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Molecular Evidence of the Role of the Red Fox (*Vulpes vulpes*) in the Epidemiology of Ungulate-Related *Sarcocystis* Species in Croatia, Lithuania, and Portugal

Naglis Gudiškis [1], Petras Prakas [1], Relja Beck [2], Ana Figueiredo [3], Evelina Juozaitytė-Ngugu [1], Linas Balčiauskas [1], Rafael Calero-Bernal [4], Ema Gagović [2], Rita T. Torres [3], Dário Hipólito [3,5], David Carmena [6,7], Vitalijus Stirkė [1] and Dalius Butkauskas [1,*]

1 State Scientific Research Institute Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania; naglis.gudiskis@gamtc.lt (N.G.); prakaspetras@gmail.com (P.P.); evelina.ngugu@gamtc.lt (E.J.-N.); linas.balciauskas@gamtc.lt (L.B.)
2 Laboratory for Parasitology, Department of Microbiology and Parasitology, Croatian Veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia; gagovic@veinst.hr (E.G.)
3 CESAM & Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal; anamfigueiredo@ua.pt (A.F.); dhipolito@ua.pt (D.H.)
4 SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Puerta de Hierro s/n, 28040 Madrid, Spain
5 Department of Biology, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia
6 Centro Nacional de Microbiologia, Laboratorio de Referencia e Investigación en Parasitología, Ctra. de Pozuelo, 28, 28222 Majadahonda, Spain
7 Centro de Investigación Biomédica en Red, Instituto de Salud Carlos III, Av. Monforte de Lemos 3-5, Pabellón 11, Planta 0, 28029 Madrid, Spain
* Correspondence: dalius.butkauskas@gamtc.lt; Tel.: +370-68-326-793

Simple Summary

Sarcocystis species are globally distributed protozoan parasites with a complex life cycle that requires two hosts. Sarcocysts develop mainly in the muscle tissues of intermediate hosts (prey), whereas oocysts and sporocysts occur in the intestinal tract of definitive hosts (predators and scavengers). One of the most widespread canid predators, the red fox (*Vulpes vulpes*), commonly acts as a definitive host, while various ungulates serve as intermediate hosts. This study investigated the diversity and prevalence of *Sarcocystis* species in red foxes from three European countries: Croatia, Lithuania, and Portugal. Overall, 164 faecal samples were analysed using molecular methods and identified using Sanger sequencing. A total of twelve *Sarcocystis* species were identified in the examined foxes, all using ungulates as intermediate hosts, with four species reported for the first time in the red fox as a definitive host. Genetic analyses indicated high similarity among detected parasites and no clear geographic structuring. These findings confirm the red fox as an important natural definitive host for multiple *Sarcocystis* species and highlight its role in the transmission of these parasites across different European ecosystems.

Abstract

Sarcocystis spp. (Apicomplexa: Sarcocystidae) are globally distributed protozoan parasites with an obligatory two-host prey–predator life cycle involving intermediate (IHs) and definitive hosts (DHs). Canids, including the red fox (*Vulpes vulpes*), often serve as DHs for species infecting various ungulates. This study identified *Sarcocystis* species in red foxes from Croatia, Lithuania, and Portugal. Between 2021 and 2024, 164 faecal samples (80 from Croatia, 50 from Portugal, and 34 from Lithuania) were analysed using nested PCR targeting the *cox1* gene. Twelve *Sarcocystis* species were detected: *S. arieticanis*, *S. capracanis*,

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S. capreolicanis, *S. cruzi*, *S. gracilis*, *S. hjorti*, *S. iberica*, *S. linearis*, *S. miescheriana*, *S. morae*, *S. rossii*, and *S. tenella*. The overall prevalence was highest in Croatia (78%) and Lithuania (62%) and lowest in Portugal (30%). Phylogenetic and haplotype analyses revealed high homogeneity and absence of geographic structuring. These results confirm the red fox as a key DH for multiple *Sarcocystis* species infecting European ungulates and underscore its epidemiological importance in parasite transmission across diverse ecosystems.

Keywords: *Vulpes vulpes*; *Sarcocystis* spp.; definitive host; molecular identification; *cox1*; epidemiology

1. Introduction

Parasites of the genus *Sarcocystis* (family Sarcocystidae) are globally distributed protozoan parasites [1], with a complex two-host prey–predator life cycle [2]. The cycle begins when the definitive host (DH) consumes tissues infected with sarcocysts. Within the intestinal epithelium of the DH, the parasite undergoes sexual reproduction, resulting in the formation of oocysts that sporulate in the gut and may also excyst, releasing the sporocysts. These are shed in faeces and contaminate the environment, including water and forage. The intermediate host (IH) becomes infected by ingesting sporocysts from contaminated sources. Once inside the IH, *Sarcocystis* parasites invade endothelial cells and undergo asexual multiplication. This process results in the development of mature sarcocysts within muscle or nervous tissues, completing the life cycle [3]. To date, more than 200 recognised *Sarcocystis* species have been described, ranging from highly host-specific to broadly host-adapted [4,5].

The severity of sarcocystosis varies between IHs and DHs, with infections in DHs typically being asymptomatic [3]. In IHs, both wildlife and livestock, the infection can cause fever, weight loss, neurological symptoms, or even death, leading to significant economic losses in livestock due to reduced meat quality and carcass condemnation [6,7]. While many *Sarcocystis* species are mildly pathogenic, acute infections by *Sarcocystis arieticanis*, *Sarcocystis capracanis*, *Sarcocystis cruzi*, and *Sarcocystis tenella* in livestock can result in reproductive failure [8–10]. Zoonotic *Sarcocystis* species can infect humans either as DHs through the consumption of undercooked meat or as IHs after ingestion of oocysts or sporocysts [3,7].

The red fox (*Vulpes vulpes*) (family Canidae) is widely distributed across the Northern Hemisphere, being the most abundant medium-sized carnivore in Lithuania, alongside the invasive raccoon dog (*Nyctereutes procyonoides*) in Portugal, along with the European badger (*Meles meles*), common genet (*Genetta genetta*), and Egyptian mongoose (*Herpestes ichneumon*), and in Croatia, along with the golden jackal (*Canis aureus*) [11,12]. In the above-mentioned countries, the number of red foxes has decreased over the last decade [12–15], a trend observed across much of Western Europe. This canid occupies a wide range of habitats all over the globe [16], from forests, farmland, and tundra to even semi-arid deserts and mountainous regions [17]. Since the 1930s, red foxes have begun colonising urban areas [18], and their population density in cities is currently increasing.

Red foxes are opportunistic omnivores [16], whose diets include small- to medium-sized mammals, birds, amphibians, reptiles, fish, insects, earthworms, fruits, seeds, carrion, and garbage [19]. They can kill prey as large as roe deer fawns, with this item being most important during spring and summer [20], but their diet usually consists of rodents, lagomorphs, and ungulate carrion [21]. Sympatry with other mesopredators or apex predators likely influences red fox access to food, such as carrion. For instance, following

the recolonisation of Sweden by the Eurasian lynx (*Lynx lynx*), red foxes increased their consumption of European roe deer. This shift was primarily due to the greater availability of lynx-killed carcasses, which allowed red foxes to replace less preferred food items, thereby narrowing their winter food niche [22].

A red fox's parasite community is shaped by both prey availability and its geographical setting. Numerous studies in Europe have demonstrated that red foxes can serve as a reservoir for zoonotic helminth species [23–25]. Due to its predatory nature, the red fox typically serves as a DH for *Sarcocystis* spp. However, the detection of two *Sarcocystis* species, *Sarcocystis arctica* and *Sarcocystis lutrae*, in red fox muscle tissue indicates that these animals can also act as IHs for certain *Sarcocystis* species [26,27]. To date, transmission experiments and molecular analysis have shown that the red fox serves as the DH for at least 14 species of *Sarcocystis*, including *Sarcocystis alces*, *S. arieticanis*, *S. capracanis*, *Sarcocystis capreolicanis*, *S. cruzi*, *Sarcocystis gracilis*, *Sarcocystis grueneri*, *Sarcocystis hjorti*, *Sarcocystis miescheriana*, *Sarcocystis pilosa*, *S. tenella*, *Sarcocystis tarandivulpes*, *Sarcocystis rangi*, and *Sarcocystis rileyi* [28–34]. Notably, apart from the Eurasian wolf (*Canis lupus lupus*), no other wild mammal has been confirmed to serve as the DH for such a high number of *Sarcocystis* species, which host at least 25 *Sarcocystis* spp. [35]. Furthermore, all the above-listed species, except *S. rileyi*, utilise domestic ungulates or cervids as their IHs [3]. Another important consideration is that, despite red foxes' dietary diversity, the observed bias in *Sarcocystis* species diversity is largely attributable to research focusing on species infecting animals used for human consumption, driven primarily by food safety concerns and the commercial importance of animal husbandry. Consequently, only three molecular studies in Europe have investigated the role of red foxes as DHs of *Sarcocystis* spp. using molecular techniques [31,32,34]. This suggests that red foxes, along with other Canidae, such as grey wolves and raccoon dogs [31,36], may play a key role in the environmental transmission of certain *Sarcocystis* species. However, molecular identification studies of *Sarcocystis* spp. in faeces or intestine scrapings of red foxes remain scarce.

Accordingly, this study aimed to identify *Sarcocystis* species using ungulates, including cervids, as IHs for which the red fox may potentially serve as the DH. To achieve this, we molecularly analysed faecal samples from red foxes collected from three ecologically and geographically distinct European regions: Croatia (Balkan region), Lithuania (Baltic region), and Portugal (Iberian Peninsula).

2. Materials and Methods

2.1. Workflow of the Study, Geographical and Ecological Context of Sample Collection

The overall workflow of this research is presented in more detail in Figure 1. Notably, faecal samples in Lithuania were specifically collected to investigate *Sarcocystis* spp., while in Croatia, the material was obtained as part of rabies control efforts. In Portugal, the purpose of sample collection was to identify other enteric microeukaryotes (*Giardia duodenalis*, *Cryptosporidium* spp., and *Enterocytozoon bieneusi*) [37,38]. Consequently, methodological uniformity was not maintained across the three countries studied.

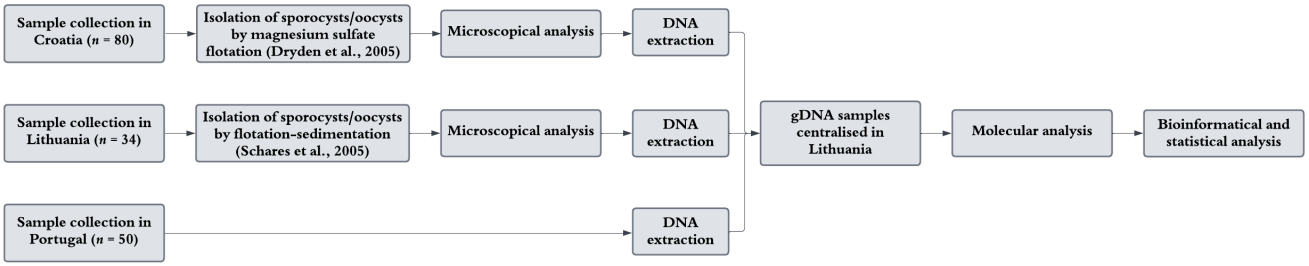

Figure 1. Workflow of the analysis of red fox faecal samples from Croatia, Lithuania, and Portugal.

A total of 164 red fox faecal samples were collected across three European countries between 2021 and 2024: 80 from Croatia, 50 from Portugal, and 34 from Lithuania. The precise geographic locations of sample collection are presented in Figure 2.

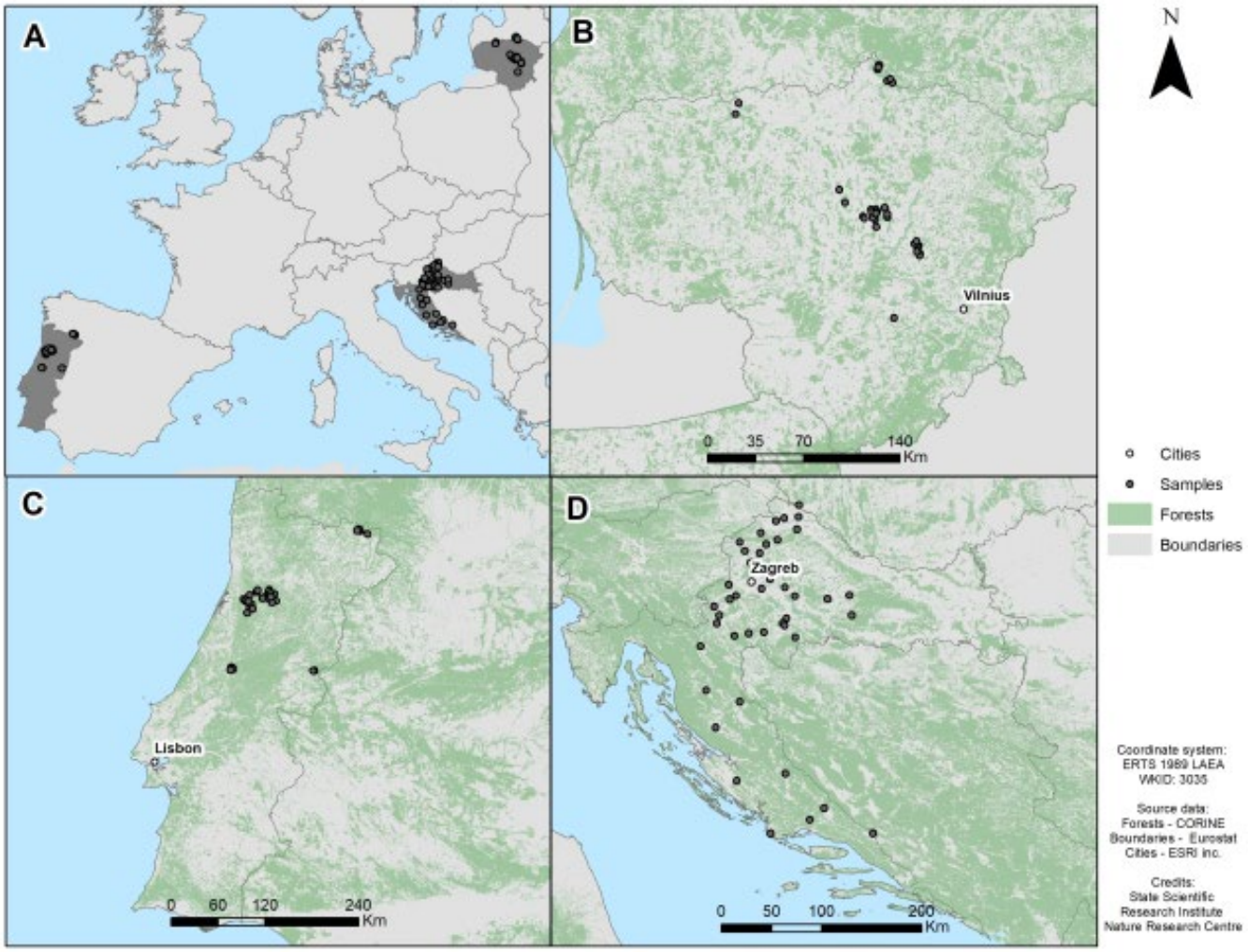

Figure 2. Map showing the locations of red fox faeces sampling sites across three European countries. (**A**) An overview of Europe with all sampling locations marked; (**B**) a detailed view of sampling sites in Lithuania, (**C**) Portugal, and (**D**) Croatia.

The ecological context of interactions between red foxes and *Sarcocystis* spp. varies across Europe, influenced by regional differences in climate, biodiversity, land use, and predator–prey dynamics [39]. The Baltic region, exemplified by Lithuania, has a temperate continental climate characterised by cold winters, diverse forests, and high densities of wild herbivores, conditions that are conducive to *Sarcocystis* transmission. The Balkan region, represented by Croatia, exhibits a transitional climate from continental to Mediterranean, with rugged terrain, patchy forests, and traditional pastoral farming practices. These factors contribute to complex wildlife–livestock interactions affecting parasite transmission. Portugal, located on the Iberian Peninsula, experiences a predominantly Mediterranean climate characterised by hot, dry summers and mild, wet winters, with an Atlantic influence and higher precipitation rates near the coast. The Iberian landscape features extensive livestock grazing, fragmented habitats, and considerable biodiversity [40,41].

2.2. Processing Stool Samples

Lithuanian red fox samples underwent prior processing for microscopic analysis. Sporocysts and oocysts of *Sarcocystis* spp. were isolated using a modified flotation–sedimentation protocol based on Schares et al. [42]. Faecal samples were homogenised in a ratio of 10 g faeces to 50 mL deionised water. After an initial settling period, the mixture was thoroughly homogenised, passed through a sieve, and the residue on the sieve was rinsed with deionised water to recover the remaining homogenate. The supernatant was allowed to settle for at least 30 min to facilitate the concentration of oocysts and sporocysts and was subsequently left undisturbed overnight (12–24 h) to maximise sedimentation. Following this, the supernatant was carefully removed to avoid disturbing the pellet, which was then resuspended in approximately 30 mL of deionised water and divided into two 50 mL centrifuge tubes. The resuspended sediments were then evaluated for the presence of oocysts/sporocysts under a Nikon ECLIPSE 80i (Nikon Corp., Tokyo, Japan). Afterwards, each portion of the precipitate was combined with 40 mL of saturated sugar solution and centrifuged at 1600× *g* for 10 min. The flotation layer (~15 mL) from each tube was collected, pooled into a single 50 mL centrifuge tube, and topped up with deionised water. This suspension was centrifuged at 1600× *g* for 8 min without braking. Up to 45 mL of the supernatant was removed, leaving only the precipitate. The precipitate was resuspended in 50 mL of deionised water, and the washing process (centrifugation, supernatant removal, and resuspension) was repeated three more times for a total of four washes. The final precipitate was used for gDNA extraction using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania).

Additional pre-processing was applied to the Croatian samples. Three grams of faeces were processed by centrifugal flotation using magnesium sulfate ($MgSO_4$; specific gravity 1.20), following the procedure described by Dryden et al. [43]. Samples were examined and photographed using an Imager M.2 microscope (Zeiss, Jena, Germany). Faecal samples from Croatia were processed by extracting DNA from 220 mg of faecal material using the NucleoSpin DNA Stool Kit (Macherey-Nagel, Düren, Germany), while for the Portuguese samples, 200 mg of faeces was used for DNA isolation with the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. Following the gDNA extraction from all collected stool samples, the samples were shipped to the State Scientific Research Institute Nature Research Centre in Vilnius, Lithuania, for further molecular analyses.

2.3. Molecular Analyses

Detection of *Sarcocystis* species was performed by amplifying the cytochrome c oxidase subunit I (*cox1*) through a nested PCR (nPCR) approach. All samples were analyzed using the nPCR method, regardless of whether they had been identified as positive by microscopic examination. The primer pairs employed for the first (genus-specific) and second (species-specific) amplification steps are detailed in Table 1. In this study, procedures for expected *Sarcocystis* species detection were selected based on previous research, which identified them as the most prevalent in Bovidae, Cervidae, and Suidae in Europe, with canids serving as their DHs. Specifically, *S. arieticanis* and *S. tenella* form sarcocysts in muscles of sheep (*Ovis aries*), *Sarcocystis bertrami* parasitizes horses (*Equus ferus caballus*), *S. capracanis* is the most common species in goats (*Capra hircus*), *S. cruzi* infects cattle (*Bos taurus*), *S. miescheriana* is the dominant *Sarcocystis* species in pigs and wild boar (*Sus scrofa*) [3], *S. capreolicanis* and *S. gracilis* are specific to the roe deer (*Capreolus capreolus*), *Sarcocystis venatoria* has been detected in red deer (*Cervus elaphus*), while *S. hjorti*, *Sarcocystis iberica*, *Sarcocystis linearis*, *Sarcocystis morae* and *Sarcocystis taeniata* infect several different cervid species [44].

Table 1. Primer pairs used for the amplification of target *Sarcocystis* species.

Sarcocystis Species	Primer Name	Sequence 5′-3′	Fragment Length, bp	IH [a]
Sarcocystis spp.	SF1 [b] SsunR3 [c]	ATGGCGTACAACAATCATAAAGAA CCGTTGGWATGGCRATCAT	913	-
S. arieticanis	Arieticanis7F [d] Arieticanis7R [d]	TAATTTCCTCGGTACTGTACTGTTTG TACTTACGCATTGCGATATTACG	441	Bovidae
S. bertrami	V2ber7 [e] V2ber8 [e]	CCCCACTCAGTACGAACTCC ACTGCGATATAACTCCAAAACCA	381	
S. capracanis	V2ca3 [d] V2ca4 [d]	ATACCGATCTTTACGGGAGCAGTA GGTCACCGCAGAGAAGTACGAT	330	
S. cruzi	V2cr7 [f] V2cr8 [f]	CAATGTGCTGTTTACGCTCCA TCGTACAGGCCCGTAGTTAG	501	
S. tenella	Tenella8F [d] Tenella8R [d]	ATACCGCTCTACGCTGGATCTAC AACCATCGTACAATCCAAAACTAAA	421	
S. capreolicanis	V2capreo1 [f] V2capreo2 [f]	CATCGTAGAGCCCCGTACTC ACCGCTATACGCTGGAGCTG	416	Cervidae
S. gracilis	V2gr9 [f] V2gr10 [f]	GTGCTCGGGGCAGTGAAC GCCAGTAGTCATCATGTGGTGT	410	
S. hjorti	V2hjo1 [f] V2hjo2 [f]	AAGGTACACGGCATTGTTCAC GAAAACTACCCTGCCGCCTA	268	
S. iberica/*S. venatoria*	V2ibeven1 [f] V2ibeven2 [f]	ATGGGCCATTATATTTACTGCTCTG GCCGCCAAAAACTACCTTACC	252	
S. linearis/*S. taeniata*	V2taelin1 [f] V2taelin2 [f]	CGTAGACTGCATGACGACTTACAA CAAAGATGGATTTGCTGCCTA	662	
S. morae	V2mor1 [d] V2mor2 [d]	GTGTGCTTGGATCGGTCAAC GCCGAATACCGGCTTACTTC	332	
S. miescheriana	V2mie5 [e] V2mie6 [e]	TCCTCGGTATTAGCAGCGTACTG ATTGAAGGGCCACCAAACAC	358	Suidae

[a] IH: The family of intermediate hosts of certain *Sarcocystis* species, [b] [45], [c] [46], [d] [5], [e] [47], [f] [44].

The first round of nPCR was performed in a total reaction volume of 25 µL, containing 12.5 µL of DreamTaq PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania), 0.5 µM of each primer (forward and reverse), 4 µL of extracted genomic DNA (gDNA), and nuclease-free water to reach the final volume. The thermal cycling program began with an initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 35 s, primer annealing at 55–68 °C (depending on the primer pair, Table 1) for 45 s, and extension at 72 °C for 55 s. A final elongation step at 72 °C for 5 min concluded the reaction. The second amplification round was set up in a similar 25 µL reaction, which included 12.5 µL of DreamTaq PCR Master Mix, 0.5 µM of each primer, 2 µL of the first-round PCR product, and nuclease-free water to adjust the final volume. The cycling parameters for the second round were identical to those used in the initial amplification. Positive and negative controls were incorporated in each run to verify the amplification reliability. Positive controls were prepared with gDNA extracted from sarcocysts of each of the examined *Sarcocystis* species, as confirmed by Sanger sequencing in our earlier studies, while negative controls contained only nuclease-free water instead of DNA.

Further, the quality of the amplified products was verified by electrophoresis on a 1% agarose gel. Successfully amplified fragments were purified using ExoI and FastAP enzymes (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to the manufacturer's instructions. Sequencing was conducted using the same primers as in the nPCR reactions. The sequencing reactions employed the BigDye® Terminator v3.1 Cycle Sequencing Kit and were analysed on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following standard protocols. The resulting sequences were manually inspected for accuracy to ensure that no double peaks or ambiguous signals were present.

2.4. Bioinformatical Analysis

To assess both intraspecific and interspecific genetic similarities, *cox1* gene sequences obtained in this study were compared with those of closely related *Sarcocystis* species using the Nucleotide BLAST version 2.17.0 [48] tool.

Several main parameters of intraspecific genetic variability, including the number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π), as well as the standard deviations (SD) of the latter two indices, were calculated using DnaSP v.6 software [49].

Phylogenetic analyses were carried out using MEGA version 11.0.13 software [50]. Sequence alignments were generated employing the MUSCLE algorithm implemented within MEGA. The aligned sequences exhibited exclusively nucleotide substitutions. Phylogenetic relationships among *Sarcocystis* species were inferred using the maximum likelihood (ML) method. Evolutionary model selection using MEGA's "Find Best DNA/Protein Models (ML)" function identified the Kimura 2-parameter model with a gamma distribution and proportion of invariable sites (K2+G+I) as the most appropriate for all datasets. The robustness of the resulting phylogenetic trees was evaluated via bootstrap analysis with 1000 replicates. The haplotype network was constructed using the median-joining (MJ) method implemented in NETWORK 10.2.0.0 software [51].

2.5. Statistical Analysis

We calculated the prevalence estimates and 95% confidence intervals (CIs) for each *Sarcocystis* species identified [52,53]. We also calculated the overall prevalence and 95% CI for all parasite species found in the investigated countries. Parasite diversity was characterised by *Sarcocystis* species richness, Shannon's diversity index H (log base e), and Simpson's dominance index c., and their upper and lower values with 95% CI calculated with bootstrap and 1000 replications. *Sarcocystis* diversity estimates were done in PAST version 5.0.2 [54].

3. Results

3.1. Microscopical Detection of Sarcocystis spp.

Sarcocystis spp. sporocysts were observed in the faeces of red foxes in both Croatian and Lithuanian samples under light microscopy (Figure 3). These parasite forms were identified in 7 out of 42 samples (16.7%) from Lithuania, and in only 1 out of 80 samples (1.3%) from Croatia. The sporocysts observed in Lithuania (Figure 3a) were thin-walled and measured 10.9–19.2 × 7.1–12.0 µm (14.8 ± 2.6 × 9.5 ± 1.6 µm, n = 90). In contrast, the sporocyst found in the single Croatian sample (Figure 3b) measured 12.4–13.2 × 8.2–8.4 µm.

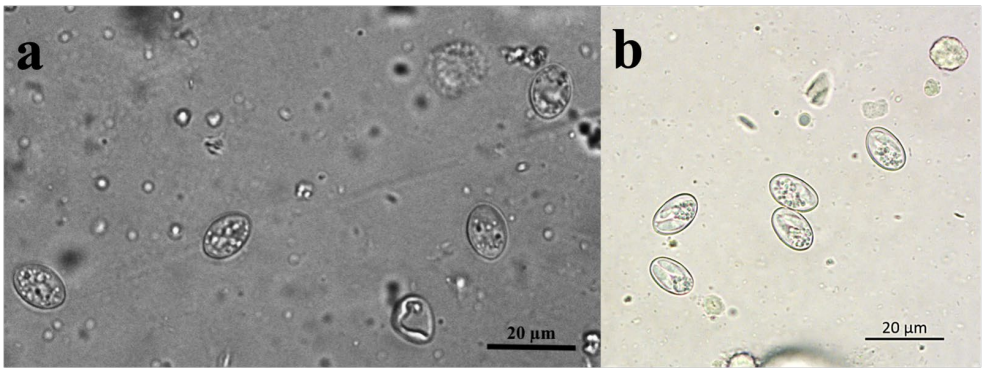

Figure 3. Sporocysts of *Sarcocystis* spp. from faecal samples of red fox. (**a**) Lithuania, (**b**) Croatia.

3.2. Distribution of Sarcocystis spp. in Three Countries Examined

The comparison of *cox1* sequences obtained revealed the presence of 12 *Sarcocystis* species: *S. arieticanis*, *S. tenella*, *S. capracanis*, *S. rossii*, *S. cruzi*, *S. miescheriana*, *S. capreolicanis*,

S. gracilis, *S. hjorti*, *S. iberica*, *S. morae*, and *S. linearis*. On the contrary, no amplicons were generated using V2ber7/V2ber8 primers in silico, theoretically designed for the identification of *Sarcocystis* spp. in equids. Using V2taelin1/V2taelin2 and V2ibeven1/V2ibeven2 primers in silico designed to amplify in the first case either *S. linearis* or *S. taeniata*, and in the second case to amplify either *S. iberica* or *S. venatoria*, we have identified *S. iberica* and *S. linearis*. Furthermore, using V2taelin1/V2taelin2 primer pair on samples from Croatia, we obtained four sequences, three of which were assigned to *S. linearis* and one to *S. rossii*. In all other cases, the primers were specific to a single *Sarcocystis* species.

The occurrence of various *Sarcocystis* species was similar across all countries (Table 2). The only significant difference was found in *S. tenella*, which had a prevalence several times higher in Lithuania than in Croatia (G = 3.80, $p < 0.05$).

Table 2. Frequency (%) of *Sarcocystis* species identified in faecal specimens of red foxes in Croatia, Lithuania, and Portugal. 95% CI presented in parentheses.

	S. arieticanis	*S. tenella*	*S. capracanis*	*S. rossii*	*S. cruzi*	*S. miescheriana*	*S. capreolicanis*	*S. gracilis*	*S. hjorti*	*S. iberica*	*S. linearis*	*S. morae*
Croatia	0.15 (0.08–0.25)	0.03 (0.00–0.06)	0.05 (0.01–0.12)	-	0.03 (0.00–0.06)	0.01 (0.00–0.05)	0.16 (0.09–0.26)	0.04 (0.01–0.11)	0.04 (0.01–0.11)	0.14 (0.07–0.23)	0.05 (0.01–0.12)	0.09 (0.04–0.17)
Lithuania	*NA*	0.15 (0.05–0.31)	*NA*	*NA*	*NA*	*NA*	0.12 (0.03–0.27)	0.06 (0.01–0.20)	*NA*	0.15 (0.05–0.31)	0.15 (0.05–0.31)	*NA*
Portugal	*NA*	*NA*	0.04 (0.01–0.13)	0.02 (0.00–0.11)	*NA*	*NA*	0.06 (0.02–0.17)	*NA*	0.04 (0.01–0.13)	0.08 (0.02–0.19)	0.06 (0.02–0.17)	*NA*

NA: not applicable.

The overall prevalence rates of *Sarcocystis* spp. also differed among these three regions, depending on the *Sarcocystis* species. The overall prevalence rates were significantly higher in Croatia (0.78, 95% CI = 0.67–0.86) and Lithuania (0.62, 95% CI = 0.44–0.78) than in Portugal (0.30, 95% CI = 0.18–0.45), with G = 27.3, $p < 0.001$ and G = 7.16, $p < 0.01$, respectively.

In all these countries, the overall prevalence of *Sarcocystis* species related to bovids was lower than that related to cervids (Croatia, G = 10.8, $p < 0.001$; Lithuania, G = 7.10, $p < 0.005$; Portugal, G = 7.21, $p < 0.005$). There were also inter-country differences in prevalence in both groups (Table 3). The summary prevalence of *Sarcocystis* species related to cervids in Croatia and Lithuania was both higher than that in Portugal (G = 7.85, $p < 0.005$ and G = 3.83, $p = 0.05$, respectively). Summary prevalence of *Sarcocystis* species related to bovids in Croatia was higher than that in Portugal (G = 9.5, $p < 0.001$).

Table 3. Summary prevalence of *Sarcocystis* species related to different host groups in red foxes in Croatia, Lithuania, and Portugal. 95% CI presented in parentheses. Superscript letters indicate significant differences between countries.

	Intermediate Hosts	
	Bovids	**Cervids**
Croatia	0.25 (0.16–0.36) [a]	0.51 (0.40–0.63) [a]
Lithuania	0.14 (0.05–0.31) [ab]	0.47 (0.30–0.65) [a]
Portugal	0.04 (0.00–0.14) [b]	0.24 (0.13–0.38) [b]

Croatia exhibits significantly higher taxonomic richness (S = 11 species) and the values of both Simpson's index and Shannon's index. These values indicate a rich and well-balanced community. Lithuania has the lowest richness (S = 5) and diversity, while Portugal has moderate richness (S = 6) and diversity (Table 4). *Sarcocystis* diversity index was highest in Croatia (t = 4.34, $p < 0.0001$ compared to Lithuania, and t = 2.48, $p < 0.02$ compared to Portugal), while dominance did not differ among countries. Therefore, overall, Croatia stands out for its high species richness and diversity of *Sarcocystis* species.

Table 4. Diversity of *Sarcocystis* species in red foxes in Croatia, Lithuania, and Portugal; 95% CI presented in parentheses. Superscript letters indicate significant differences between countries.

	Croatia	Lithuania	Portugal
Species richness, S	11 [a]	5 [b]	6 [b]
Simpson's c	0.86 (0.81–0.88) [a]	0.78 (0.68–0.79) [a]	0.81 (0.71–0.82) [a]
Shannon's H	2.13 (1.94–2.23) [a]	1.57 (1.32–1.59) [b]	1.71 (1.46–1.75) [b]

3.3. The Genetic Variability of Sarcocystis Species Identified

The intraspecific genetic variability for *Sarcocystis* species detected was evaluated. The highest number of different *cox1* haplotypes was established for *S. capreolicanis* (h = 9) and *S. linearis* (h = 8) (Table 5). In general, the highest values of haplotype diversity (Hd) and nucleotide diversity (π) were estimated for *S. tenella* and *S. capracanis* parasitising members of the subfamily Caprinae and for *S. gracilis* and *S. linearis* forming sarcocysts in muscles of cervids.

Table 5. The genetic identification of *Sarcocystis* species and their parameters of intraspecific and interspecific variability based on *cox1* sequences obtained.

Species	*n*	h	Hd ± SD	π ± SD	Intraspecific Similarity *	Intraspecific Similarity **	Interspecific Similarity ***
S. arieticanis	12	2	0.303 ± 0.147	0.00126 ± 0.00061	99.6–100%	91.3–100%	85.5–86.3% *S. hircicanis*
S. tenella	6	4	0.800 ± 0.172	0.00679 ± 0.00247	98.1–100%	96.3–100%	90.9–94.1% *S. capracanis*
S. capracanis	6	4	0.800 ± 0.172	0.00540 ± 0.00191	98.6–100%	97.2–100%	91.5–94.0% *S. tenella*
S. rossii	1	1	*NA*	*NA*	*NA*	98.2–98.7%	87.8–88.3% *S. arieticanis*
S. cruzi	2	2	1.000 ± 0.500	0.00652 ± 0.00326	99.4%	96.3–100%	92.1–93.3% *S. levinei*
S. miescheriana	1	1	*NA*	*NA*	*NA*	93.7–100%	76.2–76.9% *S. rangiferi*
S. capreolicanis	20	9	0.705 ± 0.111	0.00263 ± 0.00063	99.5–100%	99.2–100%	94.7–95.5% *S. alceslatrans*
S. gracilis	5	4	0.900 ± 0.161	0.00647 ± 0.00158	98.9–100%	98.1–100%	83.5–86.0% *S. alces*
S. hjorti	6	3	0.600 ± 0.215	0.00382 ± 0.00150	99.1–100%	97.8–100%	92.0–94.3% *S. pilosa*
S. iberica	20	2	0.268 ± 0.113	0.00130 ± 0.00055	99.5–100%	99.0–100%	95.7–97.6% *S. venatoria*
S. morae	7	2	0.286 ± 0.196	0.00098 ± 0.00067	99.7–100%	97.3–99.7%	82.9–84.3% *S. cervicanis*
S. linearis	12	8	0.939 ± 0.048	0.00700 ± 0.00151	98.1–100%	97.9–100%	96.2–97.7% *S. taeniata*

* Comparing *Sarcocystis* sequences obtained in this study; ** Comparing *Sarcocystis* sequences generated in this study and corresponding sequences retrieved from NCBI GenBank; *** Comparing *Sarcocystis* sequences obtained in this study with sequences of other *Sarcocystis* species from NCBI GenBank; *NA*: not applicable.

The intraspecific genetic similarity between our sequences compared ranged from 98.1 to 100%. Comparing our sequences with those of the same species available in GenBank, 96.3–100% similarity was observed for 10 *Sarcocystis* spp. identified, while lower genetic similarity was obtained in the case of *S. arieticanis* (91.3–100%) and *S. miescheriana* (93.7–100%). Despite such a relatively high intraspecific variation, sequences of *S. arieticanis* and *S. miescheriana* showed $\leq$86.3% and $\leq$76.9% similarity compared to other *Sarcocystis* spp. For 10 of the 12 *Sarcocystis* species detected, the difference between their lowest intraspecific similarity and highest interspecific similarity was $\geq$2.5%. For *S. iberica*, this

difference was 1.4%, and for *S. linearis* only 0.2%, as these species showed very high genetic similarity to *S. venatoria* and *S. taeniata*, respectively. Therefore, we have constructed the phylogenetic tree of the analysed *cox1* fragment, including different haplotypes of *S. linearis*, *S. taeniata* and *S.* cf. *taeniata*, and have chosen *S. morae* as the outgroup species (Figure 4). Two well-defined clusters were observed in the phylogram (bootstrap support values of 81 and 99): one cluster comprising *S. taeniata* sequences, and the other was composed of *S. linearis* sequences generated in this work and retrieved from GenBank. Thus, the non-overlapping intraspecific and interspecific genetic similarity values obtained, along with the phylogenetic results for *S. linearis*, indicate that *Sarcocystis* species were correctly identified.

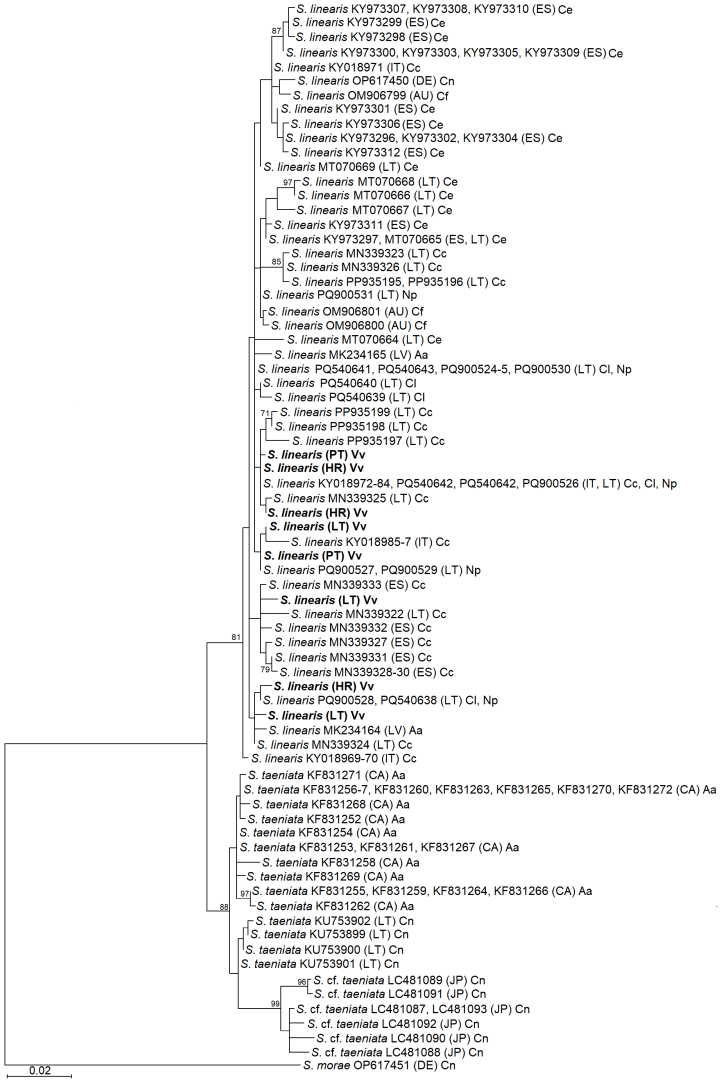

Figure 4. The maximum likelihood phylogenetic tree, based on 617 bp *cox1* sequences, shows the genetic distinctiveness of *S. linearis* from its sister species, *S. taeniata*. The tree was rooted on *S. morae* and scaled according to the branch length. Sequences generated in the current study are presented in bold font. Bootstrap support values are shown next to branches, while GenBank acc. no. are given after the species name. AU, Australia; CA, Canada; DE, Germany; ES, Spain; HR, Croatia; IT, Italy; JP, Japan; LT, Lithuania; LV, Latvia; PT, Portugal. IHs: Aa, *Alces alces*; Cc, *Capreolus capreolus*; Ce, *Cervus elaphus*; Cn, *Cervus nippon*. DH: Cf, *Canis familiaris*; Cl, *Canis lupus*; Np, *Nyctereutes procyonoides*; Vv, *Vulpes vulpes*.

In order to assess the evolutionary relatedness between isolates of *Sarcocystis* species found in red fox faeces across different geographical regions, a haplotype network analysis

was performed. In this analysis, we included *S. linearis*, *S. capreolicanis*, and *S. iberica* species, which were found in all three countries studied. For the construction of haplotype networks, both sequences obtained in the present study and all corresponding sequences available in GenBank were used. Based on the analyzed fragments, the highest number, i.e., 51 haplotypes, was identified for *S. linearis* out of 92 sequences (Figure 5a). In *S. capreolicanis* and *S. iberica*, the most common haplotypes were highly dominant (67.2–76.2% of sequences) and were detected in all or nearly all samples (Figure 5b,c). The haplotype network of *S. capreolicanis* was clearly star-shaped, while *S. iberica* haplotypes differed by one to three mutational steps. In both species, no intraspecific structuring was observed. The MJ network of *S. linearis* was the most complex (Figure 5a). Here, a small number of central haplotypes, generally of low-to-moderate frequency, were each connected to several low-frequency satellite haplotypes by one to a few mutational steps. These main haplotypes were widely distributed across countries and IHs, showing no clear genetic differentiation by host species or geographic location.

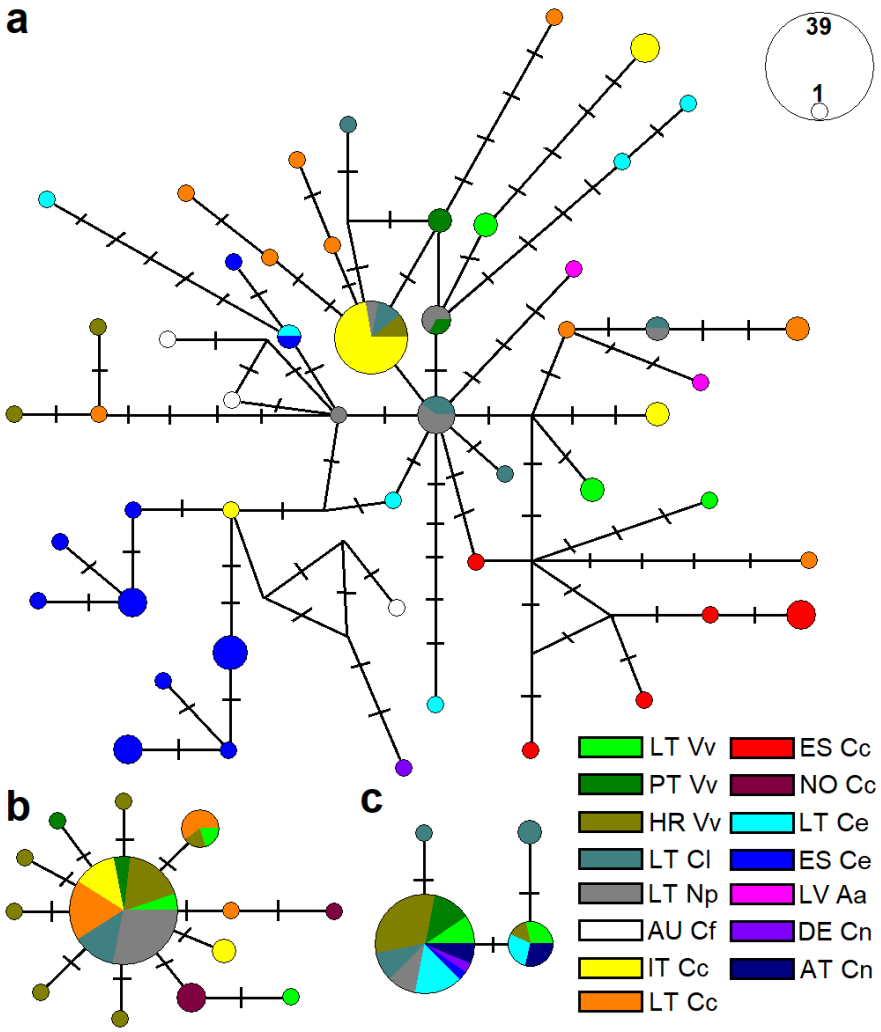

Figure 5. The MJ haplotype network of *Sarcocystis* species identified in all three countries examined. Networks were constructed on the basis of *cox1* haplotypes of *S. linearis* (**a**), *S. capreolicanis* (**b**), and *S. iberica* (**c**). Dashed lines indicate mutational events, the diameters of the pie charts are proportional to the number of sequences, ranging from 1 to 39. AU—Australia, AT—Austria, DE—Germany, ES—Spain, HR—Croatia, IT—Italy, LT—Lithuania, LV—Latvia, NO—Norway, PT—Portugal. IHs: Aa, *Alces alces*; Cc, *Capreolus capreolus*; Ce. *Cervus elaphus*; Cn, *Cervus nippon*. DH: Cf, *Canis familiaris*; Cl, *Canis lupus*; Np, *Nyctereutes procyonoides*; Vv, *Vulpes vulpes*.

4. Discussion

Generally, *Sarcocystis* species are predominantly investigated and differentiated in their IHs rather than in DHs. This is primarily due to the fact that oocysts and sporocysts found in DHs' intestines/stools often exhibit overlapping size ranges, making species-level identification difficult. In contrast, the structural characteristics of sarcocysts in IHs provide more reliable morphological features for species identification [3]. Investigations in IHs alone cannot elucidate the complete life cycle of *Sarcocystis* spp., as current ethical restrictions limit experimental studies. While DHs of certain *Sarcocystis* spp. can be determined by experimental investigation, research increasingly relies on non-invasive diagnostic approaches, with insights derived primarily from naturally occurring cases.

Among canids, the red fox is one of the most important DHs of *Sarcocystis* spp. Owing to its highly opportunistic and omnivorous feeding behaviour, it consumes a wide range of prey and food items, including small mammals (particularly rodents), invertebrates, birds, reptiles, amphibians, fish, fruits, vegetation, as well as anthropogenic resources such as garbage and pet food. Small mammals and invertebrates dominate the global diet of red foxes, but their importance shifts with latitude, elevation, and climate. Cold, high-latitude regions favour mammals and birds, whereas warmer, lower-latitude or high-elevation zones tend to have more invertebrates and fruit [19,21]. Agricultural and suburban areas increase lagomorph consumption, and urban settings add human-derived foods, thereby increasing dietary breadth and plasticity [21]. *Microtus* voles remain key year-round, bank voles (*Clethrionomys glareolus*) peak in autumn, and lagomorphs peak in summer. Carrion (e.g., deer and boar) is critical when snow limits small-mammal access [55]. Urban foxes exhibit higher $\delta^{15}N$ and lower $\delta^{13}C$ values in their whiskers, indicating that up to one-third of their diet consists of processed, C_4-based anthropogenic foods, compared to approximately 6% in rural foxes [56]. Scat, stomach content, and stable isotope analyses all indicate marked trophic flexibility, with implications for wildlife management, zoonotic disease risk, and responses to climate and land use change. In Lithuania, ungulate carrion represents a major dietary component for red foxes, particularly in winter when its contribution increases sharply from 14.6% in summer (10.8% wild + 3.8% domestic) to 41.9% (32.2% wild + 9.7% domestic), while smaller prey such as voles, hares, birds, and invertebrates makes up the remainder [57]. Similarly, in southern regions, the consumption of ungulate carrion rises from about 10.4% in summer to 28.2% in winter, coinciding with a seasonal decline in hares and other small prey [58]. Overall, ungulate carrion is a minor summer food but a major winter resource [57,58]. In Portugal, red foxes in the Iberian Peninsula primarily consume invertebrates (25.5%), followed by fruits/seeds (22.0%), small mammals (20.9%), lagomorphs (22.0%), carrion/garbage (14.2%), and reptiles (2.8%) [41]. Studies indicate that the dietary flexibility of red foxes in the Iberian Peninsula reflects the biogeographical distribution and abundance of their main food sources. In the northern and central regions of Portugal, where sampling for this study took place, there is a higher intake of small mammals and fruits/seeds, whereas in southern areas, foxes consume more lagomorphs and invertebrates. Although less common and likely consumed as carrion, large mammals reported as food items include species from the families Cervidae (red deer; fallow deer, *Dama dama*), Bovidae (cattle, goats, sheep), and Suidae (wild boar) [41]. In Croatia, the red fox diet consists primarily of rodents (32.1%), followed by slaughterhouse waste (28.7%) and domestic poultry (5.2%), with the remainder comprising pheasants, rabbits, fruits, and insects [59].

Microscopical analyses have generally shown relatively low prevalence of *Sarcocystis* spp. in red foxes, such as 16.7% in Lithuania and 1.3% in Croatia, with similarly low rates reported in Japan (1.54% [34]), Bulgaria (1.9%, [60]), Ireland (3.8%, [61]), Switzerland (10.0%, [6]), and the USA (10.1%, [62]). In contrast, molecular analyses of faecal samples

during this investigation have revealed much higher prevalence, including 26.0% in Portugal, 38.2% in Lithuania, and 57.5% in Croatia. Even higher values have been reported using other diagnostic approaches: for example, studies in the Czech Republic and Germany that examined intestinal mucosal scrapings detected prevalence rates of 38.0–38.1% [32,34], while in Canada, faecal flotation techniques revealed a prevalence as high as 84.4% [63].

The present study provides comprehensive evidence that the red fox serves as a DH for well-known *Sarcocystis* species and recently identified species, including *S. iberica*, *S. linearis*, *S. morae*, and *S. rossii*. The latter species was detected in only one specimen from Central Portugal and was originally described from an Alpine ibex (*Capra ibex*) in Austria [64]. However, the Alpine ibex is not found in Portugal; only the Western Iberian ibex (*Capra pyrenaica victoriae*) occurs in the northern part of the country [65], but its distribution does not overlap our sampling areas. Considering that the Western Iberian ibex is the only *Capra* subspecies found in Portugal, this suggests that domestic goats could also serve as a potential IH for the latter species, participating in its life cycle in Portugal. These findings further support the possibility that canids, including the red fox, act as DHs of the latter species, as previous studies had only suggested members of the Canidae family as DHs based on phylogenetic evidence [64]. Additionally, during this investigation, a statistically higher prevalence of cattle-associated *Sarcocystis* species was observed in all three study regions. The higher abundance of Bovidae may help explain this pattern compared to Cervidae and Suidae. According to official statistics, Lithuania hosts approximately 750,000 Bovidae, 520,000 Suidae, and 242,000 Cervidae [66]. Nearly all Bovidae are maintained within the husbandry sector, with only a negligible fraction roaming freely. In contrast, an estimated 50,000 Suidae are freely roaming, while Cervidae are predominantly free-living, with only a small proportion kept in husbandry [67]. In Croatia, there are about 1,023,000 Bovidae, 873,000 Suidae, and 500,000 Cervidae [68]. In Portugal, the numbers are approximately 1,439,708 Bovidae, 2,087,174 Suidae [69], and 2300 Cervidae [14]. These differences in abundance and management influence the accessibility of livestock to predators. For instance, in Lithuania, the red fox has easier access to livestock than to free-roaming cervids or Suidae. Cattle graze outdoors during the summer but are housed indoors in winter, while pigs are mostly confined indoors [70]. In Croatia, sheep and goats are primarily raised on outdoor pastures, whereas cattle are housed in barns and combine this with seasonal grazing, and pigs are generally kept indoors [71]. In Portugal, livestock systems are more pasture-based [72]. These patterns suggest that the observed differences in parasite exposure may be influenced by both the predominance of production systems and animal density.

Additionally, a higher prevalence of *S. tenella* in Lithuania than in Croatia could be explained by the active circulation and environmental detection of *Sarcocystis* species in the Baltic region. Based on the previous studies, conducted across this region, the prevalence of *S. tenella* in hay/water/pasture is relatively high, in addition to the infection rates of 100% that were observed in the previous studies in the sheep raised in Lithuania [5,46,73]. Nevertheless, definitive conclusions cannot be drawn due to the absence of comparable studies in Croatia; consequently, the circulation of *S. tenella* within the country can currently only be hypothesised as limited. Interestingly, different prevalence patterns were observed in Portugal, with red fox sampling sites showing a wide distribution of Cervidae species, including roe deer and red deer, as well as free-roaming livestock species. In two of the sampling sites, the northeast and central-west regions, an apex predator, the Iberian wolf (*Canis lupus signatus*), shares its territory with the red fox. Previous studies have demonstrated that in the northeast regions, wild ungulates are the most commonly consumed prey in wolves' diets (83%) [74], whereas in the central west, more than 94% of their prey items are Bovidae livestock species [75]. The overlap of territories between the Iberian

wolf and the opportunistic red fox provides this mesocarnivore with access to carrion from large mammals, which can increase the risk of infection with *Sarcocystis* species from the consumption of its IHs.

Comparative population genetic studies on *Sarcocystis* spp. are still limited, partly because suitable genetic markers for such analyses have not been fully established. The median-joining haplotype network during our study revealed no clustering of *Sarcocystis* spp. haplotypes based on either the IH, DH, or geographic origin. Previously, the *cox1* marker was used to investigate population connectivity in *Sarcocystis* spp. from roe deer [76,77]. In contrast to our findings, these studies suggested that intraspecific genetic variability is influenced more by the *Sarcocystis* species itself than by the genetic locus analysed or the geographic origin of the samples.

Although phylogenetic analysis revealed clustering of *S. linearis* isolates from this study with those in GenBank, and similarly for *S. taeniata*, interspecific differences between these two *Sarcocystis* species were minimal. Previous studies have shown that partial *18S* rRNA gene sequences exhibit higher identity than *cox1* sequences, indicating that *S. linearis* cannot be clearly distinguished from *S. taeniata* using only *18S* rRNA data [78]. A similar pattern occurs between the sister species *S. iberica* and *S. venatoria*, which are virtually identical at the *18S* rRNA level but differ in the *cox1* region [35,79]. Therefore, future studies should incorporate additional molecular markers to better distinguish closely related *Sarcocystis* species. Additionally, the intraspecific variability of *S. arieticanis* was notably higher compared to that observed in other *Sarcocystis* species in this study. Consequently, some of the deposited nucleotide sequences may have been incorrectly attributed to *S. arieticanis*, potentially inflating the number of isolates falsely identified as this species.

Although faecal analysis may not capture the full spectrum of *Sarcocystis* diversity due to intermittent shedding and the inherent limitations of molecular detection, it remains a robust, non-invasive approach for assessing parasite richness in wildlife populations. By examining faecal matter from red foxes in Lithuania, Croatia, and Portugal, this study provides novel comparative data that contributes to a broader understanding of *Sarcocystis* distribution in Europe, as no prior research has examined this parasite composition in red foxes across different geographical regions. Future investigations could strengthen these findings by incorporating larger and more temporally diverse sample sets, applying high-throughput sequencing to better resolve mixed infections, and extending analyses to additional DHs and IHs. Such integrative approaches would refine ecological and epidemiological interpretations, helping to clarify the role of wildlife in transmission dynamics with potential relevance for animal health and livestock production.

5. Conclusions

Based on the nested PCR of *cox1* gene sequences, this study identified 12 *Sarcocystis* species: *S. arieticanis*, *S. capreolicanis*, *S. capracanis*, *S. cruzi*, *S. gracilis*, *S. hjorti*, *S. iberica*, *S. linearis*, *S. miescheriana*, *S. morae*, *S. rossii*, and *S. tenella*. Notably, this study provides the first worldwide confirmation of the red fox as a definitive host for *S. iberica*, *S. linearis*, *S. morae*, and *S. rossii*. The observed variation in the prevalence of *Sarcocystis* spp. among the investigated countries, including higher prevalence rates in Portugal (26.0%), Lithuania (38.2%), and Croatia (57.5%), highlights the need for further studies across the European continent.

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D.H., D.C., V.S. and D.B.; supervision, N.G., P.P. and D.B.; funding acquisition, D.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements, as fecal samples were collected non-invasively without harming the animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: The *cox1* sequences of *Sarcocystis* species identified in red fox samples from Croatia, Lithuania, and Portugal during this study have been submitted to the GenBank database under accession numbers PX435148–PX435245.

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Conflicts of Interest: The authors declare no conflicts of interest.

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