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Prevalence and genetic diversity of the lung nematode *Eucoleus aerophilus* in red foxes *(Vulpes vulpes)* in Central Europe (Poland) assessed by PCR and flotation

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Eucoleus aerophilus **is the causative agent of respiratory capillariosis in wild and domestic carnivores and has sporadically affected humans. To date, this parasite has been detected in 38 countries, confirming its worldwide distribution. The aim of this study was to evaluate the prevalence of *E. aerophilus* in red fox populations from Central Europe and to describe the sequence variations in the partial *cox*1 gene of this parasite recovered from stool samples. An investigation was carried out using 342 samples of red foxes faeces investigated via seminested PCR and coproscopy. PCR results confirmed the presence of *E. aerophilus* DNA in 230 samples and *E. boehmi* DNA in 14 samples. Molecular analysis of the retrieved sequences revealed 22 haplotypes of *E. aerophilus*, EaPL1–EaPL22, and 4 haplotypes of *E. boehmi*, EbPL1–EbPL4. Coproscopic examination revealed that eggs of the Capillariidae family were most prevalent in all regions, with a mean prevalence of 73%. Furthermore, the following eggs were detected: Taeniidae (21.3%), *Toxocara* spp. (26.3%), *Toxascaris leonina* (5.3%), *Trichuris vulpis* (1.5%), trematodes (23.4%), hookworms (2.3%) and *Mesocestoides* spp. (1.8%). This study evaluated the prevalence of *E. aerophilus* in red fox populations in selected regions of Poland. To the best of our knowledge, this is the first molecular study on *E. aerophilus* in Poland.**

Keywords *Eucoleus aerophilus*, *Capillaria aerophila*, Prevalence, Haplotypes, Central Europe, Poland

Eucoleus aerophilus (syn. *Capillaria aerophila*), included in the order Trichocephalida, family Capillariidae, is the causative factor of respiratory capillariosis in wild and domestic carnivores and has sporadically affected humans[1,2]. Adult worms of this nematode live and reproduce beneath the epithelium of the bronchi, trachea, occasionally frontal sinuses and nasal passages of infected hosts[3,4]. Mature females produce nonlarvated eggs, which are coughed up, swallowed by the host and shed in faeces. The excreted eggs embryonate in the environment and remain viable for up to one year[5,6]. Hosts can acquire infections through direct incidental ingestion of embryonated eggs (with L1) or through ingestion of infected earthworms, which serve as paratenic hosts[1,7–9]. In animals, pulmonary capillariosis is considered subclinical or leads to respiratory distress ranging from mild disease to severe and potentially fatal pneumonia[5,6].

E. aerophilus indicates a cosmopolitan distribution in both, wild and domestic mammals. Investigations regarding this parasite are available worldwide (Supplementary Fig. 1, Figure S1)[10]. The prevalence of this parasite differs according to host species, region (temperature, humidity) and size of host population[11]. The range of hosts among wild animals is wide, e.g., red foxes, coyotes, wolves, martens, raccoon dogs, Arctic foxes, badgers, brown bears, crab-eating foxes, European wild cats, lynxes, ocelots, hedgehogs or jackals, as well as domestic animals, e.g., cats and dogs[10]. Among such a wide range of hosts, the red fox is acknowledged to be the main reservoir and transmitter of *E. aerophilus*[10]. The most results regarding the occurrence of this parasite come from

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studies on foxes. What is worth to mention red fox is the most widespread of all wild canid species worldwide. The population of this host has increased in recent years[12–14], which favours approaching human habitats and the possibility of contamination of the environment by the spread of infective eggs. This significantly increases the risk of transmission of this infection to companion animals and, moreover, to humans. Unlike for *Capillaria hepatica* and *C. philippiniensis*, there is no surveillance strategy against *E. aerophilus* led by the European Centre for Disease Prevention and Control (ECDC), while several cases of pulmonary capillariosis in humans have been described[2,15–17]. These data may be underestimated because the clinical symptoms are often nonspecific, and infections are, in most cases, diagnosed incidentally[2]. This could suggest that the prevalence of *E. aerophilus* in humans is underreported by national health care systems.

Based on a meta-analysis of the available literature (published from 1973 to 2022)[10], the average prevalence of *E. aerophilus* that was detected in the lungs of red foxes estimated via necropsy was 49.3% (95% CI 40.1–58.5), and that detected via faecal examination (flotation) was 43.4% (95% CI 28.0–58.7). Moreover, in domestic animals, the average prevalence in the lungs was 8% (cats), and in faeces, it was 3% and 2% (in dogs and cats, respectively). Specific coprological diagnosis of *E. aerophilus* can be difficult because of the resemblance of its egg structure to that of other parasites infecting carnivores (e.g., *E. boehmi and T. vulpis*)[4,18,19]. The development of molecular methods for use in this field has provided a solution to this problem. Although several recent studies have examined the molecular characteristics and phylogenetic analyses of *E. aerophilus* haplotypes obtained from wild and companion animals[7,20–22] in Poland, such research has not been conducted.

The aim of our study was to evaluate and compare the prevalence of *E. aerophilus* in red fox populations from selected regions of Poland (Central Europe) and to provide a deeper insight into the genetic diversity of *E. aerophilus* in the investigated regions.

Materials and methods

An investigation was carried out using 342 stool samples from red foxes (*Vulpes vulpes*) that were shot (during official hunting and a survey concerning the efficacy of antirabies vaccination) in different parts of Poland from 2021 to 2023. The studied areas included three regions: Podkarpackie Province (PK; southeastern Poland; $n=120$), Śląskie Province (ŚL; southern Poland; $n=111$) and Warmińsko-Mazurskie Province (WM; northeastern Poland; $n=111$). The study material (intestines from foxes) was sent to the laboratory of the Department of Parasitology and Invasive Diseases of the National Veterinary Research Institute in Puławy (NVRI) and stored for 7 days at < -70 °C for safety reasons before examination. Stool samples were subsequently extracted from the large intestines of red foxes and divided into two portions: the first portion of faeces, intended for molecular study, and the second, intended for coproscopic examination.

Molecular study (DNA extraction, PCR and sequencing)

DNA extraction was performed via a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for larger stool volumes. Each DNA sample was examined by PCR using two variants, namely, undiluted and 1:10 diluted (to eliminate the contigency of inhibition)[23]. Seminested PCR was performed according to the procedure of Di Cesare et al.[21] to amplify the diagnostic region within the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene of *E. aerophilus*. In the first step, Cox1NEMF (5′-CCTGAGGTTTATATTYTWRTT-3′) and Cox1NEMR (5′-CCTGTTARRCCTCCRATACT-3′) were used to amplify a 344-bp fragment, whereas in the second step, CaerInt2F (5′-GAAGCCTTAATAACTATTTCAG G-3′) and Cox1NEMR (reverse, 5′-CCTGTTARRCCTCCRATACT-3′) were used to amplify a 299-bp fragment of the *cox*1 gene.

The obtained amplicons were separated by horizontal electrophoresis on 2% agarose gels stained with Simply Safe (EURx, Gdańsk, Poland). The gels were visualized via Fusion Fx, Fusion Capt Advance software supplied by Vilber Lourmat (Collégien, France). The obtained PCR products were sequenced via Sanger dideoxy sequencing at a commercial company (Genomed S.A., Warsaw, Poland). Phylogenetic analyses of the sequenced fragments of the *cox*1 gene were performed using the Geneious Prime bioinformatics software platform. Using the ClustalW algorithm, the sequences were aligned and trimmed, after which the obtained consensus sequences were analysed and compared to the GenBank collection via the Basic Local Alignment Search Tool (BLAST) nucleotide algorithm to confirm the species affiliation. The phenogram was created by applying the Tamura–Nei genetic distance model and neighbour-joining method with 1000 nonparametric bootstrap inferences. The open reading frames were confirmed via conceptual translation via the invertebrate mitochondrial code in Geneious Prime. The nucleotide sequence data of the partial *cox*1 gene presented in this paper were added to the GenBank™ database under the following accession numbers: PP838113-PP838134 and PP838136-PP838139. Phylogenetic analysis was performed using sequences derived from GenBank[7,14,20–22].

Coproscopic examinations

Faeces were examined microscopically by flotation via the McMaster method according to Raunaud's modification[24]. As a flotation liquid, saturated magnesium sulphate with a specific gravity of 1.22 g/cm^3 was used. The eggs of the parasites were detected via stereomicroscopy at 200x magnification.

Statistical analysis

Differences in the prevalence of individual infections among regions and differences between the results that were retrieved by flotation and PCR were calculated via a chi-square test with a Bonferroni correction for multiple comparisons. The differences in all analyses were considered statistically significant when $P<0.05$. The Pearson correlation coefficient was calculated for the total results obtained from all regions with the use of both methods (flotation and PCR). Confidence intervals (95% CIs) concerning the percentages of infected foxes were calculated according to the method described by Newcombe[25]. The arithmetic mean values and coefficients of

variation were calculated for the number of eggs per gram of faeces and the intensity of infection. Statistical analyses were performed via Statistica 10 software (StatSoft Polska, Kraków, Poland).

Results

Seminested PCR

Amplification of partial sequences of the *cox*1 gene was successful in 244 foxes. A comparison of the sequencing results of the obtained amplicons using the GenBank database confirmed the presence of *E. aerophilus* DNA in 230 samples, and the mean prevalence in all investigated areas was 67.3%. The highest prevalence of *E. aerophilus* was detected in PK (71.7%), and the lowest was detected in ŚL (62.2%). The percentage of positive samples in WM was 67.6%. There were no statistically significant differences in the prevalence of *E. aerophilus* between the individual regions. Interestingly, in the case of 14 amplicons, a comparison of the sequencing results with the GenBank database revealed the DNA of *E. boehmi* (Table 1). The mean prevalence of *E. boehmi* was 4.1%. The highest percentage of *E. boehmi*-positive samples was in WM (6.3%), while in PK and ŚL, the prevalence rates were similar, at 2.5% and 3.6%, respectively. There were no statistically significant differences in *E. boehmi* prevalence rates among individual regions.

The molecular analysis of good-quality sequences of the *E. aerophilus* partial *cox*1 gene revealed 22 haplotypes, which were designated EaPL1–EaPL22 (GenBank accession numbers: PP838113–PP838134). There were no insertions or deletions in any of the sequences. The nucleotide sequence variations among all 22 haplotypes upon pairwise comparisons ranged from 0.4 to 2%. The most prevalent haplotype appearing in the investigated foxes was EaPL1, where 155 sequences were identical to each other (73.5% of positive *E. aerophilus* samples). Moreover, this partial *cox*1 gene was 100% homologous to JQ905052.1 (haplotype I), which was obtained from dogs and cats in Italy[21]. Additionally, this 256-bp fragment was 100% similar to those of KF479371.1 (haplotype IX) retrieved from red foxes in Italy[7] and KF479375.1 (haplotype XIII) from red foxes in Portugal[7]. The remaining sequence types differed from those of EaPL1 in terms of the occurrence of mutations at specific positions (Table 2). However, the EaPL2 haplotype was 100% identical to the KF479372.1 sequence (haplotype X) obtained from red foxes in Romania and beech marten in Portugal[7]. The EaPL3 haplotype exhibited 100% homology to the sequences JQ905054.1 (haplotype III)[21] and KC341991.1 that were retrieved from red foxes in Italy[22]. The EaPL4 haplotype exhibited 100% similarity to KC341988.1, which was derived from a red fox in Italy[22]. The EaPL5 haplotype exhibited 100% homology to KC341989.1, which was obtained from a red fox in Switzerland[22]. The EaPL6 haplotype displayed 100% resemblance to JQ905055.1 (haplotype IV)[21]. The EaPL10 haplotype was 100% identical to that of KC341990.1, which was acquired from red fox in Switzerland[22] (Fig. 1).

Table 2 presents the obtained haplotypes (EaPL1-EaPL22), the numbers of red foxes with specific haplotypes and the occurrences of mutations at specific positions. Translation revealed that amino acid sequences had open reading frames at the first position without a stop codon. Most nucleotide substitutions (transitions and transversions) were synonymous, except for two transversions at the 207th position (T→A), which resulted in the alteration of isoleucine in methionine (L→M).

Molecular analysis of good-quality sequences of the *E. boehmi* partial *cox*1 gene revealed 4 haplotypes, which were designated EbPL1–EbPL4 (GenBank accession numbers: PP838136–PP838139). There were no insertions or deletions in any of the sequences. The nucleotide sequence variations among all 4 haplotypes upon pairwise comparisons ranged from 0.4 to 1.2%. The most prevalent haplotype appearing in the investigated foxes was EbPL1, where 4 sequences were identical to each other. Moreover, this partial *cox*1 gene fragment was 100% homologous to KX027311.1 from a fox in Bosnia and Herzegovina[14] and to KR186213.1 from a dog in Italy[20]. The haplotype EbPL2 demonstrated 100% similarity to KX027314.1 from a fox in Bosnia and Herzegovina[14]. The differences among the remaining haplotypes are presented in Table 3. Translation revealed that the amino acid sequences had open reading frames at the first position without a stop codon, revealing synonymous intraspecific nucleotide variations (transitions: C→T and T→C).

Figure 1 shows a dendrogram of the genetic relationships based on partial cox1 mitochondrial DNA among the *E. aerophilus* and *E. boehmi* sequences included in this study and the other sequences retrieved from GenBank, with Trichuris trichiura serving as an outgroup.

Figure 2 and Table 4 present the distributions of individual haplotypes of *E. aerophilus* in the investigated areas. The dominant haplotype, EaPL1, was present in all regions, but its prevalence was significantly lower in Śląskie Province (ŚL) than in other regions. There were statistically significant differences in distribution between PK and ŚL ($P=0.0108$) and between WM and ŚL ($P=0.0025$). The EaPL2-3 and EaPL5-6 haplotypes were found in all the studied regions, EaPL8 and EaPL9 were found in two provinces, and the remaining haplotypes could

	Overall (all regions) ($n=342$)		Podkarpackie Province (PK) ($n=120$)		Śląskie Province (ŚL) ($n=111$)		Warmińsko-Mazurskie Province (WM) ($n=111$)	
	N	% Pos	N	% Pos	N	% Pos	N	% Pos
Eucoleus spp.	244	71.3	89	74.2	73.0	65.8	82	73.9
Eucoleus aerophilus	230	67.3	86	71.7	69.0	62.2	75	67.6
Eucoleus boehmi	14	4.1	3	2.5	4.0	3.6	7	6.3

Table 1. The prevalence rates (%) of *Eucoleus* species in red foxes in different regions of Poland estimated via PCR (including sequencing). *n* number of investigated samples; *N* number of positive samples; *% Pos* percentage of positive samples

	Nucleotide at position[b]																						
Haplotype	n[a]	9	27	33	42	51	54	72	78	90	99	105	108	111	129	132	144	150	159	201	207	213	231	255
EaPL1	**155**	**A**	**A**	**T**	**A**	**C**	**T**	**T**	**T**	**C**	**T**	**T**	**A**	**C**	**T**	**T**	**C**	**A**	**C**	**G**	**T**	**C**	**G**	**C**
EaPL2	11	G	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
EaPL3	8	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
EaPL4	5	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•
EaPL5	4	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T
EaPL6	3	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•
EaPL7	3	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•
EaPL8	3	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•
EaPL9	2	•	•	•	•	•	•	C	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•
EaPL10	2	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•
EaPL11	1	•	G	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
EaPL12	1	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•
EaPL13	1	•	•	•	G	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•
EaPL14	1	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
EaPL15	1	•	•	•	•	•	C	C	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
EaPL16	1	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•
EaPL17	1	•	•	•	•	•	•	C	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•
EaPL18	1	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•
EaPL19	1	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•
EaPL20	1	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	T	•	•	•	T	•	•
EaPL21	1	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	A	•	•	•
EaPL22	1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	A	•

Table 2. Residue positions of single nucleotide polymorphisms in haplotypes EaPL2 to EaPL22 compared with haplotype EaPL1 of *E. aerophilus* isolated from red fox faeces from different regions of Poland. [a] A dot indicates that the nucleotide is the same as that in haplotype EaPL1. [b] Number of samples belonging to the indicated haplotype.

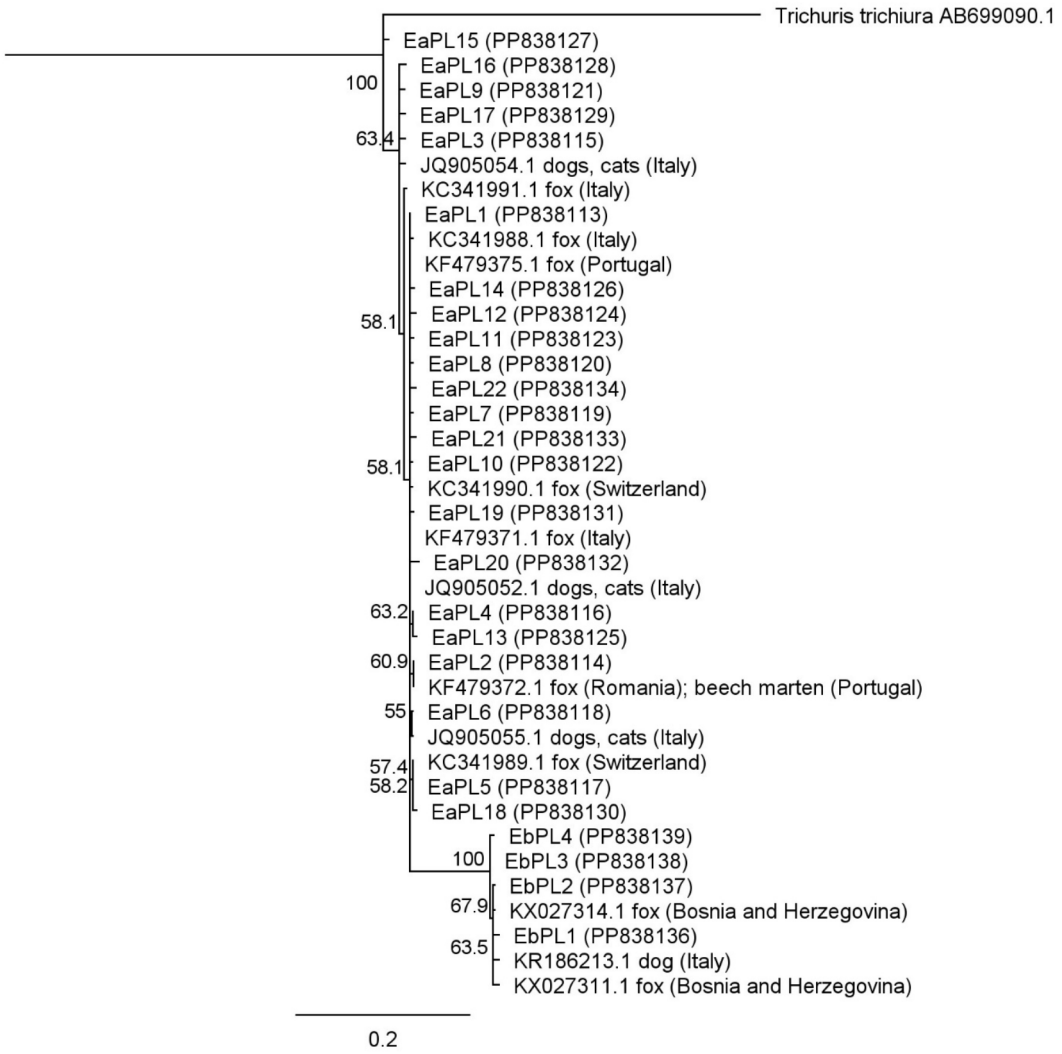

Fig. 1. Dendrogram based on neighbour-joining analysis of partial *cox*1 sequence data for *E. aerophilus* (haplotypes EaPL1-EaPL22; PP838113-PP838134, from this study) and *E. boehmi* (haplotypes Eb1-Eb4; PP838136-PP838139, from this study) compared with sequences retrieved from GenBank. The values on the tree nodes are bootstrap proportions (%).

only be observed in individual provinces. The most common PK haplotypes were EaPL4, EaPL10, EaPL13, and EaPL19. There were only 3 haplotypes within WM: EaPL11, EaPL14 and EaPL20. ŚL was the most diverse in terms of the number of haplotypes; only in this area were there 8 haplotypes: EaPL7, EaPL12, EaPL15-18, and EaPL21-22.

The most prevalent *E. boehmi* haplotype (EbPL1) was found in WM and ŚL, whereas EbPL2 was present in all investigated areas. On the other hand, the haplotype EbPL3-4 was present exclusively in the WM area (Table 5).

Coproscopic examinations (flotation)

In general, coproscopic examinations revealed that parasites were present in 288 (84.2%) stool samples from red foxes. The eggs that were most prevalent in all regions were eggs of the Capillariidae family, with a mean prevalence of 73.4% (95% CI 68.5–77.8). Because of the similarity of the eggs in this group (*E. aerophilus* and *E. boehmi*), they were classified into one category (Capillariidae), although we focused on *E. aerophilus* in further analyses. A statistically significant difference in the prevalence in this group was detected between ŚL (62.2%) and WM (82.0%) ($P=0.0017$). In addition, seven different types of parasite eggs were detected: Taeniidae, *Toxocara* spp., *Toxascaris leonina*, *Trichuris vulpis*, Trematoda, hookworms and *Mesocestoides* spp. Eggs of the Taeniidae family and *Toxocara* spp. were found in all regions in relatively high percentages of samples, 21.3% and 26.3%, respectively. Moreover, a statistically significant difference in the prevalence of Taeniidae-type eggs was detected between the PK (30.0%) and ŚL (10.8%) groups ($P=0.0006$). A comparison of the prevalence of *Toxascaris leonina* revealed statistically significant differences between PK (2/120; 1.7%) and WM (15/111; 13.5%) ($P=0.0014$) and between ŚL (1/111; 0.9%) and WM (2/111; 13.5%) ($P=0.0007$). Statistically significant differences in the prevalence of trematoda-type eggs were also found between PK (2.5%) and WM (66.7%)

	Nucleotide at position[b]			
Haplotype	n[a]	18	84	90
EbPL1	**4**	**C**	**T**	**T**
EbPL2	3	•	•	C
EbPL3	1	T	•	C
EbPL4	1	T	C	C

Table 3. Residue positions of single nucleotide polymorphisms in haplotypes EbPL2 to EbPL4 compared with haplotype EbPL1 of *E. boehmi* from fox faeces isolates from different regions of Poland. [a]A dot indicates that the nucleotide is the same as that in haplotype EbPL1. [b]Number of samples belonging to the indicated haplotype.

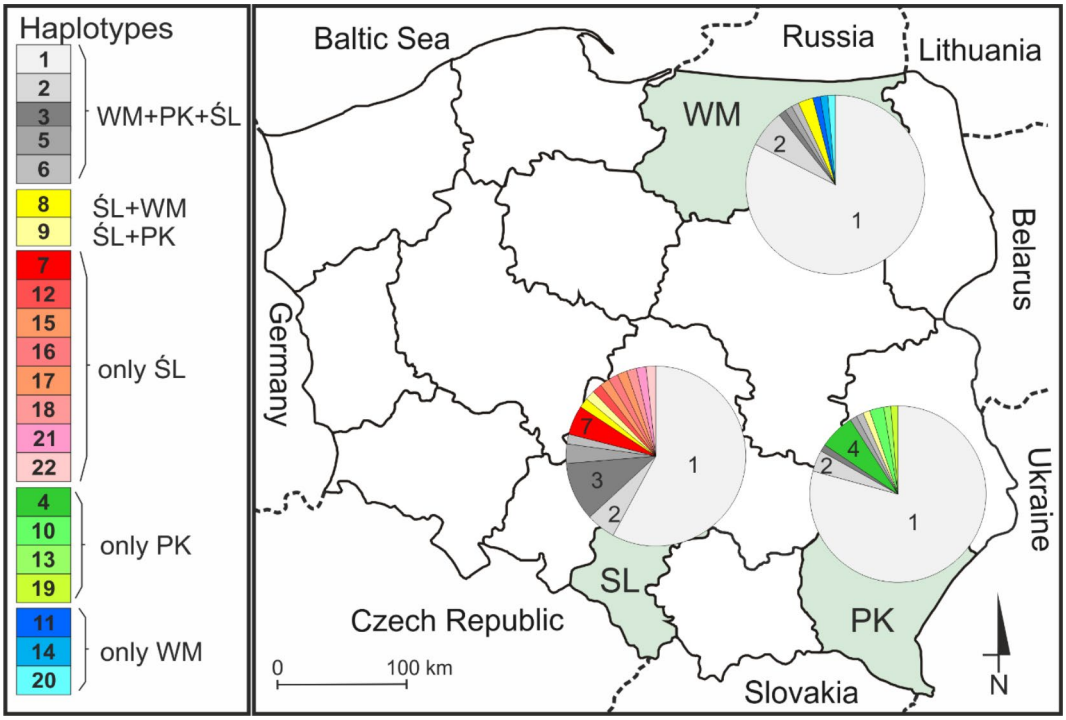

Fig. 2. Distribution of *E. aerophilus* haplotypes included in the investigation. The numbers (1–22) in the diagrams refer to the numbers of haplotypes (EaPL1-EaPL22); individual haplotypes and their distributions are marked in different colours. PK – Podkarpackie Province, ŚL – Śląskie Province, WM – Warmińsko-Mazurskie Province.

($P=0.00001$) and between ŚL (2.7%) and WM (66.7%) ($P=0.00001$). The remaining types of parasite eggs were found in relatively low percentages of the samples: *Trichuris vulpis*, 1.5%; *Mesocestoides* spp., 1.8%; and hookworms, 2.3%. The detailed results are presented in Table 6.

Comparison of seminested PCR and flotation methods

A comparison of the results concerning the prevalence of parasites obtained with seminested PCR and coproscopic examinations is presented in Table 7. For this analysis, the overall PCR results (*E. aerophilus* and *E. boehmi* combined) were considered. Despite the very similar percentages of positive results revealed by both methods, the correlation factor concerning the overall results showed a moderate positive correlation ($r=0.41$). There were 207 samples (60.5%) that yielded positive results via both methods. Almost 11% of the isolates produced positive results solely via PCR, and almost 13% produced positive results solely via flotation. The *r* value of the correlation factor concerning the overall results obtained with both methods (PCR and flotation).

Discussion

The present study focused on evaluating and comparing the prevalence of *E. aerophilus* in red fox populations from selected regions of Poland (Central Europe). To our knowledge, this is the first molecular study confirming the prevalence of *E. aerophilus* in Poland. The mean prevalence of *E. aerophilus* in all investigated areas estimated via PCR in this study was 67.3%. The highest prevalence was detected in Podkarpackie Province

E. aerophilus haplotypes	% of individual haplotypes			
	PK ($n=76$)	WM ($n=74$)	ŚL ($n=58$)	Total ($n=208$)
EaPL1 (PP838113)	78.9	82.4	58.6	74.5
EaPL2 (PP838114)	3.9	6.8	5.2	5.3
EaPL3 (PP838115)	1.3	1.4	10.3	3.8
EaPL4 (PP838116)	6.6	0.0	0.0	2.4
EaPL5 (PP838117)	1.3	1.4	3.4	1.9
EaPL6 (PP838118)	1.3	1.4	1.7	1.4
EaPL7 (PP838119)	0.0	0.0	5.2	1.4
EaPL8 (PP838120)	0.0	2.7	1.7	1.4
EaPL9 (PP838121)	1.3	0.0	1.7	1.0
EaPL10 (PP838122)	2.6	0.0	0.0	1.0
EaPL11 (PP838123)	0.0	1.4	0.0	0.5
EaPL12 (PP838124)	0.0	0.0	1.7	0.5
EaPL13 (PP838125)	1.3	0.0	0.0	0.5
EaPL14 (PP838126)	0.0	1.4	0.0	0.5
EaPL15 (PP838127)	0.0	0.0	1.7	0.5
EaPL16 (PP838128)	0.0	0.0	1.7	0.5
EaPL17 (PP838129)	0.0	0.0	1.7	0.5
EaPL18 (PP838130)	0.0	0.0	1.7	0.5
EaPL19 (PP838131)	1.3	0.0	0.0	0.5
EaPL20 (PP838132)	0.0	1.4	0.0	0.5
EaPL21 (PP838133)	0.0	0.0	1.7	0.5
EaPL22 (PP838134)	0.0	0.0	1.7	0.5

Table 4. Distributions of individual *E. aerophilus* haplotypes in the investigated areas of Poland.

E. boehmi haplotypes	% of individual haplotypes			
	PK ($n=1$)	WM ($n=6$)	ŚL ($n=2$)	Total ($n=9$)
EbPL1 (PP838136)	0.0	4.1	1.7	1.9
EbPL2 (PP838137)	1.3	1.4	1.7	1.4
EbPL3 (PP838138)	0.0	1.4	0.0	0.5
EbPL4 (PP838139)	0.0	1.4	0.0	0.5

Table 5. Distributions of individual *E. boehmi* haplotypes in the investigated areas in Poland.

(PK), and the lowest was detected in Śląskie Province (ŚL); however, there were no significant differences among provinces. Several studies from other countries have reported the presence of *E. aerophilus* in red foxes via postmortem lung examinations (to detect adult worms) or coproscopy (to detect eggs of this parasite in faeces). A systematic review and meta-analysis of available world data via a random effects model[10] revealed that the average prevalence of *E. aerophilus* based on lung examinations was 49.36% (95% CI 40.11–58.53) and that on the basis of coproscopy was 43% (95% CI 28.00–28.71). The prevalence in individual countries ranges from 0.5% to 97.1%[26–56]. Our results are mostly in agreement with those of studies conducted in Bosnia and Herzegovina (69.7%)[14], Canada (67-69%)[48,53], Germany (69%)[52], and Hungary (64%)[50]. In addition to the general analysis, some authors have reported significant differences between the prevalence rates determined by lung examinations and those determined by coproscopy. In their study, Al-Sabi et al.[55] reported 32% lower results in coprological examinations than in postmortem recoveries of this nematode from the lungs of foxes. On the other hand, in faecal examinations, Nevárez et al.[48] reported a 20% greater recovery of eggs from faeces than adult parasites from necropsies. In the another part of our investigations, we attempted to provide deeper insights into the genetic diversity of *E. aerophilus* from the investigated regions. We focused on the analysis of the sequence variations in the partial mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene of *E. aerophilus* extracted from the faeces of foxes. In Europe, several recent studies have focused on isolates of this parasite that were obtained from wild and companion animals[7,20–22], but in Poland, such research has not been conducted. This is the first description of the molecular characteristics and phylogenetic analysis of this nematode obtained in Poland.

The relatively large number of samples examined in this study resulted in 22 *E. aerophilus* haplotypes: EaPL1-EaPL22. The most frequent haplotype detected in our study was EaPL1; more than 73% of the positive *E. aerophilus* samples presented this haplotype, which corresponds to haplotype I from dogs and cats in Italy, as previously described by Di Cesare et al.[21]. In their study, the dominant haplotype I occurred in 74% of the

		Overall (all regions) ($n=342$)	Podkarpackie Province (PK) ($n=120$)	Śląskie Province (ŚL) ($n=111$)	Warmińsko-Mazurskie Province (WM) ($n=111$)
Capillariidae	% Pos	73.4	75.8[a, b]	62.2[a]	82.0[b]
	(95% CI)	(68.5–77.8)	(67.5–82.6)	(52.9–70.6)	(73.8–88.0)
	EPG	418.2	487.1	148.9	613.0
	(CV%)	1535.9	458.3	219.7	212.6
	Range	1-24000	1-24000	1-1950	1-9075
Taeniidae	% Pos	21.3	30.0[a]	10.8[b]	22.52 [a, b]
	(95% CI)	(17.3–26.0)	(22.5–38.7)	(6.3–18.0)	(15.7.-31.1)
	EPG	93.7	126.4	32.3	211.0
	(CV%)	469.0	477.9	538.2	130.5
	Range	1-4605	1-4605	1-1500	1-1095
Toxocara spp.	% Pos	26.3	23.3	20.7	35.1
	(95% CI)	(21.9–31.2)	(16.7–31.7)	(14.2–29.2)	(26.9–44.4)
	EPG	101.1	69.8	45.2	356.7
	(CV%)	277.6	364.4	421.6	116.0
	Range	1-2400	1-2400	1-1470	1-1875
Toxascaris leonina	% Pos	5.3	1.7[a]	0.9[a]	13.5[b]
	(95% CI)	(3.4–8.2)	(0.5–5.9)	(0.2–4.9)	(8.4–21.1)
	EPG	102.8	68.1	0.7	1136.0
	(CV%)	955.2	1026.9	1053.6	298.2
	Range	1-13350	1-7650	1–75	1-13350
Trichuris vulpis	% Pos	1.5	0.0	0.9	3.6
	(95% CI)	(0.6–3.4)	(0-3.1)	(0.2–4.9)	(1.4–8.9)
	EPG	1.0	-	0.1	48.8
	(CV%)	843.0	-	1053.6	88.4
	Range	0-105	-	1–15	1-105
Trematoda	% Pos	23.4	2.5[a]	2.7[a]	66.7[b]
	(95% CI)	(19.2–28.2)	(0.9–7.1)	(0.9–7.6)	(57.5–74.8)
	EPG	64.2	2.1	4.4	255.2
	(CV%)	475.2	761.8	906.2	227.4
	Range	1-4080	1-165	1-420	1-4080
Hookworms	% Pos	2.3	1.7	4.5	0.9
	(95% CI)	(1.2–4.6)	0.5–5.9	(1.9–10.1)	(0.2–4.9)
	EPG	13.6	13.0	11.5	-
	(CV%)	860.8	1084.9	703.2	-
	Range	0-1545	1-1545	1-750	-
Mesocestoides spp.	% Pos	1.8	0.0	0.0	5.4
	(95% CI)	(0.8–3.77)	(0-3.1)	(0-3.3)	(2.5–11.3)
	EPG	76.2	-	-	3021.7
	(CV%)	998.9	-	-	134.8
	Range	0-11200	-	-	1-11200

Table 6. Results of the faecal examinations of red foxes in selected regions of Poland. [a, b] Different superscript letters indicate statistically significant differences in the prevalence of parasites estimated by flotation among different regions of Poland ($P<0.05$). *CI*, confidence interval; EPG, eggs per gram; *CV*, coefficient of variation.

positive samples. Furthermore, the alignment of this 256-bp fragment of the *cox*1 gene was also 100% similar to that of haplotype IX, which was retrieved from a fox in Italy[7], and that of haplotype XIII, which was obtained from a Portuguese fox[7] (both trimmed to the 256-bp fragment). The second most abundant haplotype is EaPL2 (almost 5%), which corresponds to haplotype X obtained from foxes from Romania and beech martens from Portugal[7]. The EaPL3-6 and EaPL10 haplotypes correspond to *E. aerophilus* isolates extracted from dogs, cats and foxes in Italy and Switzerland (Fig. 1)[21,22]. Seven Polish haplotypes correspond to haplotypes that have been discovered in other European countries[7,14,21]. However, it should be emphasized that the remaining 15 haplotypes were identified only in Poland, which suggests a tendency for genetic diversity related to geographical location. This dependence is visible even among regions of Poland (Fig. 2). The dominant EaPL1 haplotype (also the most common in Europe[14,21]) was detected significantly less frequently in southwestern Poland (ŚL) than in the north eastern and south eastern parts of the country (WM, PK). The opposite was the case for the EaPL4 haplotype, which was significantly more common in ŚL. Numerous haplotypes occurring only in

	Overall (all regions) ($n = 342$)	
	No. of positive samples	% of positive samples
DNA of *Eucoleus* spp. (PCR)	244	71.3
Capillariidae eggs (flotation)	251	73.4
PCR (+) and flotation (+)	207	60.5
PCR (+) and flotation (-)	37	10.8
PCR (-) and flotation (+)	44	12.9

Table 7. Prevalence rates (%) of *Eucoleus* spp. in red foxes in different regions of Poland: comparison of results obtained by PCR (including sequencing results) and flotation. $r = 0.41$

individual provinces were also identified, most of which were found in ŚL. Geographical barriers are likely the main cause of parasite segregation and limited evolution of isolated genetic populations[7]. Moreover, the geographical distribution of individual parasite haplotypes determines the movement of their hosts. Poland, owing to its geographical location, is an interesting area in this respect, where there is a combined impact of migrating animals from the east (from Asia) as well as from the west and south of Europe. Such a dependence in the haplotype distribution was observed, for example, in the case of another parasite common in red foxes (*Echinococcus multilocularis*), including the detection of a haplotype characteristic of the Asian clade[57,58].

Moreover, some studies from other countries[7,14,21] confirmed the presence of the same subpopulations of *E. aerophilus* in both wild and domestic animals from the same areas. To obtain more knowledge about the distribution and migration of *E. aerophilus* populations in wild and domestic animals, further studies with more samples from Europe are needed.

E. boehmi is the causative agent of nasal capillariosis and infects the upper respiratory airways of wild and domestic canids. Knowledge of the geographic distribution of this nematode is scarce and incomplete, although infections have been reported previously in Europe and North America[14,19,20]. As mentioned before, during coprological examinations, this nematode is often misdiagnosed as eggs of the closely related species, *E. aerophilus* and *Trichuris vulpis*[4]. Furthermore, mixed infections with all of the above-mentioned parasites may occur more frequently than expected, especially where these parasites are endemic[20,21]. In our study, positive cases of *E. boehmi* were detected by testing samples with seminested PCR for *E. aerophilus*. A comparison of the sequencing results with the GenBank database revealed 14 amplicon (4.1% of all examined samples) sequences of *E. boehmi*. This may be due to the close relationship of both species, so cross-reactions cannot be excluded. Our results are in line with those obtained in Hungary, where 5% of the examined foxes were positive. On the other hand, in other countries, the percentages of infected foxes were much greater: Serbia (90%)[44], Bosnia and Herzegovina (64.6%)[14], Denmark (71%)[55], Norway (51%)[29] and Italy (30.7%)[11]. Such prevalence discrepancies may be due to differences in environmental conditions, as well as the applied technique[14]. Significantly more effective is looking for the parasites in the predilection sites (necropsy) than using indirect diagnostic methods (like faeces examination). Therefore, investigation applied necropsy in Serbia gave such high prevalence (90%)[44]. However, the scarce information on the biological characteristics of *E. boehmi* does not allow any specific conclusions to be drawn on this issue[11].

The most prevalent haplotype of *E. boehmi* (EbPL1) (from four identified haplotypes) corresponds to isolates described in Bosnia and Herzegovina (red fox)[14] and in Italy (dog)[20]. This haplotype was present in WM and ŚL, whereas EbPL2 occurred in all investigated areas. On the other hand, the haplotype EbPL3-4 appeared exclusively in the WM area. In the case of *E. aerophilus*, this investigation revealed that some subpopulations of *E. boehmi* can infect pets as well as wild animals, but further studies are needed.

Coproscopic examinations revealed that eggs of the Capillariidae family were most prevalent in all regions, with a mean prevalence of 73.4% (95% CI 68.5–77.8). As previously mentioned, owing to the overlapping morphological similarities between the eggs within this group (*E. aerophilus and E. boehmi);* they were analysed together in this study. Coprological examinations may also be difficult to conduct when mixed infections with *T. vulpis* occur[21]. Our results corresponded to those obtained previously[35,36], where the percentages of positive samples were greater than 76% and 78%, respectively. Moreover, the geographical locations of the areas from which samples were collected in the above-mentioned papers[35,36] bordered on or were the same area studied in this article. In our investigation, there were statistically significant differences in the prevalence of Capillariidae-type eggs between ŚL (62.2%) and WM (82.0%). Analysis of the percentages of the prevalence of other detected parasites also revealed statistically significant differences, as in the case of Taeniidae-type eggs, between PK (30.0%) and ŚL (10.8%). In other studies conducted in Poland, the percentages of Taeniidae-positive foxes ranged between 22.2% and 42.5% [35,36,59,60]. Analysis of *Toxascaris leonina* revealed statistically significant differences between PK (1.7%) and WM (13.5%) and between ŚL (0.9%) and WM (13.5%). The low prevalence rates reported in our studies correspond to those reported in western Poland (0.9%)[60] and in Germany[28]. On the other hand, a prevalence rate similar to that obtained in WM was recorded in the area of Berlin (12.0%)[61]. A statistically significant difference in the prevalence of trematoda-type eggs was also detected between PK (2.5%) and WM (66.7%) and between ŚL (2.7%) and WM (66.7%). Our results are in agreement with those obtained by Balicka-Ramisz et al.[60]. These findings indicate a heterogeneous distribution of these parasites in the investigated areas. It is believed that the environmental conditions in specific regions and access to intermediate hosts contribute to the parasite distributions[35,60]. Furthermore, it is supposed that a high percentage of flukes corresponds to the presence of large areas of water reservoirs[35,36,38].

A comparison of the results concerning the overall prevalence of *Eucoleus* spp. (and Capillariidae) obtained via seminested PCR and coproscopic examinations revealed a moderate positive correlation ($r=0.41$). Despite very similar percentages of positive results obtained via PCR and flotation (71.3% and 73.4%, respectively), only 61.7% of the tested foxes yielded positive results via both methods. The possible reason why 10% of the samples produced positive results only via PCR is that flotation is not perfectly effective in recovering parasite eggs in faeces[62]. Additionally, such a situation can be explained by damage to eggs during freezing and thawing before testing[63] or by irregular patterns of egg excretion[8]. On the other hand, 12% of the samples yielded positive results only during flotation. Freezing and thawing may degrade DNA from faeces, which has a negative effect on the sensitivity of further molecular analysis, especially when there are no or few parasite eggs in the sample[23]. Furthermore, false-negative results may occur when eggs are present in the stool but may contain too little DNA for efficient amplification [64,65].

Conclusions

The present study confirmed the high prevalence (67%) of *E. aerophilus* in red foxes from selected regions in Poland. To the best of our knowledge, this is the first molecular study on *E. aerophilus* in Poland. Analysis of the sequence variations in the partial cox1 gene revealed 22 haplotypes, with one dominant haplotype (EaPL1) occurring in 73% of the positive samples. Additionally, *E. boehmi* was detected in 4% of the red foxes. The high prevalence of *E. aerophilus* in red foxes should be considered when assessing the risk of transmission of this infection to domestic animals (e.g., dogs and cats) and to humans (it is a potentially zoonotic parasite). Therefore, it is advisable to continue investigating this parasite to assess its occurrence in Poland.

Data availability

Sequence data that support the findings of this study have been deposited in the GenBank of National Center for Biotechnology Information (NCBI) under accession numbers PP838113-PP838134 and PP838136-PP838139.

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Author contributions
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Declarations

Competing interests
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