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Genetic diversity of *Bartonella* spp. in rodents and fleas from Poland

Mohammed Alsarraf[1]✉, Łukasz Banasiak[2], Katarzyna Tołkacz[1,3], Maciej Grzybek[4], Jolanta Behnke-Borowczyk[5], Mustafa Alsarraf[1,7], Dorota Dwużnik-Szarek[1], Monika Juśko[1], Dina Al-qazaz[1], Paulina Drabik[1], Jerzy M. Behnke[6] & Anna Bajer[1]

Bartonella **spp. are parasites of mammalian erythrocytes and endothelial cells, and are transmitted by blood-feeding arthropod ectoparasites, including fleas. This study aimed to: (i) identify the main flea species responsible for *Bartonella* transmission and the specific *Bartonella* species they carry, (ii) evaluate how host-related factors influence the prevalence of *Bartonella*, (iii) examine the genetic diversity of *Bartonella* from different flea species and mammalian hosts, including rodents and European moles, and (iv) determine the haplotypes of *Bartonella* derived from rodents. Blood samples were collected from seven rodent species and two European moles in Poland (*n* = 994), and fleas were collected from rodents (*n* = 833). *Bartonella* spp. were identified and genotyped through *rpoB* and *gltA* genes. Phylogenetic analysis revealed two dominant *Bartonella* spp. in rodents and fleas: *B. grahamii* and *B. taylorii*. Moreover, 17 haplotypes of *B. taylorii* and 9 of *B. grahamii* were identified. The sequences of *Bartonella* sp. from *T. europaea* clustered in a unique separate group, possibly indicating a novel species. The study confirmed fleas as vectors of *Bartonella* transmission in rodents and highlighted the significant genetic diversity of *Bartonella* spp. in both fleas and rodents.**

Keywords *Bartonella*, *Ctenophthalmus.*, *Megabothris.*, *Histrichopsylla*. Fleas, Rodents, Poland, Haplotype

Background

Bartonella species are Gram-negative, facultative intracellular bacteria that infect mammalian erythrocytes and endothelial cells[1]. These infections can lead to the development of bartonellosis, an important vector-borne disease[2,3]. *Bartonella* spp. have a wide range of vertebrate hosts and can cause disease in both animals and humans[4,5]. At least 13 species/subspecies are known to be zoonotic[6]. In humans, *Bartonella* infections are responsible for conditions such as cat scratch disease (caused by *Bartonella henselae*), trench fever (*B. quintana*), and Carrion's disease (*B. bacilliformis*)[7–9]. Clinical symptoms of these infections include swollen lymph nodes (lymphadenopathy) with prolonged fever, intraerythrocytic bacteremia, neuroretinitis (inflammation of the optic nerve and retina), and endocarditis (infection of the heart's inner lining)[6].

Currently there are over 50 defined species of *Bartonella*, and more than half are known to infect rodents (National Center for Biotechnology Information Data). Small mammals act as reservoirs for *Bartonella* spp., playing a significant role in the maintenance and distribution of these pathogens[10]. High prevalences of *Bartonella* spp. have been detected in rodents, the most speciose group of mammals[11]. In Central Europe, the dominant small mammal species in woodlands are *Clethrionomys glareolus* and *Apodemus flavicollis*, while *Microtus* spp. dominate fallow lands[12]. In our previous studies we have shown that the prevalence of *Bartonella* infections in bank voles from Poland is 38.7%[13], while in *Microtus* spp., it is higher at 66.8%[14]. Globally, *Bartonella* spp. infections in rodents range from 26–67%[15–19].

[1]Department of Eco-Epidemiology of Parasitic Diseases, Institute of Developmental Biology and Biomedical Sciences, Faculty of Biology, University of Warsaw, Miecznikowa 1, Warsaw 02-096, Poland. [2]Institute of Evolutionary Biology, Faculty of Biology, Biological and Chemical Research Centre, University of Warsaw, Żwirki i Wigury 101, Warsaw 02-089, Poland. [3]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5A, Warsaw 02-106, Poland. [4]Department of Tropical Parasitology, Medical University of Gdańsk, Powstania Styczniowego 9B, Gdynia 81-519, Poland. [5]Department of Forest Entomology and Pathology, Faculty of Forestry and Wood Technology, Poznań University of Life Sciences, Poznań, Poland. [6]School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK. [7]Department of Microbiology, Molecular Genetics and Genomics, Centre for Advanced Materials and Technologies, CEZAMAT, 19 Poleczki St, Warsaw 02-822, Poland. ✉email: m.al-sarraf3@uw.edu.pl

Rodents, shrews, hedgehogs, lagomorphs, bats, carnivores, ungulates, and birds are all common hosts for fleas, which are considered the main vectors of *Bartonella* spp. among small mammals[20]. Fleas can transmit *Bartonella* through various routes, including oral transmission, regurgitation of blood meals, contaminated faecal pellets, and, in some cases, by ingestion of infected fleas by hosts[21]. Fleas can also switch hosts, potentially coming into contact with domestic animals and humans, making it important to understand which flea species are involved in *Bartonella* transmission. For example, *Ctenophthalmus felis* is a known vector for the zoonotic *B. henselae*[22]. *Bartonella* DNA has also been detected in other flea species such as *C. agyrtes*, *C. congener*, *C. sciurorum*, *C. solutus*, *Histrichopsylla talpae*, and *Megabothris turbidus*, with an overall prevalence of 22.9%[23]. The genes coding for the RNA polymerase beta subunit (*rpoB*) and citrate synthase (*gltA*) are commonly used to differentiate *Bartonella* species due to the variability in these genes[16,24]. This genetic variation can be used as a criterion for identifying new species or subspecies. *Bartonella* species from rodents in Western Europe are relatively well-studied, with *Bartonella grahamii* being one of the most widespread, occurring in *Apodemus* spp. and arvicolid voles (*Clethrionomys* spp. and *Microtus* spp.) throughout Eurasia[25–27]. Conversely, *Bartonella taylorii* is the most diverse yet least understood species in European rodents, composed of several distinct clades and capable of infecting both rodents and insectivores[25–28].

Additionally, there is significant genetic diversity within the *Bartonella* genus, with the greatest variation found in rodents and bats[29]. A recent study in India identified 13 haplotypes based on *rpoB* sequences from unidentified *Bartonella* spp. in rodents[18], while another study in Poland revealed two haplotypes of *Bartonella* from *Lipoptena fortisetosa*, also based on the *rpoB* gene[30].

However, the range of *Bartonella* species transmitted by different flea vectors remains poorly understood. We hypothesize that the genetic diversity of *Bartonella* spp. is closely linked to their invertebrate host vectors. This study aims to: (I) identify the main flea species involved in *Bartonella* transmission and the specific *Bartonella* species they carry, (II) evaluate the influence of host intrinsic and extrinsic factors on *Bartonella* prevalence in mammalian hosts, (III) characterize the genetic diversity of *Bartonella* spp. from various flea species and mammalian hosts, including rodents and European moles, and (IV) determine the haplotypes of *Bartonella* spp. found in rodents.

Methods

Definition of terms

The taxonomic classification of bank voles has undergone several revisions in recent decades. Bank voles were recently classified under the genus *Myodes*, but it was later determined that the historically recognized name *Clethrionomys* is more appropriate. Consequently, this study adheres to the nomenclature recommended by the Mammal Diversity Database[31], utilizing the designation *Clethrionomys glareolus*[32]. Additionally, recent taxonomic revisions have been reported for other vole species[33]. Within this framework, we refer to *Alexandromys oeconomus*, formerly known as *Microtus oeconomus*, in accordance with findings from Lissovsky et al. (2018)[34] and Zorenko et al. (2018)[35].

Study sites

The rodents were live-trapped in five sites, both woodland and fallow lands in Poland. The study sites have been previously comprehensively described[13,36–39]. They are located in Mazury in the north-eastern corner of Poland, in the vicinity of Lake Śniardwy and the towns of Mikołajki, Ryn, and Pisz. Site 1 is referred to as Urwitałt (53.79838, 21.64549), site 2 as Tałty (53.89202, 21.53494), site 3 as Pilchy (53.70569, 21.80617), and site 4 as Łuknajno (53.81395, 21.65199). The fifth site was Białobrzegi, in Central Poland (Mazovia), located about 75 km south of Warsaw (51.66445, 20.947) (Fig. 1).

Collection of rodent samples

Blood samples were collected during our long-term studies of rodent populations in the Mazurian Lake District of Northeastern Poland in the years 2010, 2013, 2014, and 2018[13,36–38,40]. At the field stations, all animals were identified, culled, weighed (to the nearest 0.1 g), sexed and relevant morphometric data were recorded. The necropsies of rodents were carried out under terminal isoflurane (Merck, Darmstadt, Germany) anesthesia. The rodents were placed in an anesthetic chamber with an isoflurane concentration of (3–5%) and then culled by cervical dislocation. The methodologies employed for rodent trapping, as well as the procedures for sampling and processing of captured specimens, have all been detailed in previous publications[36–39]. For the present study, a total of 994 rodents, encompassing eight species, were examined: *C. glareolus* ($n = 740$) in 2010, 2014, and 2018 from forest habitats; *Microtus arvalis* ($n = 124$), *Microtus agrestis* ($n = 19$), *Alexandromys oeconomus* ($n = 88$), and *Microtus* sp. ($n = 2$) from fallow land; and *Apodemus agrarius* ($n = 11$), *A. flavicollis* ($n = 9$), and *Apodemus sylvaticus* ($n = 1$) from forest regions in 2013, 2014, and 2017. Additionally, we obtained samples from two dog-killed moles, *Talpa europaea*, collected in Kury, approximately 50 km northeast of Warsaw (Table 1).

Collection and identification of fleas

In total, 914 fleas were collected from rodents. We identified five species of fleas: *Ctenophthalmus assimilis* ($n = 260$), *C. agyrtes* ($n = 229$), *Megabothris walkeri* ($n = 189$), *M. turbidus* ($n = 182$), and *H. talpae* ($n = 54$). Fleas were collected from voles trapped in fallow lands in 2013, 2014 and from bank voles trapped in forest areas in 2018. Fleas were fixed in 70% ethanol and identified to the species and sex levels morphologically using the key of Skuratowicz[41], and molecularly by amplification and sequencing of the *18S rDNA* gene fragment (570 bp)[14,16,40]. Most of the collected fleas (833 of 914) underwent further analysis (Table 2). The *18S rDNA* products from all fleas that tested positive for *Bartonella* DNA ($n = 26$) were sequenced, identified, and subjected to phylogenetic analysis. For the phylogenetic analysis, we used all our DNA sequences and 10 reference sequences from GenBank.

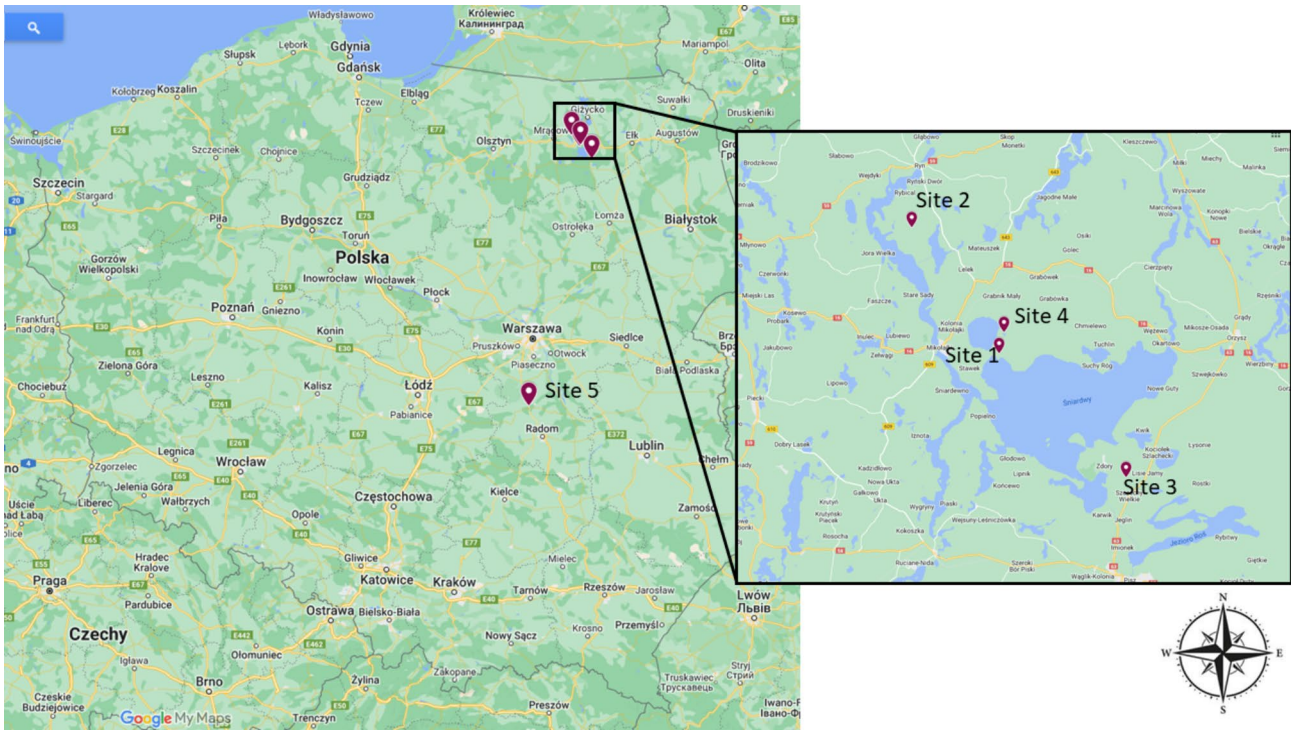


Fig. 1. Map of the study sites: (site 1 = Urwitałt, site 2 = Tałty, site 3 = Pilchy, Site 4 = Łuknajno, site 5 = Białobrzegi)

Site	Host	Year					Total +/*n* (%)
		2010 +/*n* (%)	2013 +/*n* (%)	2014 +/*n* (%)	2017 +/*n* (%)	2018 +/*n* (%)	
Urwitalt	*C. glareolus*	38/88 (43.1)	ND	25/75 (33.3)	ND	12/89 (13.4)	75/252 (29.7)
Talty	*C. glareolus*	41/95 (43.1)	ND	47/82 (57.3)	ND	23/94 (24.4)	111/271 (40.9)
Pilchy	*C. glareolus*	21/56 (37.5)	ND	33/84 (39.2)	ND	15/77 (19.4)	69/217 (31.7)
Total		100/239 (41.8)	ND	105/241 (43.5)	ND	50/260 (19.2)	255/740 (32.4)
Luknajno	*M. arvalis*	ND	39/55 (70.1)	51/69 (93.9)	ND	ND	90/124 (72.5)
	M. agrestis	ND	8/14 (57.1)	2/3 (66.6)	ND	ND	10/17 (58.8)
	A. oeconomus	ND	14/19 (73.6)	31/57 (54.3)	ND	ND	45/76 (59.2)
Total		ND	61/88 (69.3)	84/129 (65.1)	ND	ND	145/217 (66.8)
Bialobrzegi	*A. agrarius*	ND	ND	ND	2/11 (18.2)	ND	2/11 (18
	A. flavicolis	ND	ND	ND	5/9 (55.5)	ND	5/9 (55.5)
	A. sylvaticus	ND	ND	ND	1/1 (100)	ND	1/1 (100)
	M. agrestis	ND	ND	ND	2/2 (100)	ND	2/2 (100)
	A. oeconomus	ND	ND	ND	10/12 (83.3)	ND	10/12 (83.3)
	Microtus sp.	ND	ND	ND	1/2 (50)	ND	1/2 (50)
Total		ND	ND	ND	21/37 (56.7)	ND	21/37 (56.7)
Overall total		100/239 (41.8)	61/88 (69.3)	189/370 (51)	21/37 (56.7)	50/260 (19.2)	421/994 (42.3)

Table 1. Prevalence (%) of *Bartonella* spp. Based on host identity, study site, and years of study. *ND = Not Determined.

Blood collection and DNA extraction

Blood samples (200 µl) were collected from euthanized animals into tubes containing 0.001 M EDTA and subsequently frozen at − 20 °C. In cases where individuals were found deceased in traps, the entire heart was excised and homogenized in 400 µl of 0.001 M EDTA. Genomic DNA was extracted from both whole blood and heart homogenates utilizing the AxyPrep MiniPrep Blood Kit (AxyGen, USA) and the DNeasy Blood and Tissue Kit (Qiagen, Germany), with all samples stored at − 20 °C. The extracted DNA was subjected to specific polymerase chain reaction (PCR) protocols as outlined in previous studies[14,16,40]. For the detection of *Bartonella* spp., the *rpoB* gene was amplified using two fragments of 333 bp and 860 bp, along with the *gltA* gene (810 bp).

Site	Host	Vector	Year			Flea total (%)
			2013 +/*n* (%)	2014 +/*n* (%)	2018 +/*n* (%)	
Urwitalt	*C. glareolus*	*C. agyrtes*	ND	ND	2/42 (4.7)	2/42 (4.7)
		C assimilis	ND	ND	0/7 (0)	0/7 (0)
		M. turbidus	ND	ND	2/12 (16.6)	2/12 (16.6)
		M. walkeri	ND	ND	0/4 (0)	0/4 (0)
		H. talpae	ND	ND	1/2 (50)	1/2 (50)
Total					5/67 (7.4)	5/67 (7.4)
Talty	*C. glareolus*	*C. agyrtes*	ND	ND	10/51 (19.6)	10/51 (19.6)
		C assimilis	ND	ND	0/3 (0)	0/3 (0)
		M. turbidus	ND	ND	9/26 (34.6)	9/26 (34.6)
		M. walkeri	ND	ND	2/5 (40)	2/5 (40)
		H. talpae	ND	ND	1/5 (20)	1/5 (20)
Total			ND	ND	22/90 (24.4)	22/90 (24.4)
Pilchy	*C. glareolus*	*C. agyrtes*	ND	ND	2/43 (4.6)	2/43 (4.6)
		C assimilis	ND	ND	1/3 (33.3)	1/3 (33.3)
		M. turbidus	ND	ND	3/14 (21.4)	3/14 (21.4)
		M. walkeri	ND	ND	1/4 (25)	1/4 (25)
		H. talpae	ND	ND	1/6 (16.6)	1/6 (16.6)
Total			ND	ND	8/70 (11.4)	8/70 (11.4)
Luknajno	*M. arvalis*	*C. agyrtes*	3/23 (13)	8/34 (23.5)	ND	11/57 (19.2)
		C assimilis	5/109 (4.5)	16/92 (17.3)	ND	21/201 (10.4)
		M. turbidus	0/38 (0)	6/41 (14.6)	ND	6/79 (7.5)
		M. walkeri	0/16 (0)	5/17 (29.4)	ND	5/33 (15.1)
		H. talpae	0/13 (0)	3/7 (42.8)	ND	3/20 (15)
Total			8/199 (4)	38/191 (19.8)	ND	46/390 (11.7)
Luknajno	*A. oeconomus*	*C. agyrtes*	0/4 (0)	1/9 (11.1)	ND	1/13 (7.6)
		C. assimilis	0/8 (0)	4/20 (20)	ND	4/28 (14.2)
		M. turbidus	0/5 (0)	5/25 (20)	ND	5/30 (16.6)
		M. walkeri	0/21 (0)	10/90 (11.1)	ND	10/111 (18)
		H. talpae	0/8 (0)	0/2 (0)	ND	0/10 (0)
Total			0/46 (0)	20/146 (13.6)	ND	20/192 (10.4)
Luknajno	*M. agrestis*	*C. agyrtes*	1/8 (12.5)	0	ND	1/8 (12.5)
		C. assimilis	0/7 (0)	0	ND	0/7 (0)
		M. turbidus	0/5 (0)	0	ND	0/5 (0)
		M. walkeri	0/26 (0)	0/1 (0)	ND	0/27 (0)
		H. talpae	0/6 (0)	0	ND	0/6 (0)
Total			1/52 (1.9)	0/1 (0)	ND	1/53 (1.8)
Luknajno	*A. agrarius*	*C. agyrtes*	0	1/5 (20)	ND	1/5 (20)
		C. assimilis	0	2/8 (25)	ND	2/8 (25)
		M. turbidus	0	1/3 (33.3)	ND	1/3 (33.3)
		M. walkeri	0	1/2 (50)	ND	1/2 (50)
		H. talpae	0	2/3 (66.6)	ND	2/3 (66.6)
Total			0 (0)	7/21 (33.3)	ND	7/21 (33.3)
Overall Total (%)			9/297 (3)	65/359 (18.1)	35/227 (15.4)	109/833 (11.3)

Table 2. Prevalence (%) of *Bartonella* spp. Isolated from fleas collected from rodents, based on study sites and year of study. *ND = Not Determined.

Additionally, for the identification of flea species, the 18S rDNA (570 bp) was targeted. The primers utilized in this study are detailed in Table 3. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and visualized using Midori Green stain (Nippon Genetics Europe GmbH, Germany).

Sequencing and molecular typing

PCR products were sequenced by private companies in both directions (Genomed S.A., Poland, and Eurofins Genomics, Germany). Both reads were checked for quality, and then aligned and edited to form a consensus sequence using BioEdit[42]. All sequences for each marker were checked in BLAST to identify the donor species. For each marker all obtained sequences were aligned with reference sequences from GenBank using the ClustalW in MEGA11: Molecular Evolutionary Genetics Analysis version 11[43], and AliView[44]. DnaSP 6.12.03[45] were used

Species	Gene target	Primer	Sequence 5'→3'	Annealing temperature (°C)	Fragment size (bp)	Thermal profile	References
Bartonella spp.	*rpoB*	rpoBR	CGCATTATGGTCGTATTTGTCC	52	333	95 °C for 5 min., followed by 40 cycles (95 °C for 45 s., 52 °C for 45 s., 72 °C for 45 s.), 72 °C for 9 min.	27
		rpoBF	GCACGATT(C/T)GCATCATCA TTTTCC				
		1400 F	CGCATTGGCTTACTTCGTATG	55	860	95 °C for 5 min., followed by 40 cycles (95 °C for 45 s., 55 °C for 45 s., 72 °C for 45 s.), 72 °C for 7 min.	79
		2300R	GTAGACTGATTAGAACGCTG				
	gltA	CS140F	TTACTTATGATCC(GT)GG(CT) TTTA	54	810	95 °C for 2 min., followed by 40 cycles (95 °C for 1 min., 54 °C for 2 min., 72 °C for 2 min.), 72 °C for 7 min.	80
		CS1137R	AATGCAAAAAGAACAGTAA ACA				81
Fleas	*18S rDNA*	18SaiF	CCTGAGAAACGGCTACCACATC	56	570	95 °C for 5 min., followed by 40 cycles (95 °C for 45 s., 56 °C for 30 s., 72 °C for 1 min.), 72 °C for 5 min.	82
		18Sb2-5R	TCTTTGGCAAATGCTTTCGC				

Table 3. Gene markers, primers, and PCR conditions.

to calculate the number of haplotypes in the *rpoB* gene sequences. Minimum Spanning Network analysis was performed for the *rpoB* gene of *Bartonella* spp. DNA sequences using PopART 1. [46], and confined to sequences obtained in this study.

Phylogenetic analysis

Phylogenetic relationships among the *rpoB* and *gltA* gene sequences, representing samples of various *Bartonella* species, were assessed using Bayesian Inference, implemented in MrBayes v3.2.6[47]. Due to the large size of the dataset, we fitted a GTR + G model with all six rate parameters free and with variation in the rate of evolution among sites in the alignment. Two independent runs of 10 000 000 generations were sampled every 1000 generations and 25% of the initial samples were discarded as a burn-in phase. The convergence of two runs and effective sample sizes for the model's parameters were checked using Tracer v. 1.7[48].

The Maximum Likelihood method implemented in MEGA 11[49,50] was used to infer phylogenetic relationships among *18S rDNA* sequences obtained from fleas positive for *Bartonella* spp. We assumed a Kimura's 2-parameter model[49,50] and branch support was evaluated by 1000 bootstrap replicates summarized on the best scoring tree.

Statistical analysis

Prevalences were estimated based on PCR screening and microscopic analyses and were analysed by maximum likelihood techniques based on log-linear analysis of contingency tables in the software package SPSS (version 16.0.1, SPSS, Inc., Chicago, IL, USA). We first fitted full factorial models that incorporated flea species (five levels), sex of the flea (two levels, males and females), year of study (four levels), and study sites (five levels) as factors, with infection as a binary factor (presence/absence [P/A] of parasite). Beginning with the most complex model, which involved all possible main effects and interactions, and starting with the highest-level interaction those combinations that did not contribute significantly to explaining variation in the data were eliminated in a stepwise fashion (backward selection procedure in SPSS). Eventually, a minimum sufficient model was obtained, for which the likelihood ratio of χ^2 was not significant. This indicated that the model was sufficient in explaining the data and, where relevant, these values are provided in the legends to the figures. The importance of each term (i.e. interactions involving P/A of infection) in the final model was assessed by the probability that its exclusion would significantly alter the model, and both χ^2 and *P* values for these interactions are given in the text.

Ethical statement

All procedures were conducted under a license from the First Warsaw Local Ethics Committee for Animal Experimentation in Poland (Ethical license numbers: 73/2010, 148/2011, 304/2012 and 706/2015). All rodents were culled by Prof. Anna Bajer (authorized to implement experimental procedures and the culling of animals for scientific objectives by the Polish Laboratory Animal Science Association, (License number, 13/2015)

This study was carried out in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the Polish National Ethics Committee on Animal Experimentation, and according to the Polish national law for field studies involving the trapping and culling of unprotected vertebrates for scientific purposes (Resolution No. 12/2022 of the Polish National Ethics Committee on Animal Experimentation, 11 March 2022). The study was performed according to the ARRIVE guidelines 2.0.

Results

Prevalence of infection and infestation

Based on our screening of blood smears and the subsequent molecular analysis (PCR), the overall prevalence of *Bartonella* spp. infections in rodents was 42.3% (421/994) (Table 1) while, the overall prevalence of *Bartonella* spp. in fleas was 11.3% (109/833) (Table 2). Moreover, the total prevalence and mean abundance of fleas on rodents were 60.4% (303/509) and 2.94 fleas per individual, respectively (Table 4). The most common flea species was *C. assimilis* ($n = 260$), while the mole flea *H. talpae* was the rarest ($n = 54$).

Host	Year			Total (%)	Intensity
	2013 +/*n* (%)	2014 +/*n* (%)	2018 +/*n* (%)		
C. glareolus	ND	ND	124/266 (46.6)	124/266 (46.6)	257/129 (0.82)
A. flavicolis	1/1 (100)	ND	ND	1/1 (100)	1/1 (1)
M. agrestis	10/12 (83.3)	1/4 (25)	ND	11/16 (68.7)	53/14 (3.78)
M. arvalis	47/57 (82.4)	45/68 (66.2)	ND	92/125 (73.6)	390/92 (4.23)
A. oeconomus	14/19 (73.6)	48/58 (82.7)	ND	62/77 (80.5)	193/61 (3.16)
A. agrarius	0	13/24 (54.1)	ND	13/24 (54.1)	21/14 (1.5)
Total (%)	72/89 (80.9)	107/154 (69.4)	124/266 (46.6)	303/509 (60.4)	915/311 (2.94)

Table 4. Flea infestations (%) on rodents in the three years of study. ND = Not Determined

Effect of intrinsic and extrinsic factors on *Bartonella* prevalence

In our fieldwork in 2013 and 2014 we trapped *Microtus* spp. (two species) and one *Alexandromys* sp. from open areas (fallow land), while in 2018 we trapped *C. glareolus* from forest areas. Hence, we performed statistical analyses separately for these two areas. In the *Microtus* spp. dataset, we investigated how host species, host sex, flea species, and flea sex (intrinsic factors) and the year of study (an extrinsic factor) impact the prevalence of *Bartonella* spp. infections. The log-linear analysis revealed a significant interaction between flea species, year of study, and P/A of *Bartonella* spp. infections ($\chi^2_4 = 10.1$, $P = 0.037$). In 2013, *Bartonella* spp. were detected only in two flea species (*C. assimilis* and *C. agyrtes*), whereas in 2014, *Bartonella* was identified in all species of fleas with varying prevalence (Table 2; Fig. 2A).

The second significant interaction was between flea sex, year of study and P/A of *Bartonella* spp. infections ($\chi^2_1 = 5.6$, $P = 0.018$). In 2013 only, males of *C. assimilis* and *C. agyrtes* tested positive for *Bartonella* spp. whereas in 2014 males of *C. agyrtes*, *M. walkeri* and *M. turbidus* showed higher prevalence of *Bartonella* spp. than females. However, there was no significant effect of host species on the prevalence of *Bartonella* spp. (Fig. 2B).

Analysis of *Bartonella* spp. prevalence in *C. glareolus* (2018), revealed a significant effect of study site on the prevalence of *Bartonella* spp. infections ($\chi^2_2 = 9.7$, $P = 0.007$), the highest prevalence of 24.7% being recorded in Tałty (Fig. 2). Furthermore, prevalence of *Bartonella* spp. differed significantly between flea species ($\chi^2_4 = 9.5$, $P = 0.045$); the highest prevalence of *Bartonella* spp. infections was in *M. turbidus* (26.9%) while the lowest was in *C. agyrtes* (9.7%) (Fig. 2D).

Moreover, there was a significant interaction between the age class of bank voles, year of the study and infection with *Bartonella* spp. ($X^2_4 = 15.4$, $P = 0.004$). The highest prevalence of *Bartonella* spp. was 43.5% in 2014 (Table 2; Fig. 2E). In years 2014 and 2018 the highest prevalence of *Bartonella* spp. was recorded in the third age class (the oldest) of bank voles, whereas in 2010 the highest was in the first age class (Table 2; Fig. 2E).

Identification of *Bartonella* species from rodents, moles and fleas

Following sequencing of *rpoB* gene PCR products (333–860 bp), we obtained 104 sequences of *Bartonella* spp. from seven species of rodents, 53 sequences from fleas and 2 sequences from moles (Table 5). Of the 104 sequences from rodents, 67 were identified as *B. grahamii*, which was present in four species of rodents (*A. flavicollis*, *A. sylvaticus*, *A. oeconomus*, and *C. glareolus*). Thirty-three sequences were identified as *B. taylorii*, which was present in five species of rodents (*A. flavicollis*, *A. sylvaticus*, *M. agrestis*, *Microtus* sp. and *C. glareolus*; Table 6). Three isolates showed 96–98% homology to a newly described species, *Bartonella bilalgolemii*[51], and clustered in one subgroup containing *B. rochalimae* from dogs in the USA (DQ676489) and a *Bartonella rochalimae-like* isolate from *M. arvalis* from Poland (MG839175), (Fig. 3A; Table 6). Additionally, two *Bartonella* sequences from moles were identical and could not be identified to species level, because they showed only a low homology of 95% to *B. taylorii* from *A. sylvaticus* from the UK (CP083693).

Moreover, we obtained 19 DNA sequences of *B. grahamii* from four species of fleas: *C. assimilis*, *M. turbidus*, *M. walkeri* and *H. talpae*. Seventeen sequences of *B. taylorii* were obtained from all five species of fleas and one unidentified sequence/species of *Bartonella* isolated from *H. talpae* (Table 6).

Phylogenetic analysis of the *rpoB* gene

Phylogenetic analyses were conducted on 135 obtained sequences of *Bartonella* spp. and 64 reference sequences from GenBank (Fig. 3A–C). Phylogenetic analysis by the MrBayes method, revealed five major clades: Clade I, which included *B. grahamii*, contained seven diverse branches/groups related to the country of origin, with our sequences clustered on three of these branches (Fig. 3B). One branch/group contained Polish *B. grahamii* sequences from *C. glareolus*, *A. oeconomus*, *M. arvalis*, *A. flavicollis*, *M. turbidus*, and *M. walkeri*. The second group contained isolates from different hosts and countries and showed more diversity than the first group. The third group contained two sequences from *(A) sylvaticus* that clustered with one sequence from *C. glareolus*, all from Poland (Fig. 3B). Interestingly, the *B. grahamii* clade also clustered with *B. elizabethae*, a zoonotic pathogen from the UK (Fig. 3A–C).

Sequences of *B. taylorii* formed clade II and showed higher diversity than *B. grahamii*. This clade contained 6 branches/groups, one of which contained identical sequences of *B. taylorii* from Poland and Lithuania, from the flea *C. agyrtes*, and three species of rodents (*A. flavicollis*, *M. agrestis* and *M. arvalis*), and formed a sister branch with two subgroups of *B. taylorii* from *C. glareolus*, *Microtus* spp., and *C. agyrtes* (Fig. 3C). The second group of *B. taylorii* contained two subgroups; one sequence from *C. glareolus* and the second *B. taylorii* from

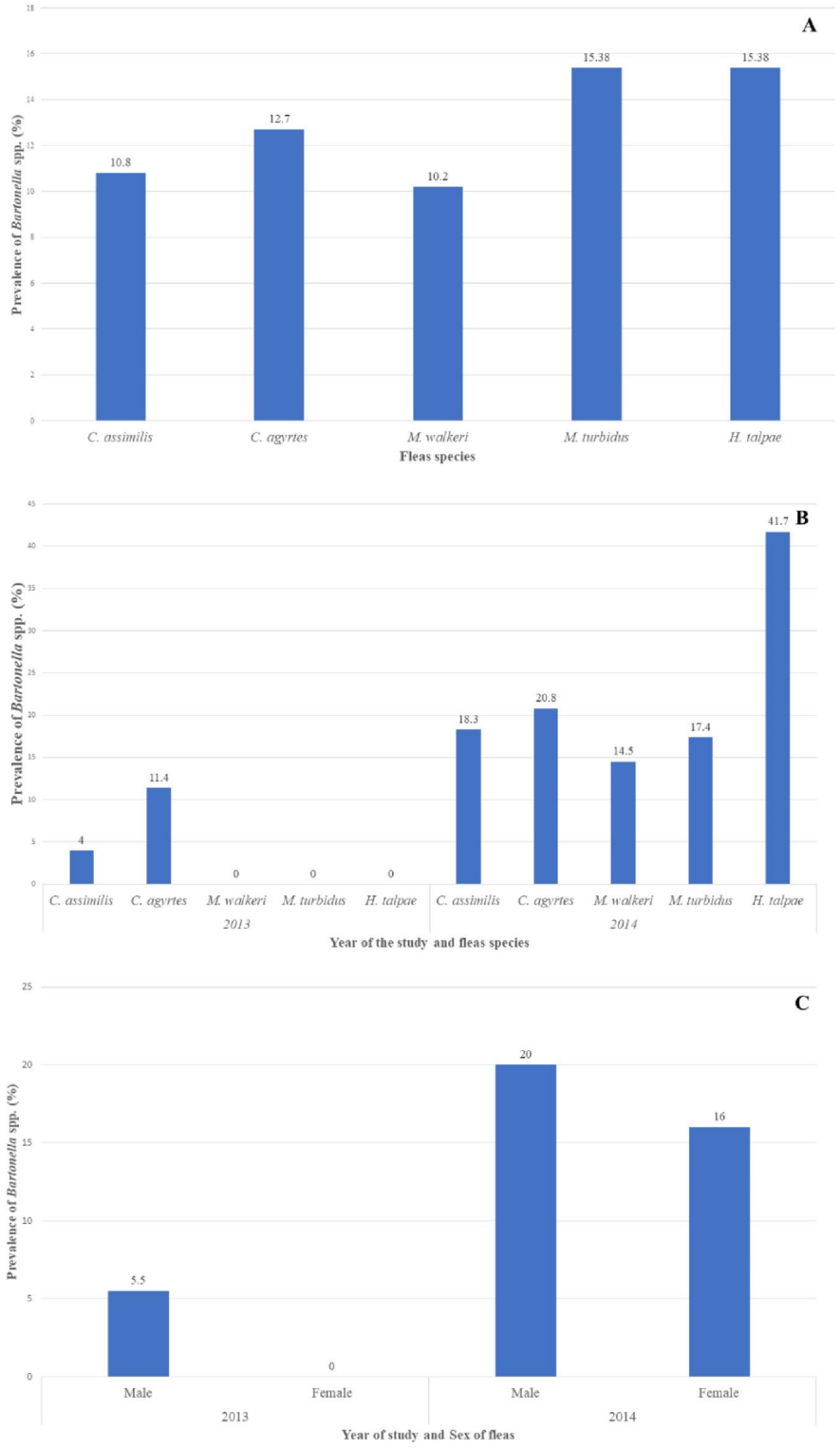


Fig. 2. (**A**) Prevalence of *Bartonella* spp. (%) in flea species. (**B**) Prevalnce of *Bartonella* spp. (%) by flea species and year of study. (**C**) Prevalence of *Bartonella* spp. (%), by flea sex and year of study. (**D**) Prevalence of *Bartonella* spp. (%), by study sites. (**E**) Prevalence of *Bartonella* spp. (%), by age class of host (rodents) and year of study.

Microtus spp. Interestingly, the third group of *B. taylorii* contained four sequences from fleas *C. assimilis* and *C. agyrtes* which grouped with sequences from *M. arvalis* obtained previously by our team from the same place-Łuknajno lake area[14,27] (Fig. 3C). The next subgroup of *B. taylorii* sequences from *C. glareolus* was located as a sister branch to the subgroup of *B. taylorii* sequences from fleas (*C. assimilis* and *C. agyrtes*). The last subgroup

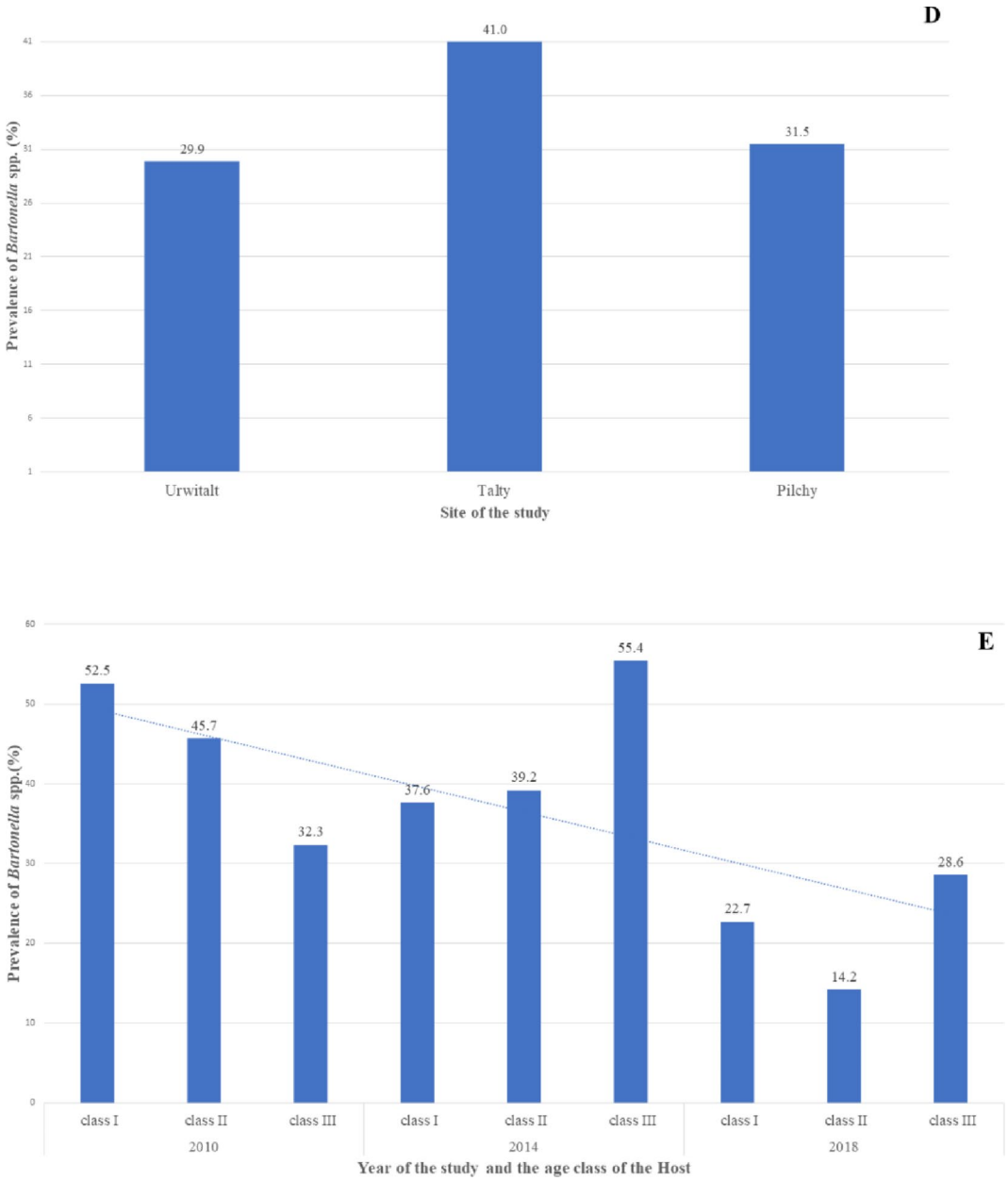


Figure 2. (continued)

of *B. taylorii* sequences from *A. flavicollis* from Poland and Lithuania clustered with *B. taylorii* from *C. agyrtes* fleas collected from *C. glareolus* from Poland. The two *Bartonella* sequences from *T. europaea* and one from *A. sylvaticus* formed two separate branches close to the *B. taylorii* group, possibly indicating a novel species of *Bartonella* (Fig. 3A–C). Three sequences of *Bartonella* sp. isolated from *A. oeconomus* and *A. agrarius* from Białobrzegi and one *Bartonella* sp. from *M. arvalis* formed a sister group to *B. bilalgolemii* recently described in *Apodemus uralensis* from Turkey[51], furthermore these groups closely clustered to *B. rochalimae* from *Canis lupus familiaris* from the USA and from *Homo sapiens* from Peru (Fig. 3A–C). Clades III, IV and V contained reference sequences of *Bartonella* spp. from different hosts and countries.

Phylogenetic analysis of the *gltA* gene

Phylogenetic analysis of the *gltA* gene, by the MrBayes method was conducted on 11 DNA sequences of *Bartonella* spp. obtained from fleas (collected in 2018) and 21 reference sequences from GenBank and revealed three clades/ groups (Fig. 4).

Clade I encompassed *B. taylorii* sequences and consisted of three subgroups. In the first subgroup four sequences of *B. taylorii* obtained from *C. agyrtes* clustered with *B. taylorii* sequences from Sweden and Poland. In the second subgroup, one *B. taylorii* sequence from *H. talpae* clustered with *B. taylorii* from Russia, China,

Host	Vector	Year						Total
		2010	2013	2014	2017	2018		
		rpoB	rpoB	rpoB	rpoB	rpoB	gltA	
Clethrionomys glareolus		61	ND	ND	ND	29	9	99
	Ctenophthalmus agyrtes	ND	ND	ND	ND	6	4	10
	Ctenophthalmus assimilis	ND	ND	ND	ND	1	0	1
	Hystrichopsylla talpae	ND	ND	ND	ND	1	2	3
	Megabothris turbidus	ND	ND	ND	ND	9	4	13
	Megabothris walkeri	ND	ND	ND	ND	2	1	3
Microtus arvalis		ND	ND	ND	ND	ND	ND	0
	Ctenophthalmus agyrtes	ND	1	ND	ND	ND	ND	1
	Ctenophthalmus assimilis	ND	ND	2	ND	ND	ND	2
	Megabothris turbidus	ND	2	ND	ND	ND	ND	2
	Megabothris walkeri	ND	4	2	ND	ND	ND	6
Microtus agrestis		ND	ND	ND	ND	ND	ND	0
	Ctenophthalmus agyrtes	ND	1	ND	ND	ND	ND	1
	Ctenophthalmus assimilis	ND	1	ND	ND	ND	ND	1
	Megabothris walkeri	ND	1	ND	ND	ND	ND	1
Alexandromys oeconomus		ND	ND	ND	5	ND	ND	5
	Ctenophthalmus agyrtes	ND	1	ND	ND	ND	ND	1
	Ctenophthalmus assimilis	ND	2	1	ND	ND	ND	3
	Megabothris turbidus	ND	2	ND	ND	ND	ND	2
	Megabothris walkeri	ND	1	1	ND	ND	ND	2
Apodemus flavicollis		ND	ND	ND	4	ND	ND	4
Apodemus agrarius		ND	ND	ND	1	ND	ND	1
	Ctenophthalmus assimilis	ND	ND	1	ND	ND	ND	1
Apodemus sylvaticus		ND	ND	ND	3	ND	ND	3
Microtus sp.		ND	ND	ND	1	ND	ND	1
Talpa europaea		ND	ND	ND	ND	2	2	4
Total		61	16	7	14	50	22	170

Table 5. Number of sequences of *Bartonella* spp. Isolated from rodents, moles, and fleas. ND = Not Determined

Rodent species / flea species	*B. grahamii*		*B. taylorii*		*Bartonella* sp.		Total
	rpoB	gltA	rpoB	gltA	rpoB	gltA	
A. agrarius	0	ND	0	ND	1	ND	1
A. flavicolis	1	ND	3	ND	0	ND	4
A. sylvaticus	2	ND	1	ND	1	ND	4
M. agrestis	0	ND	1	ND	0	ND	1
A. oeconomus	3	ND	0	ND	2	ND	5
Microtus sp.	0	ND	1	ND	0	ND	1
C. glareolus	61	3	27	ND	0	6	97
Total rodents	67	3	**33**	ND	4	6	113
C. assimilis	2	0	1	0	0	0	3
C. agyrtes	0	0	6	4	0	0	10
M. turbidus	9	4	2	0	0	0	15
M. walkeri	2	1	2	0	0	0	5
H. talpae	1	0	0	2	0	1	4
Total fleas	**4**	5	11	6	0	1	37
T. europaea	0	0	0	0	2	2	4
Overall Total	81	8	44	6	6	9	154

Table 6. Molecular identification of *Bartonella* spp. In rodents, European moles, and fleas. ND = Not Determined

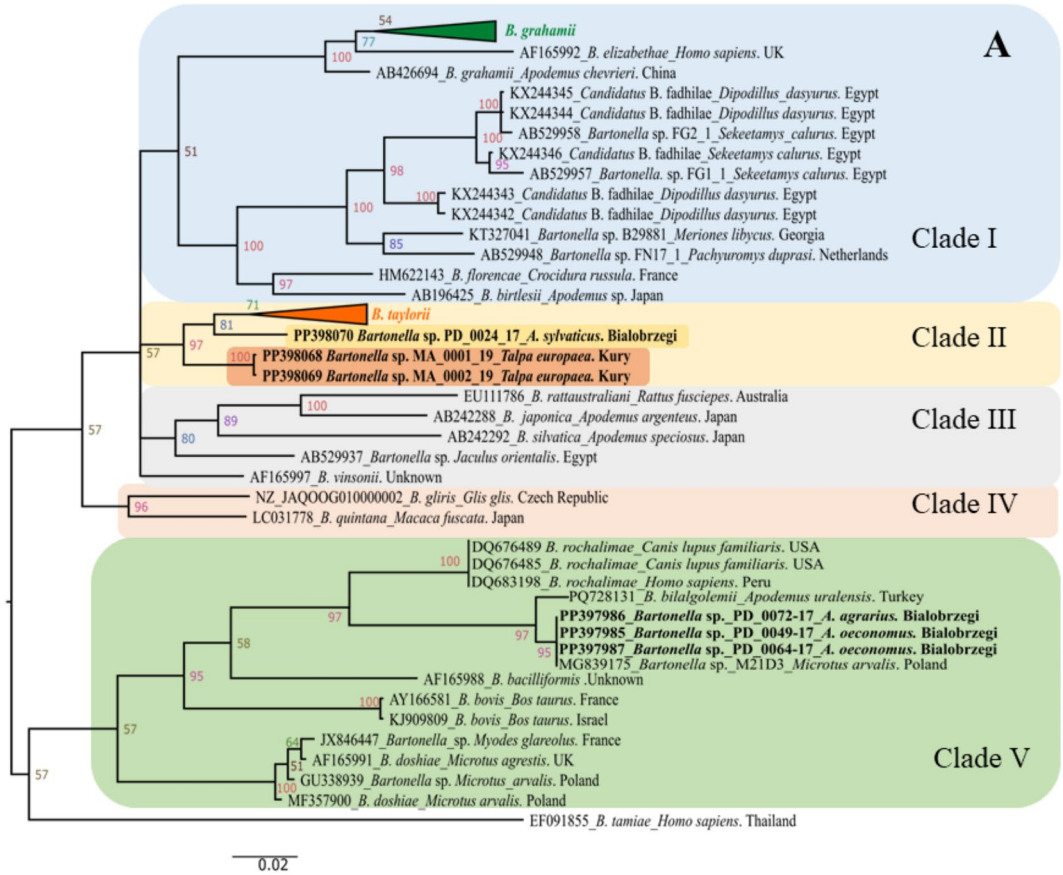


Fig. 3. (**A**) The phylogenetic tree of *Bartonella* spp. inferred from sequence variation of 860-bp *rpoB* gene fragment. The tree is 50%-majority rule consensus obtained using MrBayes (Bayesian Inference). Numbers along nodes represent *a posteriori* probability. The clades of *B. taylorii* and *B. grahamii* were collapsed into simplified forms for clarity. Custers of sequences obtained from Białobrzegi and Kury are highlighted in green and orange, respectively. (**B**) The phylogenetic tree of *Bartonella* spp. inferred from sequence variation of 860-bp *rpoB* gene fragment. The *B. grahamii* clade is shown in greater detail. (**C**) The phylogenetic tree of *Bartonella* spp. inferred from sequence variation of 860-bp *rpoB* gene fragment. The *B. taylorii* clade is shown in greater detail.

and Japan. Interestingly, our *Bartonella* sequences obtained from *T. europaea* formed the third subgroup with *Bartonella* sp. from *T. europaea* from the Netherlands (Fig. 4).

Clade II is divided into two subgroups; one contained *B. rattaustraliani*, *B. coopersplainsensis* and *B. henselae* sequences from Australia and the second subgroup contained six unidentified *Bartonella* spp., which are related to unidentified species of *Bartonella* from different countries.

Clade III encompasses *B. grahamii* sequences, including three sequences of *B. grahamii* obtained from *M. turbidus* fleas, which formed a separate sister subgroup to *B. grahamii* from *A. agrarius* from Russia (Fig. 4). Moreover, one *Bartonella* sequence from *H. talpae* formed a separate branch within the *B. grahamii* clade (Fig. 4).

Association between flea and rodent infections with *Bartonella*

We compared the genetic identity of *Bartonella* detected in fleas, and infection status and species of the rodent hosts from which the fleas were collected. In seven out of 22 *Bartonella* -positive fleas the same species of *Bartonella* was identified in both fleas and rodent hosts (Table 7). The DNA of *B. grahamii* and *B. taylorii* was detected in *C. assimilis*, *M. turbidus*, *M. walkerii* and *H. talpae* fleas, while only *B. taylorii* DNA was found in *C. agyrtes* (Table 7).

Flea identification by *18S rDNA* sequencing

To confirm morphological identification of flea species, we used a fragment of the *18S rDNA* gene (570 bp) for the molecular and phylogenetic analyses. All *Bartonella* -positive fleas used for *Bartonella* genotyping ($n = 26$) were sequenced and subjected to phylogenetic analysis (Fig. 6). For the phylogenetic analysis, all the DNA sequences from this study and 10 reference sequences from GenBank were used. The phylogenetic analysis based on the *18S rDNA* gene enabled recognition of three clades of fleas representing three genera: clade I encompassed sequences of *Ctenophthalmus* spp., clade II sequences of *Hystrichopsylla* spp. and clade III - *Megabothris* spp. No further resolution of flea species was possible, so based on morphological identification, and because of the lack

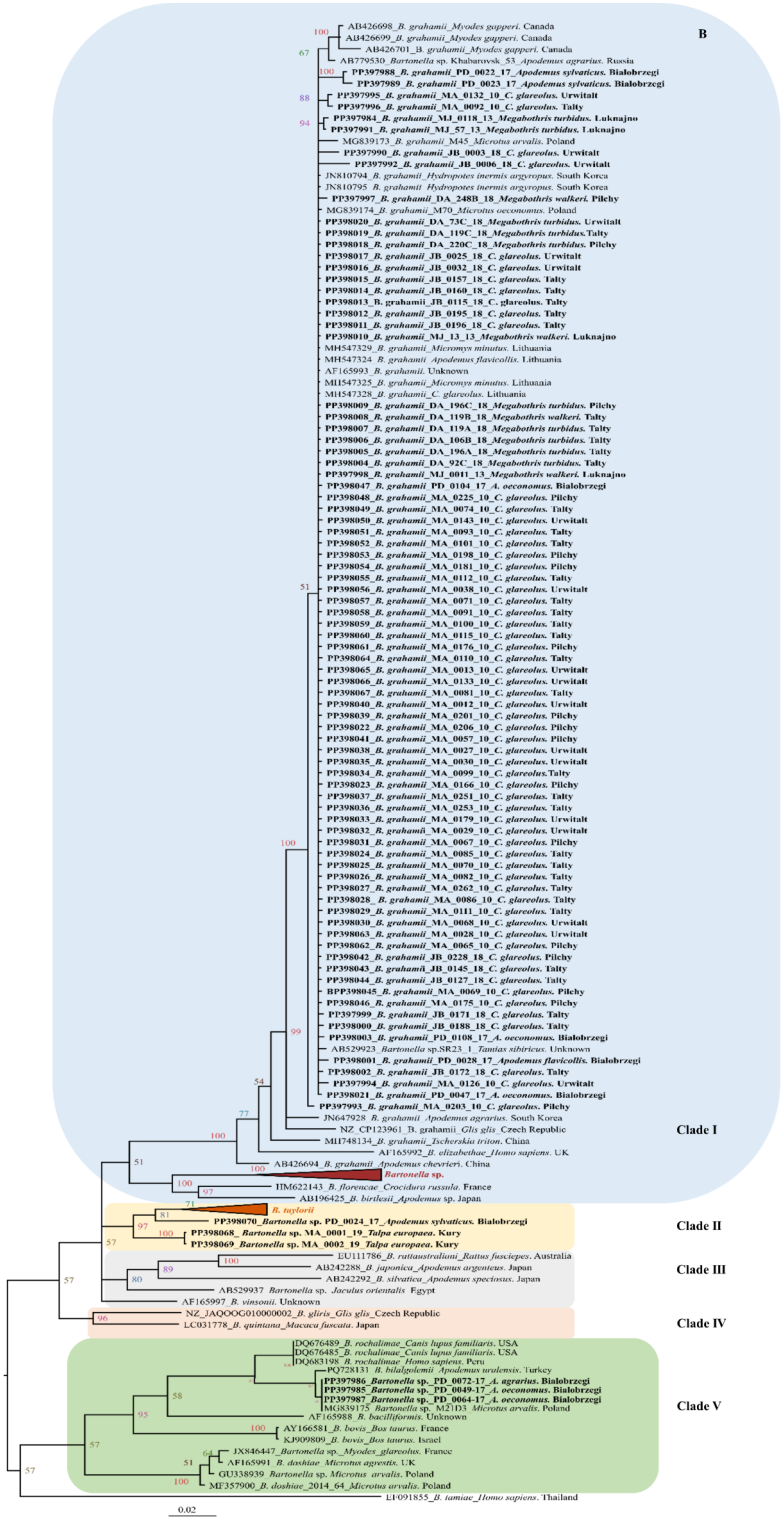
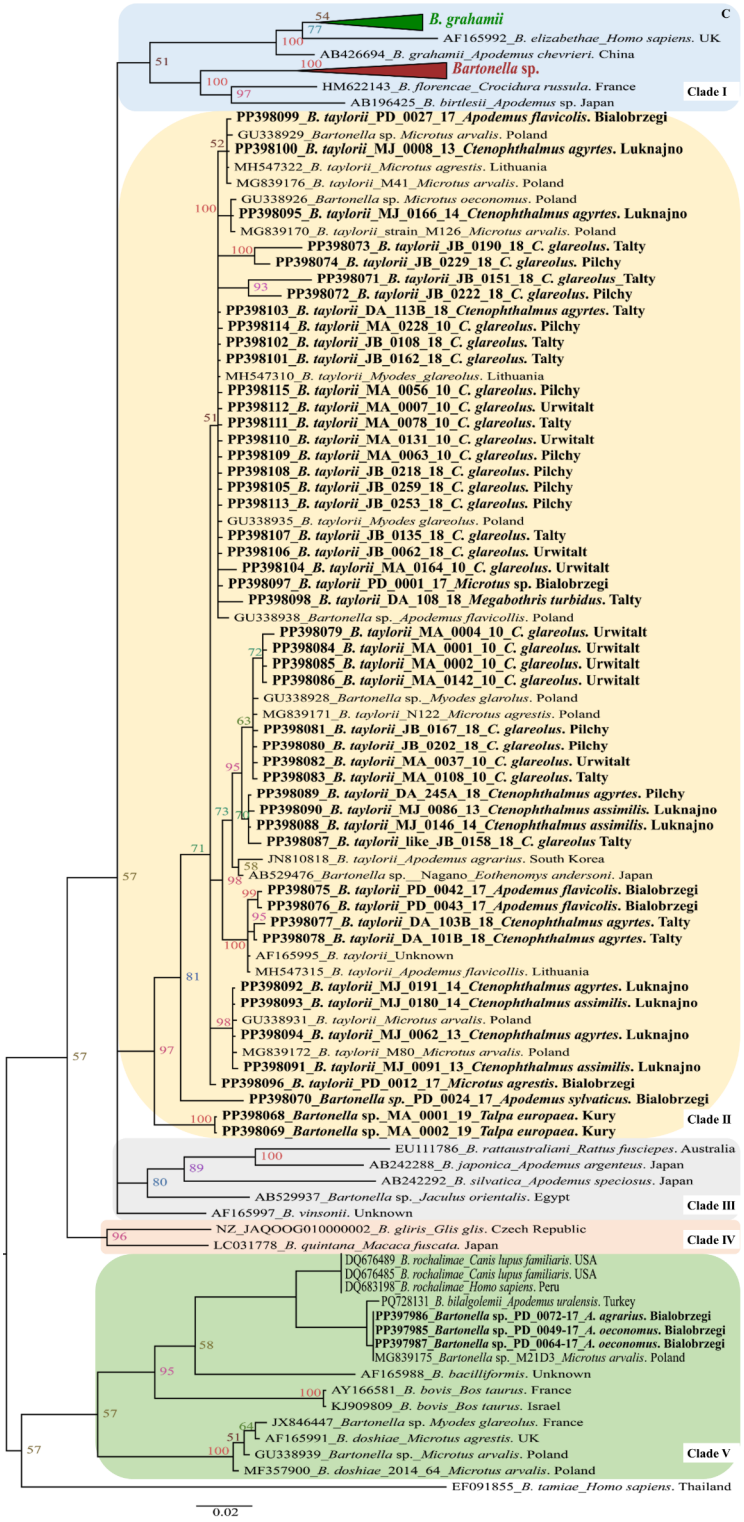


Figure 3. (continued)

of reference sequences for fleas, all the sequences obtained from fleas were deposited in the GenBank database (Fig. 5).

Haplotypes of *Bartonella grahamii* and *Bartonella taylorii*

Minimum Spanning Network analysis of the *rpoB* sequences of *B. grahamii* ($n = 81$) obtained in this study revealed nine haplotypes (Gr1-Gr9), differing by 1–4 SNPs (Additional file 1). Haplotype Gr1 was the main haplotype encompassing 70 out of 81 sequences (87.5%), identified in four species of rodents (*C. glareolus*, *A. sylvaticus*,



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A. flavicollis, and *A. oeconomus)* and in two species of fleas (*M. turbidus* and *M. walkeri*) (Fig. 6A). Moreover, haplotype Gr1 involved sequences from all study sites (Fig. 6B). Haplotype Gr2 encompassed two sequences (2.5%) from *C. glareolus* from Urwitałt and Tałty and differed by one SNP from the dominant haplotype Gr1. Interestingly, haplotype Gr3 encompassed two sequences (2.5%) from *A. sylvaticus* from Białobrzegi and differed from the dominant haplotype Gr1 by four SNPs. All other haplotypes grouped around haplotype Gr1 and were separated by 1–4 SNPs (Fig. 6A,B).

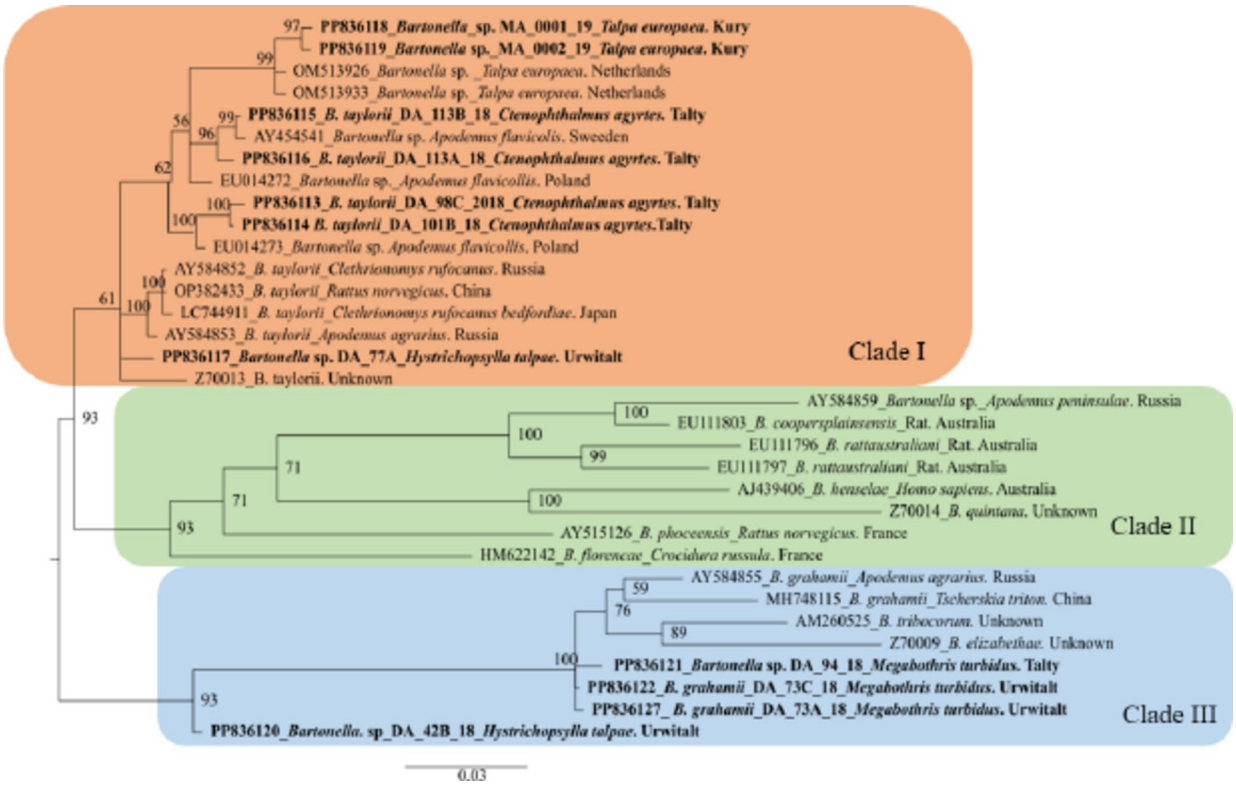

Fig. 4. The phylogenetic tree of *Bartonella* spp. inferred from sequence variation of 810-bp *gltA* gene fragment. The tree is 50%-majority rule consensus obtained using MrBayes (Bayesian Inference). Numbers along the nodes represent *a posteriori* probability. Clusters of sequences obtained from *T. europaea* from Kury are highlighted in red rectangular on the first clade.

Flea's species (*N*)	Bartonella species	Rodent species (host)	Bartonella species	Homology %	Year
M. walkeri (1)	*B. taylorii*	*A. oeconomus*	*B. taylorii*	99	2014
C. assimilis (1)	*B. taylorii*	*A. oeconomus*	*B. taylorii*	99	2014
H. talpae (1)	*B. grahamii*	*A. oeconomus*	*B. grahamii*	99	2013
C. assimilis (1)	*B. grahamii*	*M. arvalis*	*B. grahamii*	98	2013
M. turbidus (2)	*B. grahamii*	*C. glareolus*	*B. grahamii*	100	2018
M. turbidus (1)	*B. taylorii*	*C. glareolus*	*B. taylorii*	99	2018
C. agyrtes (6)	*B. taylorii*	*C. glareolus*	Negative	-	2018
C. agyrtes (1)	*B. taylorii*	*M. arvalis*	Negative	-	2014
C. assimilis (1)	*B. grahamii*	*M. arvalis*	Negative	-	2013
M. turbidus (5)	*B. grahamii*	*C. glareolus*	Negative	-	2018
M. turbidus (1)	*B. grahamii*	*M. arvalis*	Negative	-	2013
M. walkeri (2)	*B. grahamii*	*C. glareolus*	Negative	-	2018
M. walkeri (1)	*B. taylorii*	*M. agrestis*	Negative	-	2013
H. talpae (2)	*B. taylorii*	*C. glareolus*	Negative	-	2018

Table 7. Comparison of *Bartonella* spp. Isolated from the collected fleas and their hosts.

Despite this generally low genetic diversity, there was some host, vector and site-specific segregation of less common haplotypes, for example haplotypes Gr6, Gr7 and Gr8 were identified in *C. glareolus* from Urwitałt, whereas haplotypes Gr4 and Gr5 were present only in *M. turbidus* fleas from the Łuknajno site (Fig. 6A,B).

Minimum Spanning Network analysis of the *rpoB* sequences of *B. taylorii* ($n = 45$) successfully delineated 17 haplotypes (T1-T17), differing by 1–8 SNPs (Additional file 1). Haplotype T1 was the dominant haplotype encompassing 15 out of 45 sequences (33.3%) and was identified in *C. glareolus* and *C. agyrtes* from three sites, Urwitałt, Tałty and Pilchy (Fig. 6C and D). Haplotype T2 encompassed 8 out of 45 sequences (17.7%) and was also found in *C. glareolus* from three sites. Haplotype T2 differed from T1 by 5 SNPs. Interestingly, haplotype T3 was present only in the fleas *C. assimilis* and *C. agyrtes* from site 4 (Łuknajno) and was represented by 4

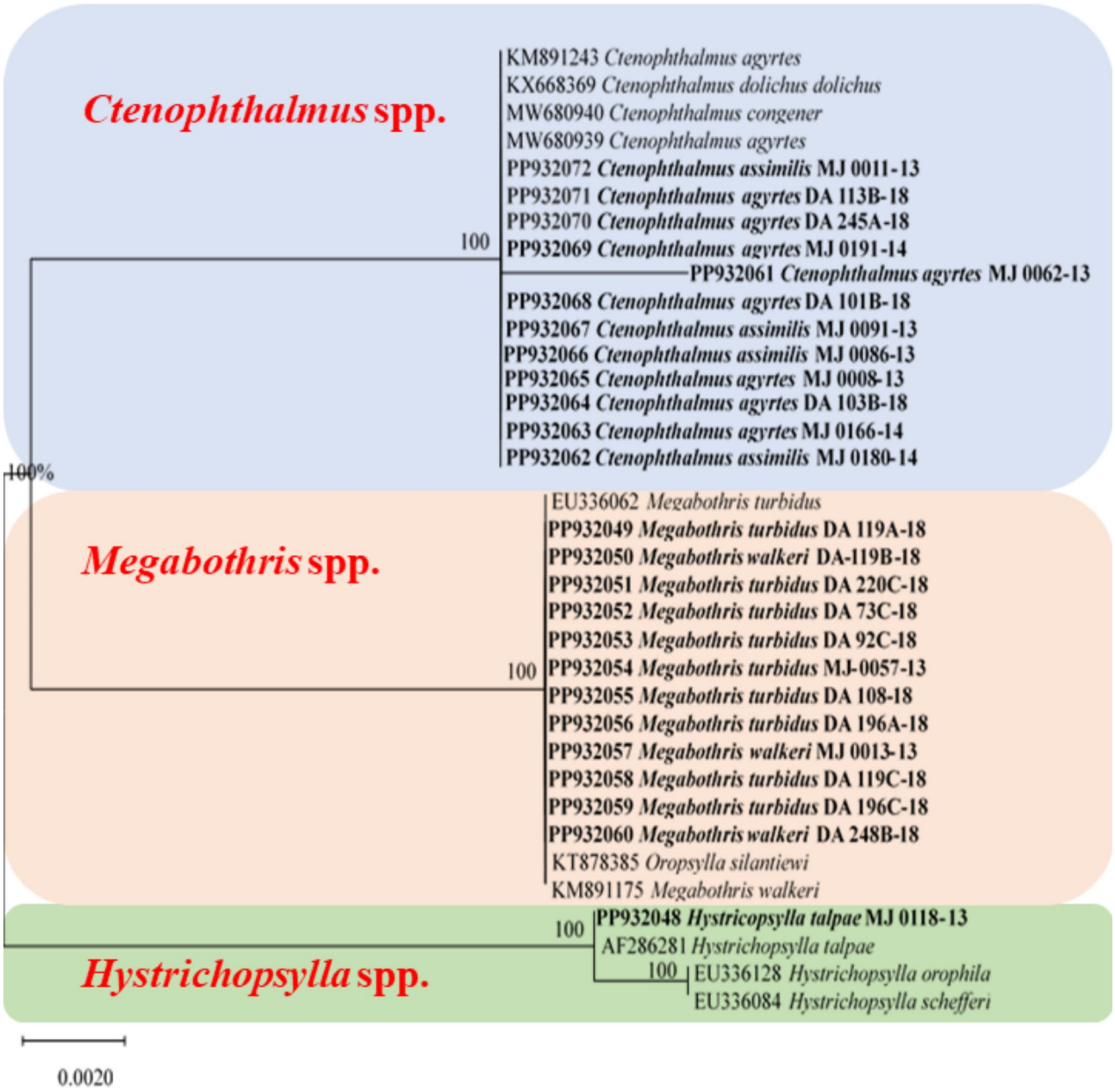

Fig. 5. The phylogenetic tree of the *18 S rDNA* of fleas was inferred by using the Maximum Likelihood method and Kimura 2-parameter model by MEGA11.

sequences out of 45 (8.8%) (Fig. 6C and D). The differences between T1, T2 and T3 were 3–5 SNPs, respectively. Moreover, haplotype T4 was isolated from the same species of fleas as haplotype T3 but was present in Pilchy and Łuknajno and differed from haplotype T2 by one single SNP. All other haplotypes of *B. taylorii* grouped around haplotypes T1 and T2 and were separated by 1–8 SNPs.

Discussion

This study examined the prevalence, diversity, and phylogenetic relationships of *Bartonella* spp. in rodent and flea communities in Northeastern and Central Poland. We identified *Bartonella* spp. in eight rodent species and five flea species. The overall prevalence of *Bartonella* spp. infection in the rodent community was found to be 42.3%, although this figure varied in comparison to previous studies and was influenced by rodent species and environmental factors (study site). In our previous investigation of haemoparasites in bank voles from NE Poland, we reported a prevalence of *Bartonella* spp. infections of 30.6% in a rodent community comprising *A. flavicollis*, *C. glareolus*, *M. arvalis*, and *A. oeconomus*[52]. Subsequent studies indicated a prevalence of 38.7% in *C. glareolus*[13] and 66.8% in the *Microtus* spp. community (comprising *M. arvalis*, *M. agrestis*, and *A. oeconomus*)[14]. In contrast, prevalence rates in rodents from other countries have been reported at 54.8% in Lithuania, 26% in

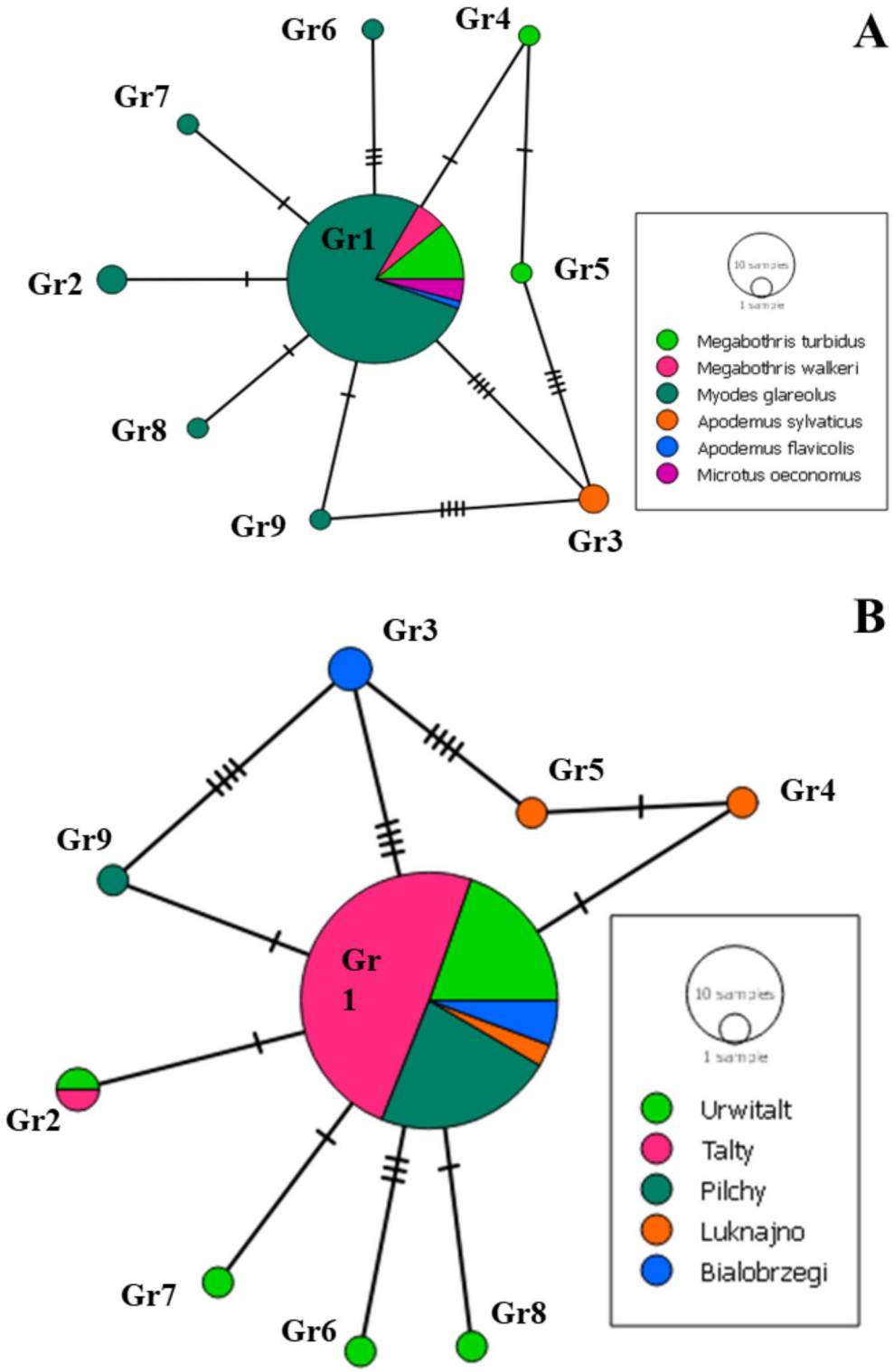

Fig. 6. The Minimum Spanning Network of the *rpoB* gene showing the relationship between the haplotypes. (**A**) *Bartonella grahamii* haplotypes based on the host and vector species; (**B**) *Bartonella grahamii* haplotypes based on the study sites; (**C**) *Bartonella taylorii* haplotypes based on the host and vector species; (**D**) *Bartonella taylorii* haplotypes based on the study sites.

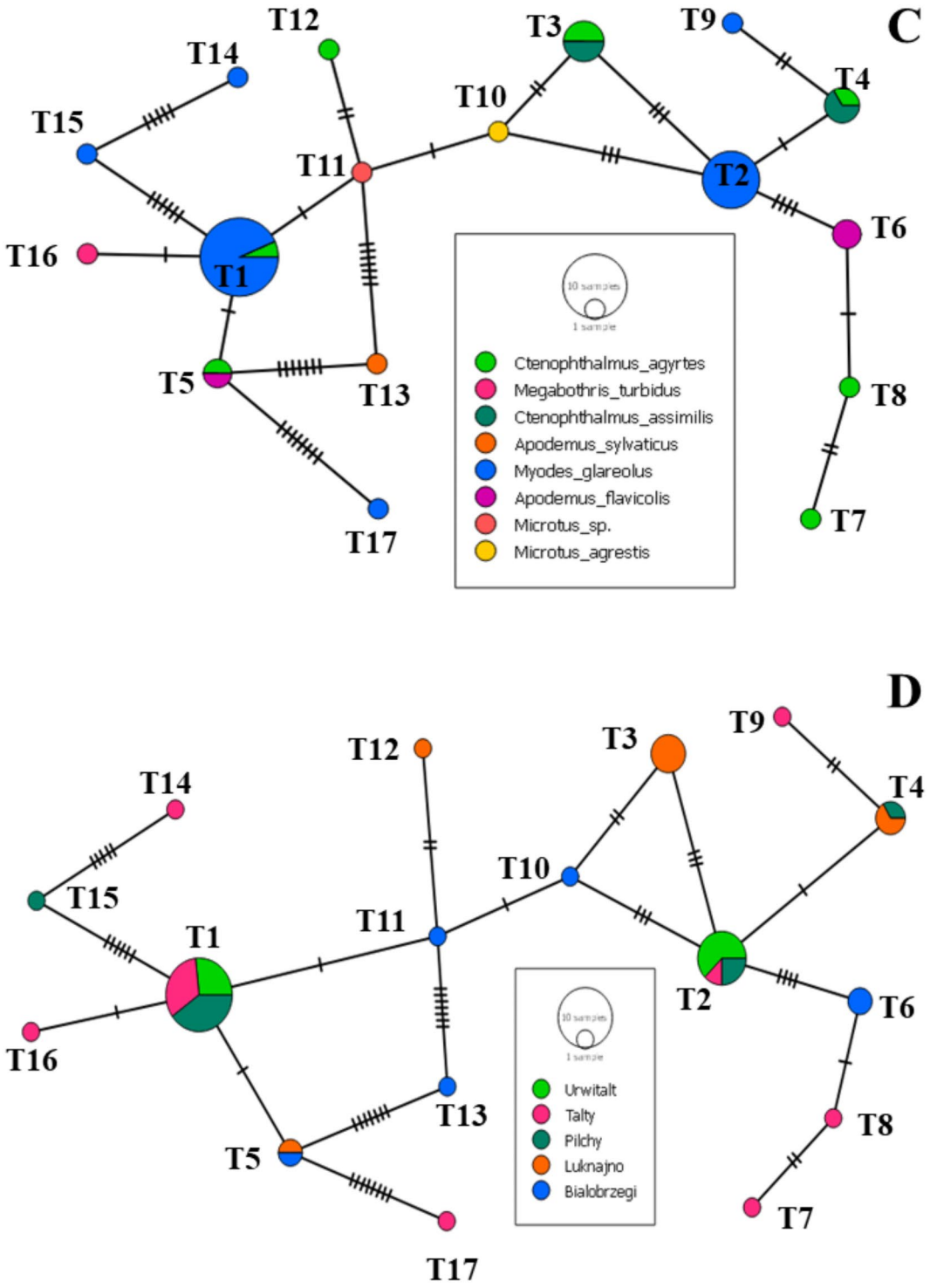

Figure 6. (continued)

the Netherlands, and 43% in Germany[17,19,53,54]. The notably high prevalence rates of *Bartonella* in rodents are significant, as these synanthropic host species are frequently found in habitats where they may come into close contact with humans, potentially posing a risk of exposure.

In our study, five flea species were collected from rodents, four of which (*C. agyrtes*, *C. assimilis*, *M. turbidus*, and *H. talpae*) are also common on rodents in Slovakia[23]. The overall prevalence of *Bartonella* spp. in flea populations associated with rodents was 11.3%, which is lower than previously reported rates of 59.4% in Germany, 34% in Slovakia, and 28% in Chile[23,54,55]. However, the intensity of flea infestation on rodents in our study was comparable to the 2.44 fleas per individual reported in Slovakia[23]. In 2013, we detected *Bartonella* spp. DNA in only two flea species, *C. assimilis* and *C. agyrtes*, paralleling findings from a previous study on

flea communities in dogs in Israel, where *Bartonella* spp. DNA was identified in two out of four flea species (*Ctenocephalides canis* and *Ctenocephalides felis*)[56].

Krasnov et al. (2006)[57] have suggested that the composition of flea species on host organisms is influenced by both host-flea and host-habitat interactions. Our analysis indicates that the year of study also may have impacted the *Bartonella*-flea-host interaction, because we detected *Bartonella* spp. in only two flea species in 2013, while in 2014 and 2018, *Bartonella* spp. DNA was identified in all five flea species collected from various hosts. This finding is consistent with previous studies conducted in Chile and Tanzania, where *Bartonella* spp. DNA was found in all tested flea species[55,58]. Interestingly, Gutiérrez et al. (2018)[20] reported only one flea species, *Synosternus cleopatrae*, with confirmed *Bartonella* spp. DNA on *Gerbillus andersoni* and *Gerbillus pyramidum* in Israel, suggesting a greater specificity of flea-host relationships compared to our findings.

In 2013, we observed a higher prevalence of *Bartonella* spp. in male fleas compared to females, potentially linked to the higher male-to-female ratio among the sampled fleas (55% vs. 45%, respectively). In contrast, no significant sex-related differences in *Bartonella* spp. prevalence were noted in 2014 and 2018 across all five flea species tested, aligning with findings from a previous study on fleas from rodents in Slovakia[23], where no significant differences in prevalence were detected between sexes among eight flea species.

Our analysis of *Bartonella* spp. prevalence in *C. glareolus* in 2018 revealed significant site-specific effects, with the highest prevalence (24.7%) recorded at site 2 (Tałty). In a previous long-term study (1999–2010) of *C. glareolus*, we reported a higher overall prevalence (45.4%) of *Bartonella* spp. at this same site, with peak levels observed in 2006. The lower prevalence of *Bartonella* spp. observed in the current study suggests changes in conditions affecting parasite survival over time, likely influenced by fluctuations in flea populations, although other unidentified factors cannot be ruled out at this stage. We note a trend of decreasing prevalence of *Bartonella* spp. infection in *C. glareolus* through the years of our study (i.e. from 2010, 2014 until 2018) and these results are consistent with our previous long-term studies on the haemoparasites from *C. glareolus* from NE of Poland, and *A. dimidiatus* from Egipt[13,59]. We hypothesised that extrinsic factors would have the major influence on haemoparasite communities, notably through the largely unpredictable long-term temporal effect, resulting in distinct between-year dynamics. Populations of rodent host species often fluctuate significantly over time, sometimes in multi-year cycles or unpredictably between years[60]. These changes impact the populations of their ectoparasites (vectors) and, consequently, the prevalence of vector-borne pathogens. Studies have found a high prevalence of *Bartonella* spp. in young voles across various species[13,61–63]. This may be due to high exposure to infections, likely through close contact with flea vectors or possible vertical transmission from infected mothers to offspring. In 2014 and 2018 the oldest bank voles showed the highest prevalence of infection (age class 2 and/or 3). The observed pattern of increased infection likelihood with increasing host age supports the idea that, as a rodent lives longer their chances of encountering an infected vector and thus contracting the infection also increase.

In Europe, *B. grahamii* and *B. taylorii* have been detected in various rodent species, including *A. flavicollis*, *A. agrarius*, *A. sylvaticus*, *Apodemus uralensis*, *Apodemus witherbyi*, *Micromys minutus*, *C. glareolus*, *M. arvalis*, *M. agrestis*, and *M. musculus*. *B. rochalimae* has been identified in *A. flavicollis*, *C. glareolus*, and *M. arvalis* (reviewed by Špitalská et al., 2017[66]), while *B. doshiae* has been detected in *A. flavicollis*, *A. agrarius*, *M. agrestis*, and *C. glareolus* (reviewed by Buffet et al., 2013[67]). In our current study, 58% of sequences derived from small rodents and fleas were classified as *B. grahamii*, 32% as *B. taylorii*, 1.9% as *Bartonella* spp., and 7.8% as an unidentified *Bartonella* species. While these findings differ somewhat from a study in Lithuania, which reported *B. grahamii* in 26% of hosts, *B. taylorii* in 42%, and lower occurrences of *B. rochalimae* and other species[17], *B. grahamii* and *B. taylorii* were clearly the dominant species in both studies. Generally, *B. taylorii* exhibited greater diversity, occurring in five rodent and five flea species, while *B. grahamii* demonstrated lower diversity, identified in four rodent and four flea species.

Phylogenetic and Minimum Spanning Network analyses based on the *rpoB* sequence of *B. taylorii* revealed 17 haplotypes associated with five rodent species and three flea species. Previous studies have reported high genetic diversity of *B. taylorii* strains in rodents from Poland, Turkey, and the Netherlands[14,27,66–68], potentially resulting from frequent recombination events, horizontal gene acquisition, and accumulation of mutations (reviewed by Gutiérrez et al.[71]). In our study, *B. grahamii* strains exhibited lower sequence diversity, with only nine haplotypes identified, fewer than the 30 haplotypes reported from Sweden[69]. Notably, five haplotypes were restricted to *C. glareolus*, and two haplotypes were confined to *M. turbidus*. The two *Bartonella* isolates from European moles (*T. europaea*) were identical and formed separate branches on phylogenetic trees for the *rpoB* and *gltA* genes. These sequences closely resembled an uncultured *Bartonella* sp. obtained from *T. europaea* in the Netherlands[66]. Currently, *Bartonella talpae* is the only species associated with this host[70], but no type strain or molecular data exist for *Bartonella* spp. from moles. Our DNA sequences cannot yet be assigned to this species until a type strain is isolated and characterized. The *Bartonella* from our study showed host specificity in moles, which aligns with reports of host specificity in certain rodent and shrew species from China[71] and rodents from Egypt[16]. In contrast, *B. grahamii* and *B. taylorii* demonstrated poor host specificity, as they were detected across all studied rodent hosts, consistent with findings from our previous work[14,27,52]. Furthermore, *B. grahamii* has been reported in 53 small mammal species to-date, while *B. taylorii* has been identified in 27 small mammal species (reviewed by Krügel et al., 2022).

Our *Bartonella rochalimae*-like sequence has previously been reported also from *M. arvalis* from the Mazurian Lake District[14]. Interestingly, our *Bartonella* sp. isolate from *A. oeconomus* and *A. agrarius* in Białobrzegi (approximately 340 km from the Mazurian Lake District) clustered together with the *Bartonella rochalimae*-like sequence from *M. arvalis* with minimal diversity. While *B. rochalimae* is generally thought to infect rodents[29,47], our phylogenetic analysis showed that sequences from a common vole (*M. arvalis*) from Poland[14], which closely resembled our sequence (99.3–99.8%), formed a sister branch to *B. rochalimae* isolates from dogs and humans in Peru and the USA, but were closer to *B. bilalgolemii* described recently from *A. uralensis* from Turkey. Thus, their

species identity needs further investigation and currently this *Bartonella* variant from rodents can be referred to only as *Bartonella* sp.

Phylogenetic analysis of *gltA* sequences from *Bartonella* spp. obtained from fleas during the 2018 fieldwork largely confirmed the results obtained from analysis of the *rpoB* gene. The *B. taylorii* clade exhibited greater diversity than the *B. grahamii* clade. However, the phylogenetic placement of the *Bartonella* sp. from *T. europaea* differed between the *rpoB* and *gltA* phylogenetic trees. The two *Bartonella* sp. *rpoB* sequences from moles clustered on separate branches distinct from other *Bartonella* species, while they clustered within the *B. taylorii* clade on the *gltA* tree. As previously noted[16,27], diversity in the *gltA* gene correlates with host specificity, suggesting that further research is needed to formally describe the isolate from European moles as a novel *Bartonella* species. One of the *gltA* sequences from the *Bartonella* sp. isolated from *H. talpae* (site 1) formed a distinct branch, exhibiting low homology (87%) to *B. taylorii*. Unfortunately, the quality of the *rpoB* sequence for this sample was inadequate for conclusive species identification. However, *H. talpae* is known to infest various hosts, including dogs[73], cats[73], dormice (*Glis)*[74] and bank voles[75,76], suggesting it may harbour a different or novel species/strain of *Bartonella*.

Remarkably, *Bartonella* spp. DNA was identified in 19 fleas collected from *Bartonella* -negative hosts, indicating a potential vector role of fleas for *Bartonella* spp. We identified *C. assimilis*, *M. turbidus*, *M. walkeri*, and *H. talpae* as vectors for *B. grahamii* and *B. taylorii*, while *C. agyrtes* was specifically associated with *B. taylorii*. These findings confirm the role of fleas as vectors for *Bartonella* spp. Although Špitalská et al. (2022) suggested that fleas act as vectors for rodent-infective *Bartonella* spp., precise information regarding the specific flea species involved and the corresponding *Bartonella* species/strains has been lacking.

Due to the absence of reference sequences for fleas in GenBank, it was challenging to confirm flea identification based on 18S rDNA. Nevertheless, we believe our morphological identifications are accurate, and thus, all obtained DNA sequences of the fleas have been deposited in the GenBank database. There is a dearth of phylogenetic studies on fleas, particularly regarding species infesting rodents; however, some research has focused on cat fleas using the Internal Transcribed Spacer 1 (ITS1) and Internal Transcribed Spacer 2 (ITS2)[77], as well as mitochondrial genes such as cytochrome c oxidase subunit I (cox1) and II (cox2)[78].

In this study, the haplotypes of the *rpoB* gene from *B. grahamii* and *B. taylorii* were analyzed, enabling a comparison of haplotype diversity, structure, and distribution between both *Bartonella* species across different sites, hosts, and vectors. Minimum Spanning Network analysis for *B. grahamii* revealed nine haplotypes, with the dominant haplotype (Gr1, 87.5%) present in four rodent species and two flea species, with sequences originating from all study sites. This result suggests that the Gr1 haplotype is the predominant haplotype found in NW Poland; however, additional *B. grahamii* sequences from other locations in Poland are necessary to corroborate this conclusion. Interestingly, among the five haplotypes of *B. grahamii* from *C. glareolus* (Gr2, Gr6, Gr7, Gr8, and Gr9), three (Gr6, Gr7, and Gr8) originating from Urwitałt, one haplotype was shared between Urwitałt and Tałty, and the last haplotype was from Pilchy. The unique haplotype (Gr3) was exclusively identified in *A. sylvaticus* from Białobrzegi. These findings support our hypothesis that host identity and study site significantly influence the genetic diversity of *Bartonella*. Among fleas, two unique haplotypes of *B. grahamii* were identified in *M. turbidus* (Gr4 and Gr5), both from Łuknajno, echoing findings from a study on *Lipoptena fortisetosa* (Diptera, Hippoboscida) in SE Poland, where two unique haplotypes of *Bartonella* sp. were detected from the same vector and site[30].

Minimum Spanning Network analysis of *B. taylorii* revealed 17 haplotypes, with two dominant haplotypes (T1 = 33.3% and T2 = 17.7%) encompassing sequences from *C. glareolus* and *C. agyrtes* from three sites: Urwitałt, Tałty, and Pilchy. The detection of haplotype T1 in both *C. glareolus* and *C. agyrtes* suggests a correlation between host, bacterium, and vector, indicating that host identity and vector specificity may influence the genetic diversity of *B. taylorii*. The remaining haplotypes (T7, T8, T9, T10, T11, T12, T13, T14, T15, T16, and T17) are unique and related to their respective hosts or vectors, with six haplotypes identified from Tałty, three from Białobrzegi, and one from Łuknajno (Additional file 1).

A previous investigation of *Bartonella* haplotypes in rodents from Poland[27] provided strong evidence for recombination within the *rpoB* gene among all *B. grahamii* haplotypes and all *B. taylorii* haplotypes, isolated from four rodent species (*A. flavicollis*, *M. arvalis*, *A. oeconomus*, and *C. glareolus*). The analysis of the *gltA* gene revealed six haplotypes of *B. grahamii* and 24 haplotypes of *B. taylorii*.

Conclusions

We identified nine haplotypes of *B. grahamii* and 17 haplotypes of *B. taylorii* from rodents and fleas at five study sites in Poland. Two *Bartonella* species, the first isolated from *T. europaea* and the other from *A. sylvaticus*, and the second from *A. agrarius* and *A, oeconomus* are proposed as novel species (Fig. 3). Furthermore, we conclude that the fleas *C. agyrtes* and *H. talpae* are vectors for *B. taylorii*, while *C. assimilis* and *M. walkeri* serve as vectors for *B. grahamii*, and *M. turbidus* is a vector for both *B. grahamii* and *B. taylorii*.

Data availability

Data availabilityAll relevant data are included in the article. All DNA sequences of Bartonella spp. and fleas have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) under accession numbers provided in main text and Additional file 1.

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Author contributions

MoA and AB, Designed the study. MoA, MuA and ŁB performed the phylogenetic analysis. MoA, MuA, KT, AB, JMB, DDS, JBB, MG, participated in a field work. JMB performed the statistical analysis and drafted the manuscript. MoA, MJ, DA, PD participated in laboratory work. All authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to M.A.

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