-time PCR screening showed a 41.4% (24/58) overall infection rate with Bartonella spp. in the tested fleas. Bats, in particular, are recognized as reliable reservoirs for novel *Bartonella* species, contributing significantly to the overall diversity observed in these bacteria. While most studies have focused on detecting Bartonella in the blood and associated ectoparasites of bats, such as ticks, mites, cimicid bugs, and bat flies [5,9,17,18,52,53], only a few have reported on the prevalence of *Bartonella* in bat-associated fleas [5,18,54]. Five genetic loci (ssrA, gltA, rpoB, groEL, and 16S-23S rRNA ITS) have been used for Bartonella characterization in this study. More detailed studies are needed to confirm fleas as competent vectors of *Bartonella* in bats. To the best of our knowledge, this is the first comprehensive study on the prevalence of *Bartonella* in fleas associated with bats in Europe. The *Bartonella* sp. has previously been detected only in *N. eusarca* fleas (7 positive out of 100 tested) in Ukraine [18], Sternopsylla texanus (Jordan and Rothschild, 1921) fleas (1 positive out of 11 tested) in the

USA [54], *Thaumapsylla breviceps* (Rothschild, 1907) fleas (4 positive out of 61 tested) in South Africa [5], *Ischnopsyllus needhami* (Hsu Yinch'i, 1935) fleas (79 positive out of 174 tested) in Japan [55], and unidentified bat fleas (2 positive pools out of 3 tested) in China [56]. The BLAST analysis and topology of the phylogenetic trees showed that the *Bartonella* sequences derived in this study were similar to other bat-associated *Bartonella* sequences from the GenBank database and shared the same clusters at all four tested loci (*gltA, rpoB, groEL,* and *16S-23S rRNA ITS*). These findings confirm many other authors' conclusions about the high host/vector specificity of bat-associated *Bartonella* [18,39,55,57–59].

## 5. Conclusions

To conclude, this study showed that the flea species diversity on Lithuanian bats is high. Herein, we report the first detection of the *I. variabilis* flea species on the *B. barbastellus* bat species and the *I. simplex* flea species on the *P. nathusii* bat species. The phylogenetic analysis of flea species indicated the necessity for additional research. In the future, Ischnopsyllidae family fleas should be further explored using additional or other genetic markers that would help confirm the taxonomy of flea species. Additionally, this is the first report of *Bartonella* infection in bat fleas in the Baltic region. More research is needed to specify *Bartonella* strains for different bats and their flea species.

**Supplementary Materials:** The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/d16040192/s1, Table S1: PCR target genes and primers that were used in this study; Table S2: Detailed PCR protocols for *Bartonella* spp. detection; Table S3: GenBank accession numbers for Bartonella sp. from Lithuanian bat fleas; Table S4: Variable nucleotide positions of *COI* gene fragments in studied bat flea species; Figure S1: *Ischnopsyllus variabilis* general view of male (a) and female (b); Figure S2: *Ischnopsyllus octactenus* general view of male (a) and female (b); Figure S3: *Ischnopsyllus simplex* general view of male (a) and female (b); Figure S4: *Ischnopsyllus elongatus* general view of male (a) and female (b); Figure S5: *Nycteridopsylla pentactena* general view of male (a) and female (b); Figure S6: *Ischnopsyllus hexactenus* general view of male; Figure S7: *Nycteridopsylla eusarca* general view of male.

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