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First report of *Sarcocystis halieti* in Asia: the genetic confirmation in muscles of the Eurasian sparrowhawk (*Accipiter nisus*) from Iran

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Previous studies showed that the Eurasian sparrowhawk *Accipiter nisus* (Linnaeus, 1758) acts as the definitive host of the *Sarcocystis halieti* (Apicomplexa: Sarcocystidae) Gjerde, Vikøren et Hamnes, 2017. Herein, we report macrocysts of *S. halieti* in the pectoral muscle of a Sparrowhawk in the west of Iran. Between September 2019 and December 2023, a total of 55 wild birds belonging to 8 orders, 9 families, 18 genera, and 21 species were examined in Hamedan province, western Iran. In one Eurasian sparrowhawk, macroscopic cysts resembling rice grains were observed in the breast muscle of the bird. None of the other examined birds were infested with macrosarcocysts. DNA extracted from sarcocysts was molecularly characterized at four nuclear and mitochondrial DNA markers (the *18 S* rRNA, *28 S* rRNA, *cox*1 genes, and the ITS1 region). Sequence analysis confirmed the identity of the macrosarcocysts as *Sarcocystis halieti*. This is the first report of *S. halieti* in Asia. Sparrowhawk proved to act as both the definitive and intermediate host of *Sarcocystis halieti*. The phenomenon of the same species of *Sarcocystis* serving as both hosts is extremely rare among birds and needs to be studied further.

Abbreviations
PCR   polymerase chain reaction
*cox*1   cytochrome c oxidase subunit I
18S rRNA   *18 S* ribosomal RNA
28S rRNA   *28 S* ribosomal RNA
ITS1   internal transcribed spacer 1

The genus *Sarcocystis* is one of the most species-rich genera in the phylum Apicomplexa, widely distributed worldwide. Members of the genus *Sarcocystis* can infect poikilotherm animals (reptiles) as well as birds and mammals, including man[1]. These parasites have a two-host life cycle, which is based on animal prey-predator relationships. Sexual multiplication of the parasite takes place in definitive hosts, while asexual development occurs in intermediate hosts. The definitive host becomes infected by consuming animal tissues infected by mature sarcocsyts, whereas the intermediate host acquires the parasite by consuming food or water contaminated with sporocysts. Sarcocysts are mainly found in muscle tissues and CNS of the intermediate host, while sporocysts develop in the *lamina propria* of the small intestine of the definitive host[2].

To date, birds serve as intermediate hosts of 32 valid *Sarcocystis* species[3]. *Sarcocystis rileyi* forms macroscopic cysts resembling rice grains in the breast and other muscles of ducks[4]. Furthermore, two species, *S. horvathi* and *S. wenzeli* can parasite chickens; the latter may induce encephalitis[2,5]. In wild birds, fatal diseases can be caused by *S. falcatula* and *S. calchasi*, which have low host specificity and can parasitize birds belonging to several distinct orders[2].

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Birds of prey are usually the definitive hosts of *Sarcocystis* species[3]. However, in the last decade, there has been an increasing number of reports of sarcocysts in tissues of birds of prey, indicating that these birds can serve as true intermediate hosts of *Sarcocystis* spp. Three species, *S. falcatula*, *S. halieti*, and *S. wobeseri* have been identified in the birds order Accipitriformes[2,3,6,7], while *S. falcatula* and *S. halieti* have been detected in the birds order Strigiformes[8,9].

Species of *Sarcocystis* are described in intermediate hosts based on morphological features of sarcocysts, including their walls, and genetic characteristics of the parasite investigated mainly by Sanger sequencing of informative loci[2,3,10]. Morphological examinations rely on light microscopy and/or complex and expensive electron microscopy. However, morphological studies alone are not sufficient to distinguish between evolutionarily close species with very similar morphological characteristics found in closely related hosts. For instance, even though four *Sarcocystis* species (*S. columbae*, *S. halieti*, *S. lari*, and *S. wobeseri*) are indistinguishable based on the usual morphological features, they have been discovered in the same species of bird, the herring gull (*Larus argentatus*)[11]. Therefore, DNA analysis is widely used in the identification and revision of *Sarcocystis* species. Notably, more than half of the *Sarcocystis* species parasitizing birds have been described in the last 15 years, largely due to the progress of molecular approaches and the increasing use of these techniques in parasitology and veterinary science[10–13]. Different loci, such as nuclear *18 S* rRNA, *28 S* rRNA, internal transcribed spacer 1 (ITS1), and mitochondrial cytochrome c oxidase subunit 1 (*cox*1), are the most widely applied for genetic characterization of *Sarcocystis* species[10,14].

Iran is the 17th largest country in the world, with 550 avian species, almost equal to the richness of birds in Europe[15,16]. However, there is limited and scanty information about their parasite fauna, especially sarcocystosis, i.e., only four documents reporting *Sarcocystis* species in chickens, ducks, and pigeons[17–20].

The Eurasian sparrowhawk *Accipiter nisus* (Linnaeus, 1758) is a relatively small bird of prey belonging to the family Accipitridae[21,22]. It is a widespread species in the temperate and subtropical parts of the Old World, with an estimated population of 1.5 million birds[23]. This species mainly preys on small forest birds[22]. In Iran, it is a fairly common breeding bird in Caspian forests and forests of northern Azarbaijan and a common winter visitor occurring throughout Iran, but chiefly in the north, central, and southwest[16]. The objective of the present study was to identify macroscopic cysts observed in the pectoral muscle of one Eurasian sparrowhawk necropsied in the west of Iran utilizing molecular methods.

Results

A total of 55 wild birds belonging to 8 orders, 9 families, 18 genera, and 21 species were examined (Table 1). In one Eurasian sparrowhawk bird examined on 13 June 2020, elongated spindle-shaped macroscopic cysts resembling rice grains were observed in the breast muscle of the bird (Fig. 1). None of the other examined birds were infested with macrocysts.

Since the sparrowhawk was presented by the Provincial Department of Environment to the lab in the frozen state, histopathology could not be performed.

Figure 1 a) The Eurasian sparrowhawk (*Accipiter nisus*) was examined on June 13, 2020. **b)** Several elongated spindle-shaped macroscopic cysts resembling rice grains (arrows) were observed in the bird's pectoral muscle.

Genetic identification and characterization of *S. halieti* from the Eurasian sparrowhawk in Iran

In the current study, partial sequences of four genetic loci of the *Sarcocystis* parasite from Iran were generated. Specifically, 255 bp *18 S* rRNA, 842 bp *28 S* rRNA, 828 bp *cox*1, and 505 bp ITS1 region (427 bp ITS1 and 78 bp *5.8 S* rRNA) sequences generated were deposited in NCBI GenBank database under accession numbers (PQ270244 for *18 S* rRNA, PQ270246 for *28 S* rRNA, PQ276104 for ITS1, PQ274874 for *cox*1).

Based on a short *18 S* rRNA sequence, the sarcocyst isolated from the Eurasian sparrowhawk in Iran showed 100% identity with numerous *Sarcocystis* spp. using predatory mammals (*S. arctica*, *S. caninum*, and *S. svanai*) or birds (*S. calchasi*, *S. cornixi*, *S. corvusi*, *S. fulicae*, *S. halieti*, *S. turdusi*, and *S. wobeseri*) as their intermediate hosts and birds as their definitive hosts. The variability of *cox*1 also appeared to be insufficient to identify the species of the isolated parasite, as 100% identity was found by comparing the sequence of the parasite studied in this work with those of *S. calchasi*, *S. columbae*, *S. corvusi*, and *S. halieti* (Table 2). Based on *28 S* rRNA, the sequences of the studied parasite showed 99.9–100% similarity with *S. halieti* and less than 99.4% similarity with other *Sarcocystis* spp. Specifically, 99.3% similarity was established when comparing the analyzed parasite with *Sarcocystis* sp. ex *Corvus corax*, *S. columbae*, and *S. corvusi*, and 98.2–98.6% similarity was obtained when compared to *S. calchasi*, *S. turdusi*, and *S. wobeseri*. The 427 bp ITS1 sequence generated in the present work showed 96.3% similarity to that of *Sarcocystis* sp. isolate Skua-2016-CH isolated from the Chilean skua (*Stercorarius chilensis*), 94.6% similarity to that of *Sarcocystis* sp. ex *Corvus corax*, 92.5% similarity to that of *Sarcocystis* sp. ex *Accipiter cooperii*, 91.8% similarity to those of *S. columbae* and 90.5% similarity to that of *S. corvusi*. In summary, the genetic analysis showed that the sarcocysts found in the muscles of the Eurasian sparrowhawk were identified as *S. halieti*.

Phylogeny of *S. halieti* from Iran

In the *28 S* rRNA phylogenetic tree, all the analyzed isolates of *S. halieti*, including the one obtained in the current study, were grouped together with a high bootstrap support (Fig. 2). However, the variability of this locus was not sufficient to determine the most closely related species to *S. halieti*. Phylogenetic relationships between *S. halieti* and three other taxa showing high sequence similarity (*Sarcocystis* sp. ex *Corvus corax*, *S. columbae*, and *S. corvusi*) were not resolved. Despite that, it was demonstrated that the above-mentioned taxa were sister species to *S. wobeseri*, *S. calchasi*, *S. kutkienae*, *S. cornixi*, *S. turdusi*, and *S. fulicae* cluster.

n birds	Host bird examined taxonomy	Host scientific name	Host vernacular name
	ACCIPITRIFORMES		
	Accipitridae		
5		*Accipiter nisus* (Linnaeus, 1758)	Eurasian sparrowhawk
3		*Aquila chrysaetos* (Linnaeus, 1758)	Golden eagle
1		*Aquila heliaca* Savigny, 1809	Asian imperial eagle
3		*Aquila nipalensis* Hodgson, 1833	Steppe eagle
1		*Aquila rapax* (Temminck, 1828)	Tawny eagle
12		*Buteo buteo* (Linnaeus, 1758)	Buzzard
3		*Circus aeruginosus* (Linnaeus, 1758)	Eurasian marsh-harrier
1		*Gyps fulvus* (Hablizl, 1783)	Griffon vulture
	ANSERIFORMES		
	Anatidae		
1		*Anas platyrhynchos* Linnaeus, 1758	Mallard
	FALCONIFORMES		
	Falconidae		
12		*Falco tinnunculus* Linnaeus, 1758	Common kestrel
	GRUIFORMES		
	Rallidae		
1		*Rallus aquaticus* Linnaeus, 1758	Water rail
	PELECANIFORMES		
	Ardeidae		
1		*Ardea cinerea* Linnaeus, 1758	Gray heron
1		*Ixobrychus minutus* (Linnaeus, 1766)	Little bittern
1		*Botaurus stellaris* Linnaeus, 1758	Great Bittern (Eurasian bittern)
	PTEROCLIDIFORMES		
	Pteroclidae		
1		*Pterocles orientalis* (Linnaeus, 1758)	Black-bellied Sandgrouse
	PICIFORMES		
	Picidae		
1		*Dendrocopos syriacus* (Hemprich & Ehrenberg, 1833)	Syrian woodpecker
	STRIGIFORMES		
	Strigidae		
2		*Asio otus* (Linnaeus, 1758)	Long-eared owl
1		*Athene noctua* (Scopoli, 1769)	Little owl
2		*Bubo bubo* (Linnaeus, 1758)	Eagle owl
1		*Otus scops* (Linnaeus, 1758)	European scops owl
	Tytonidae		
1		*Tyto alba* (Scopoli, 1769)	Barn owl

Table 1. Examined birds in this study. Names of orders are capitalized, and names of families are shown in bold.

Figure 2 The phylogenetic relationships of *S. halieti* based on *28 S* rRNA sequences. The ML tree was scaled according to the branch length and rooted on *S. rileyi*. GenBank accession numbers are given following the species name. Bootstrap support values ≥ 70 are displayed above branches.

For the ITS1 phylogenetic investigation, only sequences of *S. halieti* that differed from each other were retrieved from the GenBank. These sequences, in combination with ones belonging to *Sarcocystis* spp. characterized by bird-bird life cycle and ones of undescribed species showing high similarity to *S. halieti* were compared with *S. halieti* from Iran. Altogether, 12 sequences of *S. halieti* were placed into one cluster with 88 bootstrap support values (Fig. 3). Also, some significant grouping of different *S. halieti* ITS1 sequences was noticed and analyzed in detail in the following section. Based on the ITS1 partial fragment, *Sarcocystis* sp. ex *Stercorarius chilensis* formed a sister branch to *S. halieti*. These two taxa were sister species to *S. corvusi*, *S. columbae*, and *Sarcocystis* sp. ex *Accipiter cooperii*. Finally, *Sarcocystis* sp. ex *Corvus corax* formed a sister branch to the above-mentioned species. Other examined species using birds as their intermediate and definitive hosts (*S. wobeseri*, *S. calchasi*, *S. fulicae*, *S. turdusi*, *S. cornixi*, and *S. kutkienae*) were phylogenetically distant from *S. halieti* group and were placed in a separate cluster.

Figure 3 The phylogenetic placement of *S. halieti* based on ITS1 sequences. The ML tree was scaled according to the branch length and rooted on *S. rileyi*. The dashed line indicates that its length does not represent evolution

**Fig. 1**. Samples were collected from Hamedan provinces in Iran. The map was drawn by using ArcGIS software version 10.3 (https://enterprise.arcgis.com/en/portal/).

Species	Region				
	18 S rRNA	*cox*1	*28 S rRNA*	**ITS1 427 bp**	**ITS1 and *5.8 S* rRNA 505 bp**
S. halieti	100	100	99.9–100	98.4–100	98.4–100
S. sp. ex *Stercorarius chilensis* (isolate Skua-2016-CH)	–	–	–	96.3	96.4
S. sp. ex *Corvus corax*	100	–	99.3	94.6	94.7
S. sp. ex *Accipiter cooperii*	–	–	–	92.5	93.6
S. columbae	99.6	100	99.3	91.8	91.9
S. corvusi	100	100	99.3	90.5	90.1
S. calchasi	100	100	98.2	82.7–83.1	84.5
S. wobeseri	100	99.9	98.5–98.6	82.7–82.8	81.6–84.7
S. fulicae	100	99.9	97.4	< 80.0	< 80.0
S. cornixi	100	99.9	97.6	< 80.0	< 80.0
S. turdusi	100	99.8	98.3	< 80.0	< 80.0
S. kutkienae	99.6	–	97.7	< 80.0	< 80.0

Table 2. The percentage genetic similarity values of DNA sequences of *S. halieti* from Iran compared to other *Sarcocystis* spp.

distance. GenBank accession numbers are given after the species name. Bootstrap support values ≥ 70 are shown above branches.

Intraspecific genetic variability of *S. halieti*

We have used eighty-two 427–428 bp and one hundred forty-five 350–351 bp long ITS1 sequences of *S. halieti* for the analysis of the intraspecific genetic variability of the species under investigation. In total, 11 (A1-A11) and 12 (B1-B12) different sequence variants of 427–428 bp and 350–351 bp fragments were ascertained. These sequence variants differed by up to eight and nine single nucleotide polymorphisms (SNPs), respectively. The median-joining network analysis showed that the most common A1 and A2 genotypes were found in 16 of 20 examined bird species which served as intermediate and definitive hosts of *S. halieti* (Fig. 4a). Based on both analyses ITS1 fragments relatively remote from most common genotypes A3 and A6 as well as B3, B4, and B12 were identified only in intermediate hosts of the parasite. Furthermore, all sequences from Brazil made
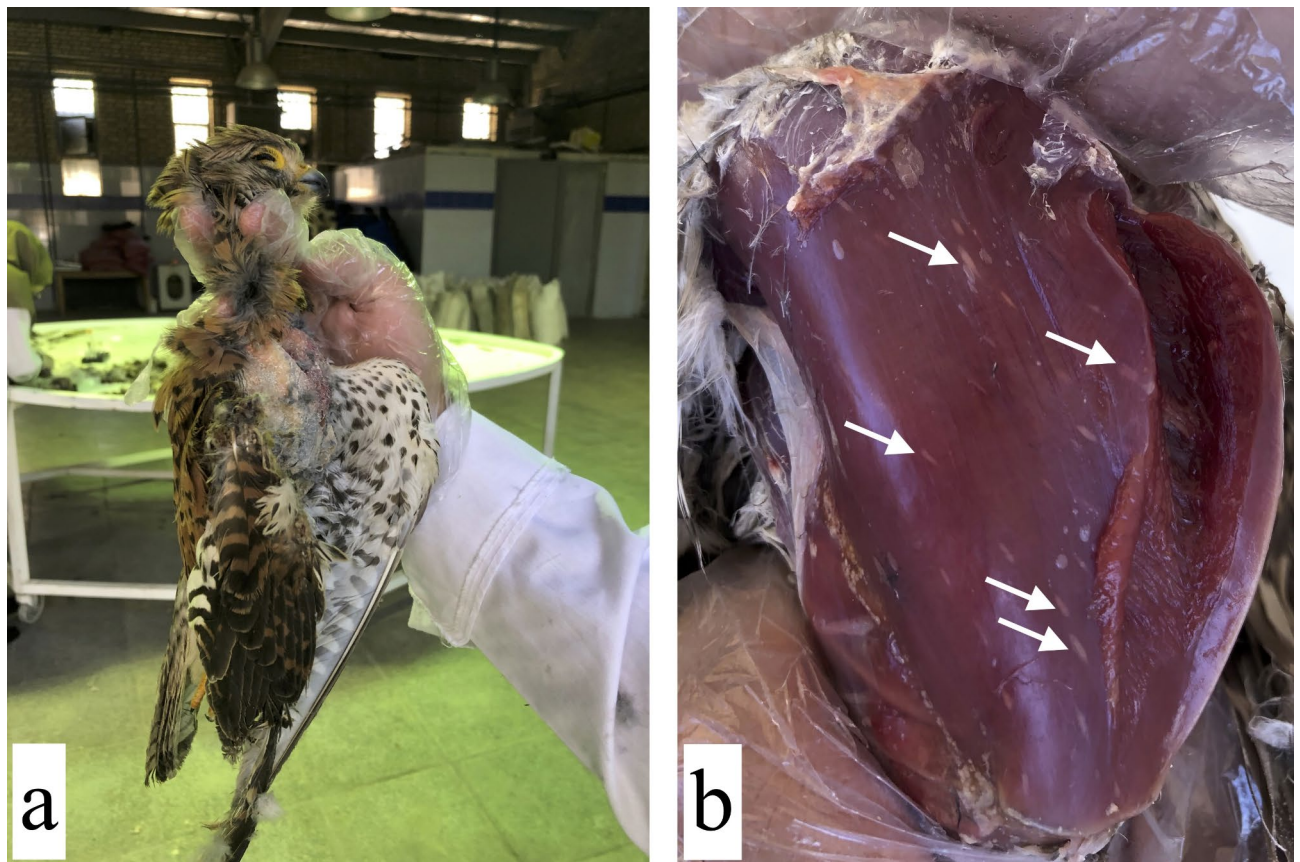
Fig. 2. **(a)** The Eurasian sparrowhawk (*Accipiter nisus*) examined on June 13, 2020. **(b)** Several macroscopic cysts resembling rice grains (arrows) were observed in the bird's pectoral muscle.

A3, A6 or B3, B12 genotypes which differed from the other genotypes by at least four SNPs and apparently formed a distinct evolutionary lineage (Fig. 4). Based on the larger fragment analyzed (427–428 bp), *S. halieti* sequence from the Eurasian sparrowhawk in Iran demonstrated 100% identity with *S. halieti* isolates obtained from sarcocysts of the common starling (*Sturnus vulgaris*) (GenBank accession no.: MZ333538) and sporocysts of the red kite (*Milvus milvus*) (GenBank accession no.: MZ333537), both from Check Republic (Fig. 4b). The genotype established in Iran were closely related to genotypes detected in birds of the families Accipirtidae, Corvidae, Laridae, and Phalacrocoracidae from Lithuania and Spain and serving as intermediate hosts of *S. halieti*.

Figure 4A median-joining network of *S. halieti* based on 351 bp (a) and 428 bp (b) ITS1 fragments. Hypothetical not-determined genotypes are represented by black rectangles. The circle area is proportional to the genotype frequency (A1 was identified for 97 sequences, A6-A11, B7-12 were detected for a single sequence each). Dashes display mutational steps. The different colors represent different hosts and geographic origins. BR Brazil, ES, Spain, LT Lithuania, CZ Czech Republic, IR Iran, and NO Norway.

The comparison of *S. halieti* ITS1 sequences originating from Brazil with those originating from Eurasia resulted in lower interspecific variability values (Table 3). However, such results should be considered with caution due to the small sample size from Brazil. Slightly higher genetic variability values were obtained when comparing the sample consisting of sequences obtained from definitive to those obtained from intermediate hosts ($K = 2.29474$ vs. 2.07192, $Hd \pm SD = 0.585 \pm 0.034$ vs. 0.457 ± 0.068, $\pi \pm SD = 0.00656 \pm 0.00045$ vs. 0.00592 ± 0.00095). However, strong differences in genetic variability were observed when comparing the variation of *S. halieti* in different bird families. Higher variation was observed in birds of the family Accipitridae when compared to those of the family Laridae serving as intermediate hosts of *S. halieti*, as well as in members of the family Accipitridae when compared to those of the family Corvidae serving as definitive hosts of the parasite. However, the Accipitridae as intermediate host samples were made up of only 4 sequences.

The genetic differentiation analysis based on F_{ST} pairwise calculations showed substantial genetic differences between some of the samples compared. Very high and significant genetic differentiation was obtained between Eurasia and Brazil samples ($F_{ST} = 0.569$, $P < 0.001$), Accipitridae and Laridae intermediate hosts samples ($F_{ST} = 0.523$, $P = 0.003$), and Accipitridae and Corvidae definitive hosts samples ($F_{ST} = 0.351$, $P < 0.001$). Moderate and significant genetic differentiation was assessed between intermediate hosts and definitive hosts samples ($F_{ST} = 0.073$, $P = 0.001$).
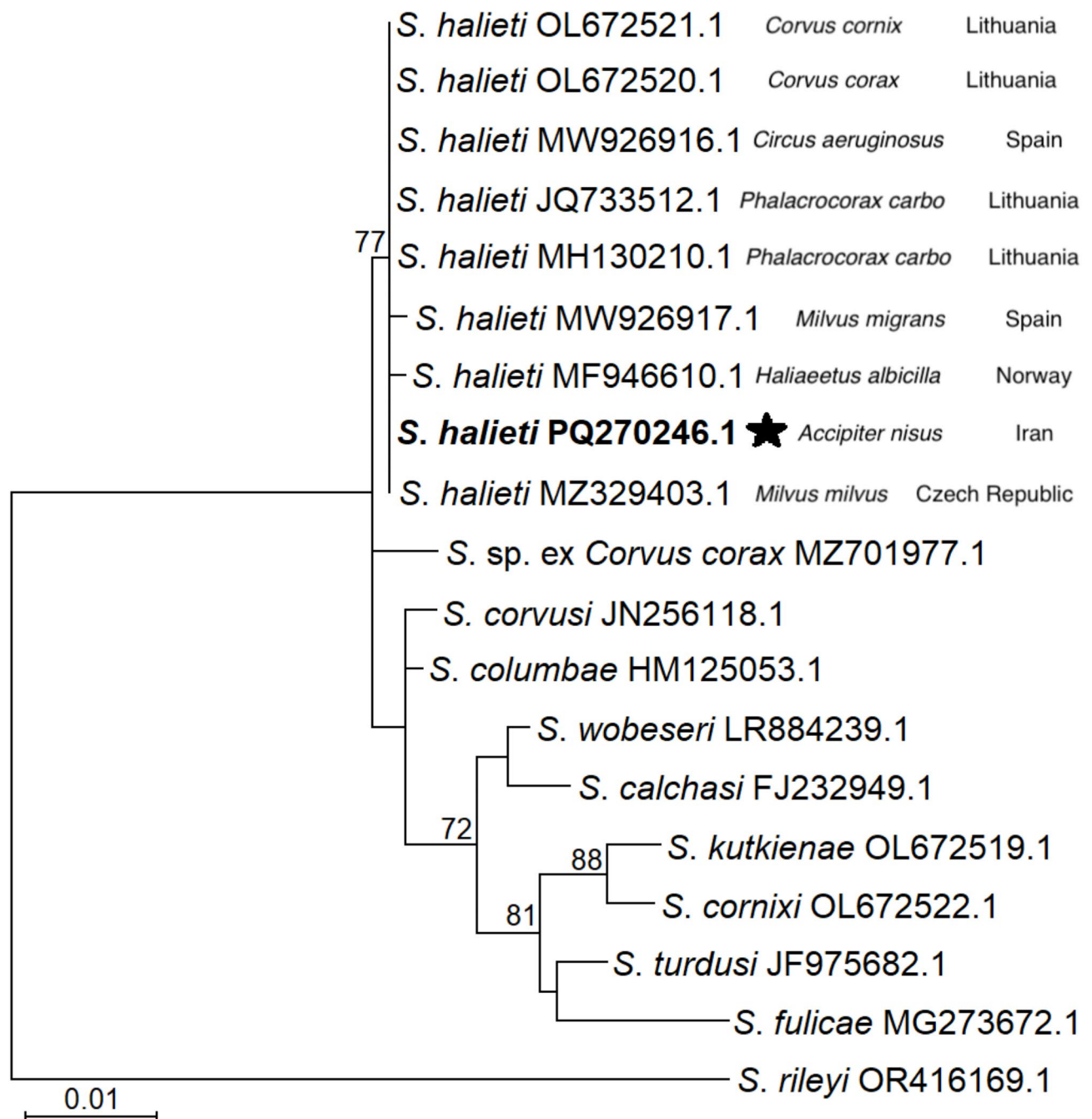
Fig. 3. The phylogenetic relationships of *S. halieti* based on *28 S* rRNA sequences. The ML tree was scaled according to the branch length and rooted on *S. rileyi*. GenBank accession numbers are given following the species name. Bootstrap support values ≥ 70 are displayed above branches.

Discussion

Herein, we report the first data about the presence and, henceforth, possible circulation of *S. halieti* in Asia as confirmed by *18 S* rRNA, *28 S* rRNA, *cox*1, and ITS1 sequences obtained from macroscopic cysts in the pectoral muscle of the Eurasian sparrowhawk. We also prove that sparrowhawk acts as both the definitive and intermediate host of the parasite. In Iran, although numerous studies reported *Sarcocystis* species infecting food animals[24,25], our study is the first research work on sarcocystosis in wild birds. In the only four previous studies from the country, researchers examined domestic birds, i.e. pigeons *Columbia livia domestica* (3 studies), chickens *Gallus gallus domesticus*, and ducks *Anas platyrhynchos domesticus* (1 study). For the first time, Khordadmehr and colleagues reported that two of 94 pigeons in Yazd province located in the central desert were infected with microscopic *Sarcocystis* cysts in the tunica muscularis mucosa of the gizzard[19]. Shortly after that, Shahbazi and her team performed the first PCR-based study on pigeons' microcysts in a northwestern region and reported a 99% genetic similarity with *S. calchasi*[17]. In the same region, Khordadmehr and colleagues
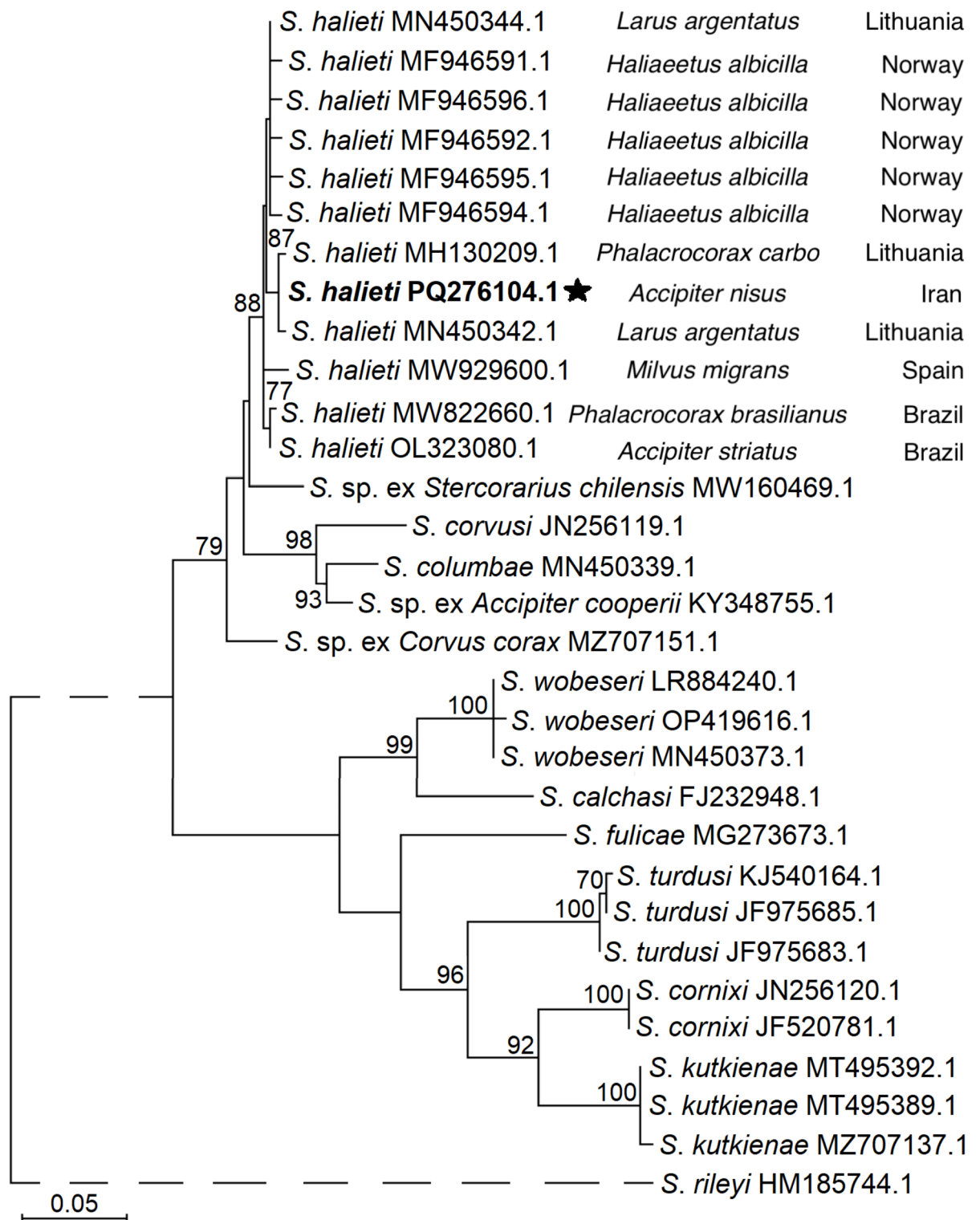
Fig. 4. The phylogenetic placement of *S. halieti* based on ITS1 sequences. The ML tree was scaled according to the branch length and rooted on *S. rileyi*. The dashed line indicates that its length does not represent evolution distance. GenBank accession numbers are given after the species name. Bootstrap support values ≥ 70 are shown above branches.

reported *Sarcocystis* DNA in the brain and the gizzard of 1 / 100 examined pigeons[18]. In that study, sequencing of the *28 S* rRNA gene product was not successful, but consensus sequences of *cox*1 displayed 99.1% nucleotide similarity and 96% query cover with two sequences available in the GenBank i.e., *S. columbae* ex. common wood pigeon *Columba palumbus* from Lithuania (accession number: MH138312) and *S. corvusi* ex. Eurasian jackdaw

Samples	n	h	S	K	Hd ± SD	π ± SD
Overall	156	11	18	2.27734	0.549 ± 0.036	0.00651 ± 0.00051
Europe	150	9	16	2.13647	0.516 ± 0.037	0.00610 ± 0.00051
Eurasia	151	9	16	2.14834	0.519 ± 0.036	0.00614 ± 0.00050
Brazil	5	2	1	0.40000	0.400 ± 0.237	0.00114 ± 0.00068
IH	73	6	11	2.07192	0.457 ± 0.068	0.00592 ± 0.00095
DH	83	9	16	2.29474	0.585 ± 0.034	0.00656 ± 0.00045
IH Accipitridae	4	3	8	4.50000	0.833 ± 0.222	0.01286 ± 0.00381
IH Laridae	62	5	11	1.32522	0.268 ± 0.073	0.00379 ± 0.00107
DH Accipitridae	35	9	16	2.56134	0.593 ± 0.092	0.00732 ± 0.00130
DH Corvidae	48	2	4	1.24468	0.311 ± 0.071	0.00356 ± 0.00081

Table 3. The intraspecific variability of *S. halieti* within 350–351 bp long ITS1 fragment. IH intermediate host, DH definitive host, n the number of sequences, h the number of haplotypes, S the number of polymorphic sites, K the average number of nucleotide differences, Hd the haplotype diversity, π the nucleotide diversity, SD the standard deviation.

Corvus monedula from Lithuania (accession number: MH138314). In another study, microscopy examination following the digestion method revealed *Sarcocystis* bradyzoites in the breast muscle of 100% of 18 backyard ducks and 94.78% of 37 backyard chickens in Mazandaran, the northern part of Iran[20]. Considering the shortage of information about the occurrence, prevalence, and distribution of *Sarcocystis* species infecting avian hosts in Iran, further studies, especially PCR-sequencing studies on wild birds, are recommended.

Based on the primer pairs used for the genetic characterization of *Sarcocystis* species at the four most popular loci, i.e., *18 S* rRNA, *28 S* rRNA, ITS1, and *cox*1, we obtained partial length sequences of the amplified fragments. This was due to relatively faint PCR fragments, which could be the result of improperly preserved biological material[26]. Indeed, the infected sparrowhawk was found dead in nature (unknown time-lapse between the death to collection by the Provincial Department of Environment) and was presented to the lab frozen. Nevertheless, the 842 bp and 505 bp long fragments of the *28 S* rRNA and ITS1 region, respectively, were sufficiently variable to enable accurate identification of the *Sarcocystis* species as *S. halieti* (Table 2; Figs. 2 and 3). In contrast, using *18 S* rRNA and *cox*1 sequences it turned out that the parasite belonged to the genus *Sarcocystis* and was most closely related to species using birds and predatory mammals as their intermediate hosts. Previously, it was also shown that mitochondrial *cox*1, as well as the RNA polymerase B gene of the apicoplast genome, are not variable enough for the separation of *Sarcocystis* species employing birds as their intermediate and definitive hosts[10,14,27]. Nonetheless, the *cox*1 marker is suitable for the distinguishment of *Sarcocystis* species *S. anasi*, *S. albifronsi*, *S. rileyi*, and *S. wenzeli*, which spread between birds as intermediate hosts and placental mammals as definitive hosts[4,5,28,29]. The *cox*1, suggested as a barcoding marker for animals, is used as a golden standard for the identification of *Sarcocystis* spp. forming sarcocysts in ungulates[30–32]. However, this locus is not variable enough for the separation of *Sarcocystis* from small mammals and predatory mammals[33,34]. Thus, it is clear that *Sarcocystis* from birds using different definitive hosts (birds or mammals) have evolved independently of each other at different rates. Studies by other researchers have also confirmed that ITS1 is most suitable for the identification of *Sarcocystis* spp. characterized by a bird-bird lifecycle[12–14,35]. Also, *28 S* rRNA can be used for the diagnosis of these groups of parasites[10,36]. In the current work, it was demonstrated that ITS1 is more suitable than *28 S* rRNA for understanding the phylogenetic relationships of analyzed species. However, it should be noted that due to large ITS1 length variability in different *Sarcocystis* species, it is hard to generate ITS1 alignment. Therefore, the ITS1 is more suitable for uncovering phylogenetic relationships between closely related taxa than for making phylogenetic inferences about more remote groups[10]. Based on ITS1 and/or *28 S* rRNA, sequences of *S. halieti* had the highest similarity with several undescribed *Sarcocystis* species. The phylogenetic analysis showed that *S. halieti* clearly represents different species compared to *Sarcocystis* sp. ex *Accipiter cooperii* and *Sarcocystis* sp. ex *Corvus corax* (Fig. 3). Despite the very high 96.3% ITS1 sequence similarity of *Sarcocystis* sp. ex *Stercorarius chilensis* with *S. halieti*, *Sarcocystis* sp. formed a separate branch in the phylogenetic tree and further investigations are needed to resolve the taxonomic status of this parasite.

The historical confusion on the *S. halieti* parasite should also be clarified here. Recently, *Sarcocystis* species have been characterized generally on the basis of the morphology of sarcocysts found in intermediate hosts and the comparison of DNA sequences of several genetic loci[13,37]. However, this was not the case with *S. halieti*. Initially, the *18 S* rRNA, *28 S* rRNA, and ITS1 sequences of *Sarcocystis* sp., named *Sarcocystis* sp. ex *Phalacrocorax carbo*, appeared in the GenBank database in 2012 with accession numbers JQ733511–JQ733513. Sarcocysts of this parasite were isolated from the muscles of the great cormorant collected in Lithuania. However, the name of the parasite was not given, as a morphological characterization of sarcocysts was not performed[10]. Subsequently, Norwegian researchers identified several *Sarcocystis* species using DNA sequencing in the intestinal mucosa of one white-tailed sea eagle (*Haliaeetus albicilla*)[14]. The comparison of *cox*1, *18 S* rRNA, *28 S* rRNA, and ITS1 sequences showed the identity of one of the detected parasites with *Sarcocystis* sp. ex *Phalacrocorax carbo*, and this organism was named *S. halieti*. Only later, the morphology of sarcocysts of *S. halieti* was characterized in detail with the help of light and transmission electron microscopy[10].

Previously, it was reported that the ITS1 marker shows a high level of intraspecific variability for *Sarcocystis* spp. using ungulates as intermediate hosts[38]. Furthermore, for some *Sarcocystis* species of this group, intraspecific and

Species	Order	Family	Isolation source	Country	Continent	GenBank accession number	Reference
Intermediate host							
Accipiter nisus	Accipitriformes	Accipitridae	Breast muscle	Iran	Asia	PQ276104	**This study**
Accipiter striatus	Accipitriformes	Accipitridae	Muscles	Brazil	South America	OL323080	[47]
Circus aeruginosus	Accipitriformes	Accipitridae	Leg muscles	Spain	Europe	MW929599	[6]
Gypaetus barbatus	Accipitriformes	Accipitridae	Breast muscles	Spain	Europe	OR059468-9	[42]
Milvus migrans	Accipitriformes	Accipitridae	Breast muscles	Spain	Europe	OR059466-7	[42]
Larus argentatus	Charadriiformes	Laridae	Leg muscles	Lithuania	Europe	MN450340–56, OP419641–4, OP419649–64	[11,40]
Larus canus	Charadriiformes	Laridae	Leg muscles	Lithuania	Europe	OP419605–8, OP419613, OP419621–4	[40]
Larus dominicanus	Charadriiformes	Laridae	Brain	Brazil	South America	MW822661–2	[35]
Larus ridibundus	Charadriiformes	Laridae	Leg muscles	Lithuania	Europe	OP419625–38	[40]
Corvus corax	Passeriformes	Corvidae	Leg muscles	Lithuania	Europe	MZ707148	[41]
Corvus cornix	Passeriformes	Corvidae	Leg muscles	Lithuania	Europe	MZ707149	[41]
Sturnus vulgaris	Passeriformes	Sturnidae	Muscles	Czech Republic	Europe	MZ333538	[48]
Puffinus puffinus	Procellariiformes	Procellariidae	Pectoral muscles	Brazil	South America	MW822661	[35]
Athene noctua	Strigiformes	Strigidae	Brain	Germany	Europe	100% identity with JQ733513	[9]*
Nannopterum brasilianum	Suliformes	Phalacrocoracidae	Pectoral muscles	Brazil	South America	MW822663	[35]
Phalacrocorax carbo	Suliformes	Phalacrocoracidae	Muscles	Lithuania	Europe	JQ733513	[10]
Definitive host							
Accipiter gentilis	Accipitriformes	Accipitridae	Intestine	Lithuania	Europe	OQ848696–705	[46]
Accipiter nissus	Accipitriformes	Accipitridae	Intestine	Lithuania	Europe	OQ848706–710	[46]
Buteo buteo	Accipitriformes	Accipitridae	Intestine	Lithuania	Europe	PP937502–12	unpublished
Buteo jamaicensis	Accipitriformes	Accipitridae	Intestine	USA	North America	OK576418, OK576450, OK576458	[51]*
Halietus albicila	Accipitriformes	Accipitridae	Intestine	Norway	Europe	MF946589–96	[14]
Milvus milvus	Accipitriformes	Accipitridae	Intestine	Czech Republic	Europe	MZ333537	[48]
Coleus monedula	Passeriformes	Corvidae	Intestine	Lithuania	Europe	OK481240–6	[49]
Corvus corax	Passeriformes	Corvidae	Intestine	Lithuania	Europe	OK481202–19	[49]
Corvus cornix	Passeriformes	Corvidae	Intestine	Lithuania	Europe	OK481220–39	[49]
Pica pica	Passeriformes	Corvidae	Intestine	Lithuania	Europe	OK481247–9	[49]

Table 4. Summarized data on molecular identification of *S. halieti* in different intermediate and definitive hosts based on ITS1 sequences. * Based on *28 S* rRNA.

interspecific differences with ITS1 overlap[39]. Thus, ITS1 is not an appropriate locus for the genetic characterization of *Sarcocystis* spp. from ungulates[30,31,39]. In contrast, the intraspecific variability of avian *Sarcocystis* spp. within ITS1 is relatively low and does not hinder species identification. In general, no or minor (up to 0.1%) intraspecific variation of avian *Sarcocystis* spp. was observed within rRNA and *cox*1 loci. Whereas intraspecific differences of these *Sarcocystis* species in ITS1 do not exceed 3%. Furthermore, based on ITS1, the minimum interspecific difference between two valid *Sarcocystis* species that are characterized by a bird-bird life cycle is not less than 5%[41]. So, ITS1 is a suitable locus for the discrimination of these *Sarcocystis* species. Notably, the highest variability in ITS1 among avian *Sarcocystis* spp. was observed particularly for *S. halieti*[10,11,40–42]. These findings enabled us to perform a detailed population genetic analysis of *S. halieti* samples based on the aligned ITS1 sequences retrieved from GenBank. The median-joining network analysis indicated that several sequences of *S. halieti* from Brazil represent separate evolutionary lineages (Fig. 4). Furthermore, some evolutionary lineages were observed only in intermediate hosts of *S. halieti*. Analyses of genetic variability and genetic differentiation also showed that some samples of *S. halieti* genetically differ depending on geographical and host factors. In general, investigations on the population genetics of *Sarcocystis* species are limited. The intraspecific genetic variability of these parasites has been examined using amplified fragment length polymorphism (AFLP), microsatellites, and DNA sequence data[43–45]. The dependence of genetic differences on the geography of *Sarcocystis* parasites has been reported previously[1,2,4]. Furthermore, it was shown that *Sarcocystis* species parasitizing the same host may differ in the level of genetic variability[11]. However, the genetic variability pattern of the same *Sarcocystis* species in different hosts is inadequately understood and should be further examined. More parasite isolates from the same geographical area but from different hosts would be desirable for such studies.

Recording the Eurasian sparrowhawk as the intermediate host of *S. halieti* is of significance since this bird species was confirmed as the definitive host of *S. halieti* very recently[46]. In Lithuania, 5/16 (31.3%) of the intestinal scraping samples of Eurasian sparrowhawks were positive by molecular analysis for sporocysts/oocysts of *S. halieti*. In addition to the Eurasian sparrowhawk, sarcocysts of *S. halieti* were also found in the muscles of five more species of birds of prey belonging to the families Accipitridae and Strigidae[6,9,42,47]. To the best of our

knowledge, including this study, *S. halieti* has been detected in 16 species of birds belonging to seven families and six orders (Table 4)[6,9–11,35,40–42,47,48]. Our study presents the first identification of *S. halieti* in Asia while it was previously found in the intestines of 10 birds from the families Accipitridae and Corvidae in Europe and North America (Table 4)[14,27,46,48–50]. However, in the case of corvids, oocysts but not sporocysts were observed in the intestines of birds[49]. Since the genus *Sarcocystis* differs from other members of the family Sarcocystidae, such as *Toxoplasma*, *Besnoitia*, *Hammondia*, *Neospora*, etc., by endogenous sporulation of oocysts in definitive hosts[2,51], therefore, transmission experiments are needed to clarify whether corvids are true definitive hosts of *S. halieti* and several other *Sarcocystis* spp. forming sarcocysts in the muscles of birds. Intriguingly, three bird species, Eurasian sparrowhawk, common raven (*Corvus corax*), and hooded crow (*Corvus cornix*), might potentially serve as both intermediate and definitive hosts of *S. halieti*[41,46,49].

Indeed, the phenomenon of the same species of *Sarcocystis* serving as both hosts is extremely rare among birds and was previously described only for corvid-associated *S. cornixi* and *S. kutkienae*that were found in both the muscles[37,41,52]and intestines[49] of these omnivorous birds. As discussed above, the latter findings should be checked using transmission experiments. Nevertheless, the phenomenon that some *Sarcocystis* species might use the same bird species to complete their life cycle, encompassing both sexual and asexual phases, should be addressed in further studies.

Since the description of *S. halieti*in 2017[14], new host records have proliferated over several years in different countries and avian families (Table 3). Since this species can form cysts not only in muscles but also in the brain of intermediate hosts and encephalitis, most likely caused by *S. halieti* has been reported in the little owl (*Athene noctua*) in Germany[9], further research on *S. halieti*, covering host diversity, intraspecific variability, and histopathology, are needed.

There were some limitations in the present study, such as molecular detection and characterization of *Sarcocystis* only for the macrocysts and not in different muscle and brain tissues and feces because of resources, and also the impossibility of histopathological and electron microscopy because of the bird's condition. However, the strength is that we could establish a multi-loci PCR system for the detection of *Sarcocystis* in domestic and wild animals and birds for the first time in Iran that can facilitate future molecular-based studies in the country and region.

Conclusions
Herein, we report the first data about the presence and possible circulation of *S. halieti* in Asia as confirmed by *18 S* rRNA, *28 S* rRNA, *cox*1, and ITS1 sequences obtained from macroscopic cysts in the pectoral muscle of the Eurasian sparrowhawk in Iran. We also prove that sparrowhawk acts as both the definitive and intermediate host of the parasite. The phenomenon of the same species of *Sarcocystis* serving as both hosts is extremely rare among birds and needs more future investigations. Considering the shortage of information about the occurrence, prevalence, and distribution of *Sarcocystis* species infecting avian hosts in Iran, further studies, especially PCR and DNA sequencing studies on wild birds, are recommended.

Methods
Study area and sampling
Birds included in this study were examined between September 2019 and December 2023 in Hamedan, Iran (34.7983° N, 48.5148° E) (Fig. 5). Hamedan has a hot-summer Mediterranean climate (Köppen-Geiger classification Csa) with a yearly rainfall of ca. 443 mm. The birds were either euthanized by the Provincial Department of Environment because of general health failure or were found dead in the environment. The time lapse from death to collection of birds could not be estimated; however, only fresh carcasses were examined. Individual birds were sent to the Laboratory of Parasitology, Faculty of Veterinary Medicine, Bu-Ali Sina University, in sealed plastic bags for examination. The bird identifications were made using the reference book *Atlas of Birds of Iran*[15]. The birds underwent a standard examination for searching chewing lice (Published elsewhere)[53] and then were skinned for the observation of macroscopic sarcocysts. Detected macrosarcocysts were cut with a 1–2 mm margin with sterile scalpel blades and preserved in sterile Eppendorf tubes at −20 C until further examination.

DNA extraction, PCR protocols, and sequencing
Tissue specimens (ca. 200 mg) were minced using sterile surgical scalpel blades in disposable plastic Petri dishes, then transferred into 1.5 Eppendorf microtubes and homogenized using disposable plastic applicators, followed by three freeze/thaw cycles. Finally, the homogenates were vortexed with sterile glass beads. Genomic DNA was extracted using FavorPrep™Tissue Genomic DNA Extraction Mini Kit (Favorgen, Pingtung, Taiwan) according to the manufacturer's instructions. DNAs were tested for the presence of *Sarcocystis* spp. DNA by conventional PCRs targeting fragments of the *18 S* rRNA of apicomplexan parasites, e.g., *Toxoplasma gondii*, *Neospora caninum*, eimeriid coccidia, isosporid coccidia, *Sarcocystis*, *Cryptosporidium* and *Hammondia*[51], *cox*1[32], *28 S*rRNA[29,36], and ITS1[29,50]. The primers and conditions utilized in this study are described in Table 5. PCRs were performed using 2 μL from the prepared DNA as the template with the Taq DNA Polymerase Master Mix RED (Ampliqon, Odense, Denmark) in a SimpliAmp thermal cycler (Applied Biosystems, Waltham, MA, USA). For all reactions, the DNA of *Sarcocystis bovifelis* isolated from one naturally-infected cattle slaughtered in the Hamedan industrial abattoir served as a positive control, and distilled water as the negative control.

The PCR amplification products were documented using a UV Imager (Transilluminator, Vilber Lourmat, France) after electrophoresis in a 1% agarose gel (SinaClon, Tehran, Iran) gels stained with DNA Safe Stain (SinaClon, Tehran, Iran) at 100 V for 60 min. The cPCR amplicons were sequenced by an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, MA, USA) in Codon Genetics Laboratory (Tehran, Iran).
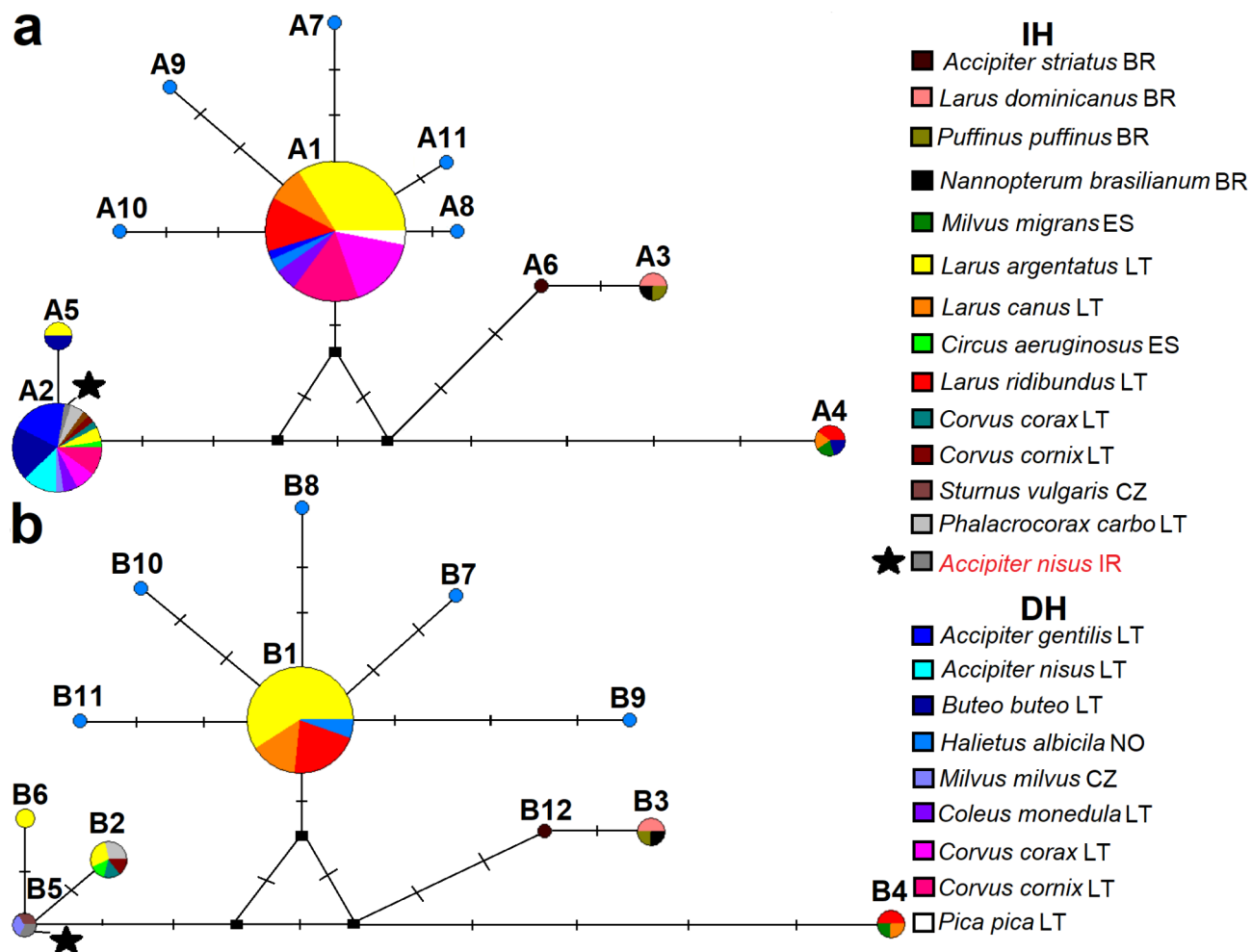
Fig. 5. A median-joining network of *S. halieti* based on 351 bp (a) and 428 bp (b) ITS1 fragments. Hypothetical not-determined genotypes are represented by black rectangles. The circle area is proportional to the genotype frequency (A1 was identified for 97 sequences, A6-A11, B7-12 were detected for a single sequence each). Dashes display mutational steps. The different colors represent different hosts and geographic origin. BR Brazil, ES, Spain, LT Lithuania, CZ Check Republic, IR Iran, NO Norway.

Pathogen	Target	Primers	Reference
Apicomplexa	*18 S* rRNA	COC-1: 5'- AAGTATAAGCTTTTATACGGCT-3' COC-2: 5'- CACTGCCACGGTAGTCCAATAC-3'	51
Sarcocystis spp.	*cox*1	SF1: 5'- ATGGCGTACAACAATCATAAAGAA-3' SR5: 5'-TAGGTATCATGTAACGCAATATCCAT-3'	32
Sarcocystis spp.	*28 S* rRNA	KL1: 5'- TACCCGCTGAACTTAAGC-3' LS2R: 5'- GACTTCATTCTACTCAGGCATA-3'	36 29
Sarcocystis spp.	ITS1	SU1F: 5'-GATTGAGTGTTCCGGTGAATTATT –3' 5.8SR2: 5'-AAGGTGCCATTTGCGTTCAGAA-3'	29
Sarcocystis halieti	ITS1	GsShalF: GATAATTGACTTTACGCGCCATTAC GsShalR2: CCATCCCTTTTTCTAAAGGAGGTC	49

Table 5. Oligonucleotides used for the identification of the *Sarcocystis* isolated from the Eurasian sparrowhawk (*Accipiter nisus*).

Phylogenetic analysis

The generated DNA sequences were checked for their quality with the help of Chromas 2.6.5 (https://technelysium.com.au/wp/chromas/). Afterward, sequences obtained with forward and reverse primers were assembled into consensus sequences using freely available CLC Sequence Viewer v. 8.0 (QIAGEN, Aarhus, Denmark). The resulting sequences were compared with those of various *Sarcocystis* spp. available in NCBI GenBank using

the nucleotide BLAST, Mega BLAST, and discontiguous Mega BLAST options (http://blast.ncbi.nlm.nih.gov/, accessed on 16 August 2024)[54].

The phylogenetic analysis of the *28S*rRNA and ITS1 region was performed using MEGA11 v. 11.0.13 software[55]. Based on BLAST comparison, it was demonstrated that our sequences showed the highest similarity with those of *Sarcocystis* spp. using birds as their intermediate and definitive hosts. Therefore, these sequences have been taken as ingroup. Meanwhile, sequences of *S. rileyi* forming macrocysts in the muscles of ducks and transmitted via predatory mammals were chosen as the outgroup for both phylogenetic analyses. The ClustalW algorithm incorporated into MEGA11 was used to get multiple alignments. The final alignment of the partial *28S*rRNA fragment contained 19 sequences and 846 nucleotide positions, including gaps. The alignment of the partial ITS1 region lacking the 5' part consisted of 31 sequences and 528 nucleotide positions, including gaps. The phylogenetic trees were reconstructed using the Maximum Likelihood (ML) method. The Tamura 3 parameter + G (gamma distribution) evolutionary model[56] was suggested by MEGA11 based on the calculated lowest Bayesian Information Criterion values of checked models. The robustness of the resulting phylogenies was tested using the bootstrap test (1000 bootstrap replicates).

The analysis of intraspecific genetic variability

To assess geographic, intermediate, and definitive host factors for the intraspecific evolution of the identified *Sarcocystis* species, we performed a phylogenetic network analysis using NETWORK v. 10.2.0.0 software (https://www.fluxus-engineering.com/sharenet.htm, accessed on 16 August 2024). DnaSP v. 6 software[57] was used for the estimation of intraspecific genetic variability of the identified *Sarcocystis* species. The number of haplotypes (h), the number of polymorphic sites (S), the average number of nucleotide differences (K), the haplotype diversity (Hd), the nucleotide diversity (π), and the standard deviation (SD) for Hd and π were calculated for several subsets divided by the hosts and geographic origin of the parasite. The genetic differentiation between selected sample pairs was evaluated with Arlequin v. 3.5.2.2[58] using fixation index (F_{ST}). The significance of the resulting F_{ST} values was tested by 10,000 permutations at the 95% confidence level.

Data availability

The datasets generated and analyzed during the current study are available in the NCBI—GenBank—Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession numbers: PQ270244 for *18 S* rRNA, PQ270246 for *28 S* rRNA, PQ276104 for ITS1, and PQ274874 for *cox*1. Any additional data are available from the corresponding author, [AS] on request.

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Declarations

Consent for publication
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Consent to participate
Not applicable.

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