



# First Histopathological and Molecular Characterization of Giant Thorny-headed Worm, *Macracanthorhynchus hirudinaceus* (Pallas, 1781) (Acanthocephala: Oligacanthorhynchidae) in Wild Boars, *Sus scrofa* Linnaeus, 1758 from Eastern Türkiye

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## Abstract

**Background** *Macracanthorhynchus hirudinaceus* (Pallas, 1781) is a zoonotic acanthocephalan that parasitizes the small intestine of wild boars. It is a pathogenic that causes economic losses, and poses a public health threat due to increased emergence.

**Purpose** The aims of this study is describes histopathologically the damage caused by *M. hirudinaceus* in the small intestine of wild boar *Sus scrofa* Linnaeus, 1758, and molecularly characterize this parasite (sequences, haplotypes, phylogeny) for the first time in Elazig city, Türkiye.

**Results** A high prevalence of infection was obtained. Upon separating the worms, it was discovered that there were ulcers resembling craters in the center, of the small intestine mucosa, surrounded by edema. The intestine wall where the parasite attached was damaged, with the villi epithelium and lamina propria in the mucosa being destroyed. The genomic DNA was isolated from all *M. hirudinaceus* samples, and PCR amplified the 489 bp gene fragments were sequenced and confirmed that all 21 sequences were *M. hirudinaceus*. The haplotype analysis of the sequences revealed the presence of a central star-shaped haplotype, in addition to four other haplotypes.

**Conclusion** After conducting sequence analysis, the genetic differences between the *M. hirudinaceus* sequences obtained in this study and those reported from Europe and Japan suggest that this parasite is endemic to Türkiye's local wild boar population. Also, four haplotypes were identified, distinguishing it from other haplotypes by 1–5 mutation steps. It is essential to consider the worm's sequences and the formation of haplotypes, since these intrinsic characteristics may impact in the epidemiology and pathology of the worm in the future.

**Keywords** *Macracanthorhynchus hirudinaceus* · Wild boar · Sequence · Haplotype

## Introduction

Wild boars, *Sus scrofa* Linnaeus, 1758 are recognized as one of the most hostile animal in the world due to their impact on the environment, agriculture, and natural habitats. They are also significant reservoirs of infectious diseases that can be transmitted to animals and humans. Pigs are omnivorous and can also host many parasitic infections as they consume all animals they can easily capture, including arthropods, birds, mice, and small amphibious reptiles [1].

Wild boars are distributed throughout Eurasia, some areas of North Africa and America [2]. It is estimated that the wild boar population has been steadily increasing, particularly in the last decade from Türkiye [3]. The giant

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acanthocephalan, also known as the giant thorny-headed worm *Macracanthorhynchus hirudinaceus* (Pallas, 1781), is a parasite that can affect many animals, including canids, swine, birds, and humans [4]. The final hosts are pigs and wild boars. Female parasites excrete eggs containing larval acanthocephalan worms in their faeces. The insect, usually the dung beetle, acts as an intermediate host, eating the eggs from which the acanthor develops into an acanthocephalan and then a cystacanth. The final host is infected by eating insects or their parts, which contain cystacanths that become adults. Carnivores and primates, including humans, can become infected [5, 6]. Acanthocephaliasis causes abdominal discomfort and digestive problems. This is because the parasite's proboscis damages the intestinal wall [7]. The attachment of *M. hirudinaceus* to the intestinal wall in the host is done using its proboscis, which may cause inflammation and development of granulomas at the attachment site. Severe infections may cause catarrhal enteritis or lead to ulcerative necrosis accompanied by inflammation of the submucosa [8].

The length of female worms can reach up to 40 cm, and males measure up to 10 cm long [9]. In severe cases, perforation of the intestinal wall may cause fatal peritonitis [10, 11]. This parasite is frequent in free-range pigs, considerably affecting animal health and production [12, 13]. Over the past two decades, there has been increasing recognition of a zoonotic parasitic disease linked to unsanitary practices [4]. Wild boar populations have increased recently, particularly in Europe, encroaching on crops, pastures, and peri-urban areas. In these regions, the animals may be susceptible to infection with the parasite, which could result in the transmission of the parasite to other animals and humans [14, 15].

Despite Türkiye's vast wild boar population and wide distribution, research on parasites remains scarce. A study by Merdivenci (1964) revealed the presence of three *M. hirudinaceus* in the small intestine of a domestic pig that had been slaughtered in Istanbul [16]. In a study by Senlik et al. (2011), 27 wild boars in the Bursa province of Türkiye were examined and found positive for *M. hirudinaceus* with a prevalence of 19% [17]. However, there is a lack of information regarding the molecular characteristics of this parasite in wild boars from Türkiye. Therefore, it is crucial to identify the distribution of zoonotic parasites in wildlife. This study aims to determine the prevalence of *M. hirudinaceus* in wild boars, reveal its histopathological characteristics, and perform molecular characterization of this species from Türkiye.

## Materials and Methods

### Study Area and Sample Collection

This study examines wild boars collected by hunters during the hunting season in rural areas of three districts in Elazığ province, Türkiye between January 2022 and December 2023. No Ethics Committee approval was required as wild boars were not sacrificed for research. Immediately after the wild boars were hunted, the location was shared with the research team, and the team arrived in the area within 12 h at the latest and carried out the systemic necropsy there. After conducting a general organ examination of the wild boars, of which all were adults, digestive tracts were collected in individual bags and, then transported to the Parasitology Laboratory of Firat University's Faculty of Veterinary Medicine. In the laboratory, the intestines were cut longitudinally with the help of a pair of scissors and all adult parasites were removed from the intestine as a whole, placed in falcons and washed several times with distilled water. The adults of *M. hirudinaceus*, which were cleaned from faeces and other tissue parts, were stored in 70% ethanol at -20 °C until they were used in the molecular studies. Then, for histopathological examinations, tissue samples from lesioned intestinal sections were taken and placed in 10% formalin.

### Histopathological Examination

After the tissues in formalin are washed with tap water, dehydrated, embedded in paraffin and sectioned at 5 µm following standard histological techniques. Sections were stained with haematoxylin–eosin (H&E). The sections were examined under a light microscope. All techniques used were based on standardized protocols of the Firat University, Faculty of Veterinary Medicine, Pathology Laboratory [18].

### Molecular Studies

Tissue samples were obtained from smaller pieces of individual worms. Subsequently, the pieces were transferred to 1.5 mL eppendorf tubes and washed at least five times with 600 µL of 1X PBS solution. Genomic DNA (gDNA) was isolated using the Hibrigen Tissue Kit (Ankara, Türkiye) with minor modifications. Initially, 500 µL of DHP Buffer (lysis buffer) was added to the eppendorf tubes containing the parasites, which were then mixed using a vortex. Subsequently, 20 µL of proteinase K and 200 µL of DA buffer were added. Subsequently, the mixture was incubated in a water bath at 65 °C for a period of 24 hs. We obtained 100 µL of gDNA solution and stored at -20 °C until the analysis, according to the manufacturer's instructions. The

gDNA was amplified by the polymerase chain reaction (PCR) using newly designed oligo-nucleotide primers from the mitochondrial cytochrome oxidase subunit 1 (mt-CO1) sequence of *M. hirudinaceus* (OR168977). The primer sequences are Forward: Mh-F (5'-TAACAGTTCCGGTGT TTGGCA-3'), Reverse: Mh-R (5'-TCGACACACAATAA CCCCCG TC-3'). The PCR reaction components were as follows: to prepare the PCR mix, add 5 µl of 10X PCR buffer, 2 µl of 50 mM MgCl<sub>2</sub>, 400 µM of each dNTP, 20 pmol of each primer, 0.2 µl of TaqDNA Polymerase (1.25 IU) and 31.8 µl of PCR-grade water. Then, add 5 µl of each gDNA sample to the PCR mix. The thermal profile for the PCR reaction was as follows: a pre-denaturation step at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 50 s, annealing at 52 °C for 50 s, and extension at 72 °C for 50 s. The reaction was completed with a final extension at 72 °C for 10 min. We then checked the PCR products using electrophoresis on a 1.4% agarose gel. Finally, we performed one-way (using with forward primer) DNA sequence analysis on the amplified PCR products using BM Labosis (Ankara, Türkiye).

### Phylogeny and Haplotype Analyses

Sequence results were observed in ab1 format using the 1.4.0 tool in FinchTV (Geospiza Inc., Seattle, Washington, USA) (<http://www.geospiza.com>). Sequences here obtained were compared with haplotypes of mt-CO1 sequences of

**Table 1** Haplotypes of mt-CO1 sequences of *Macracathorhynchus Hirudinaceus* (Pallas, 1781), (Acanthocephala: Oligacanthorhynchidae) from the wild boar *Sus scrofa* Linnaeus, 1758 province, Türkiye and accession numbers of isolates forming groups

No	Haplotype name	Number of Isolates	Isolate Codes (Accession Numer)
1	Hap1	15	Mh01(PP112603) Mh04(PP112606) Mh06(PP112608) Mh08(PP112610) Mh09(PP112611) Mh10(PP112612) Mh13(PP112615) Mh14(PP112616) Mh15(PP515291) Mh16(PP515292) Mh17(PP515293) Mh18(PP515294) Mh19(PP515295) Mh20(PP515296) Mh21(PP515297)
2	Hap2	4	Mh02(PP112604) Mh05(PP112607) Mh07(PP112609) Mh11(PP112613)
3	Hap3	1	Mh03(PP112605)
4	Hap4	1	Mh12(PP112614)

*M. hirudinaceus* occurring in wild boar from different geographical regions (Table 1). The concatenated alignments were performed using MAFFT Version 7 software (<https://mafft.cbrc.jp/alignment/server/index.html>). Phylogenetic molecular analyses were conducted on the aligned mt-CO1 sequences and were inferred by both Maximum-Likelihood (ML) method using MEGA10 [19]. The phylogenetic tree model was determined using the reference sequence of this species (MZ683370, OR168977, NC\_019808, FR856886 and LC350021) and *Oncicola luehei* (JN710452) was included as an outgroup. Regarding ML, to determine the nucleotide substitution model that gave the best fit to our data set, the MEGA 10 software which held the Automatic (Neighbor-joining tree) test analysis was employed, with model selection based on the Akaike information criterion (AIC). The sequence alignment was conducted using ClustalW within the MEGA 10 program. Results indicated that the Hasegawa-Kishino-Yano model (HKY) was the most appropriate. The percentage of trees in which the associated taxa clustered together is shown above/below the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites (+G, parameter=5 rate categories). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches). This analysis involved 454 bp nucleotide sequences, and there. The ML statistical method was employed, with a bootstrap test of 1000 replicates was used to generate the evolutionary tree [19]. The phylogenetic analysis matched other statistical data and the haplotype network. Hap2, Hap3, and Hap4 are other groups that are relatively distantly related.

Haplotype analysis was done using the DnaSP6 program [20]. Population diversity index values were calculated, including haplotype number (h), nucleotide diversity ( $\pi$ ), and haplotype diversity (Hd). Neutrality indices were also calculated, including Tajima's D and Fu's statistics (Table 2). MSN was used to create the network with PopART-1.7 software [21]. The nucleotide diversity was studied by comparing with the reference sequences using phylogenetic analysis.

## Results

### Histopathological Description

A total of 25 wild boars were necropsied for this study, and adult *M. hirudinaceus* was found in 21 of them. Between 1 and 5 adult parasites were obtained from each infected animal.

Macroscopically, yellow-white coloured, hard, nodule-like formations with diameters ranging from 2 to 6 mm were

**Table 2** Diversity and neutrality indices obtained using nucleotide data of the *Macracanthorhynchus hirudinaceus* (Pallas, 1781), (Acanthocephala: Oligacanthorhynchidae) from the wild boar *Sus scrofa* Linnaeus, 1758 from Elazığ Province, Türkiye mt-CO1 gene (454 bp)

mt-DNA	n	hn	hd ± S.D	πd ± S.D	Tajima's D	P value	Fu's F	P value	FLD	P value	FLF	P value
COI	21	4	0,471 ± 0,116	0,00155 ± 0,00059	-1,48564	P > 0.10	-0,751	0,198	-2,05593	0.10 > P > 0.05	-2,18914	0.10 > P > 0.05

n: number of isolates; hn: number of haplotypes; hd: haplotype diversity; πd: nucleotide diversity; πd: nucleotide diversity; πd: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F statistics test. Not significant, P > 0.10; Not significant, 0.10 > P > 0.05

observed on the serosal surface of the small intestine, surrounded by a red halo caused by haemorrhage (Fig. 1A). Upon opening the intestinal lumen, it was observed that the worm bodies were free in the luminal cavity (Fig. 1B) and adhered to the intestinal mucosa with their proboscis thinner than the body (Fig. 1C). Upon separation of the worms, the mucosa was found to have crater-like ulcers with oedematous surroundings and crater-like ulcers in the centre.

It was observed that the worm had penetrated the submucosa and muscularis mucosae layers. At the site of attachment, fat cell infiltration, giant cell formations, capillary congestion and fat cell infiltration were observed in all intestinal layers, with the exception of the serosa (Fig. 1D). It was observed that the structure of the intestine at the site of parasite attachment was disrupted, and that the villi and epithelium of the intestinal mucosa were completely destroyed (Fig. 1E). In the nodular formations observed in the small intestine, a severe inflammatory reaction with necrosis and calcification was observed in the centre, surrounded by lymphocytes and histiocytes, with a lesser presence of eosinophils (Fig. 1F).

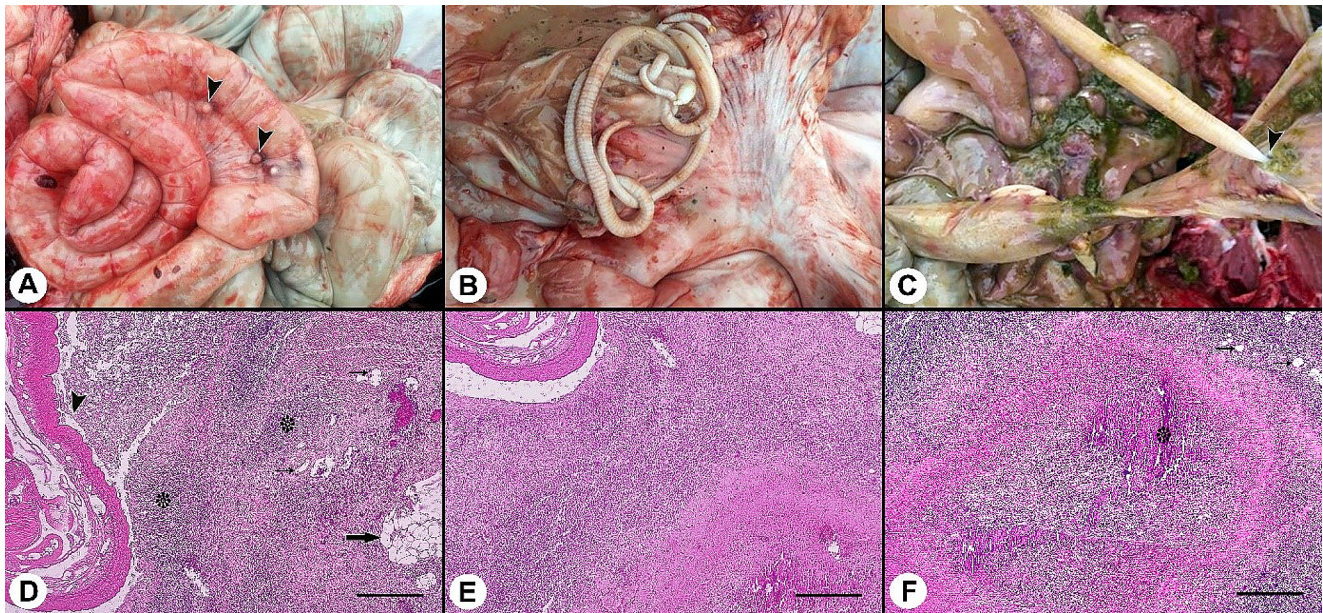
## Molecular Analysis

The genomic DNA was isolated from all *M. hirudinaceus* isolates, and PCR amplification of mt-CO1 gave 489 bp band for each one. Following the forward sequence analysis, sequence ends were trimmed, and all were aligned to 454 bp. BLAST analysis of the sequences confirmed that all 21 sequences were *M. hirudinaceus*. The nucleotide sequences obtained were submitted to GenBank and the accession numbers registered were PP112603 - PP112616 (Mh01-Mh14) and PP515291 - PP515297 (Mh15-Mh21) (Table 1).

The haplotype analysis of CO1 sequences obtained from *M. hirudinaceus* isolates revealed the presence of a central star-shaped haplotype, in addition to four other haplotypes. These four haplotypes were identified as distinct from the central star-shaped haplotype by a single to five mutation steps, and collectively accounted for 71.42% (15/21) of all samples (Fig. 2). While five polymorphic sites were detected in these sequences, one was detected as parsimony informative. Two of the four haplotypes formed a single haplotype (Table 2). Genetic divergence between the haplotypes identified in this study and previously published sequences was determined by pairwise analysis and shown in Table 3.

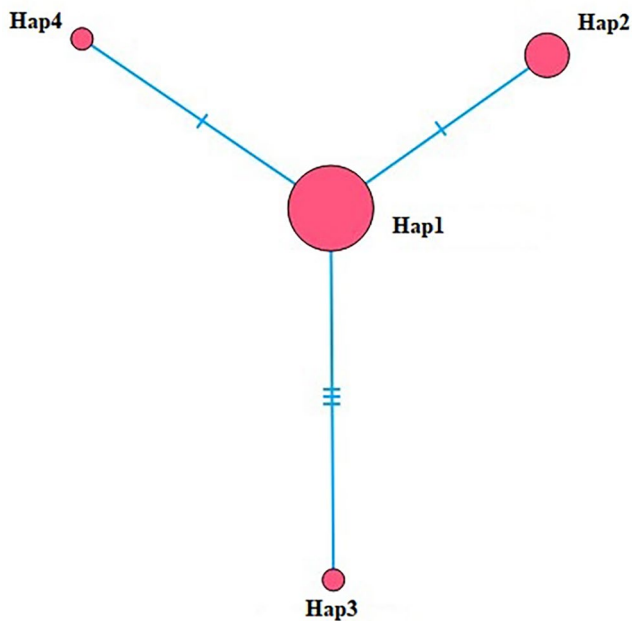
The phylogenetic tree view, created by aligning *M. hirudinaceus* mt-CO1 gene sequences, is presenting in Fig. 3.





**Fig. 1** A. Nodule formations (arrow heads) on the serosal surface of the small intestines, B. Adults *Macracanthorhynchus hirudinaceus* (Pallas, 1781), (Acanthocephala: Oligacanthorhynchidae) from the small intestine lumen of wild boar *Sus scrofa* Linnaeus, 1758 from Elazig province, Türkiye, C. *Macracanthorhynchus hirudinaceus* attached (arrow head) in the intestinal mucosa, D. Severe cell infiltra-

tion (asterix) and lipid cell infiltrations (big arrow) in muscular and submucosal layers, inset: giant cell formations E. Destruction of villus epithelium (inset) and capillary congestions in lamina propria (small arrows) F. Severe inflammatory reaction with necrosis and calcification (asterix) in nodule, Bar = 500  $\mu$ m, HE x 20



**Fig. 2** The appearance of mt-CO1 haplotypes of wild boar isolates of *Macracanthorhynchus hirudinaceus* (Pallas, 1781), (Acanthocephala: Oligacanthorhynchidae) from the wild boar *Sus scrofa* Linnaeus, 1758 from Elazig province, Türkiye. The size of the circles is related to the haplotype frequency. Small circles indicate additional mutational areas. The numbers in the figure indicate the number of mutations

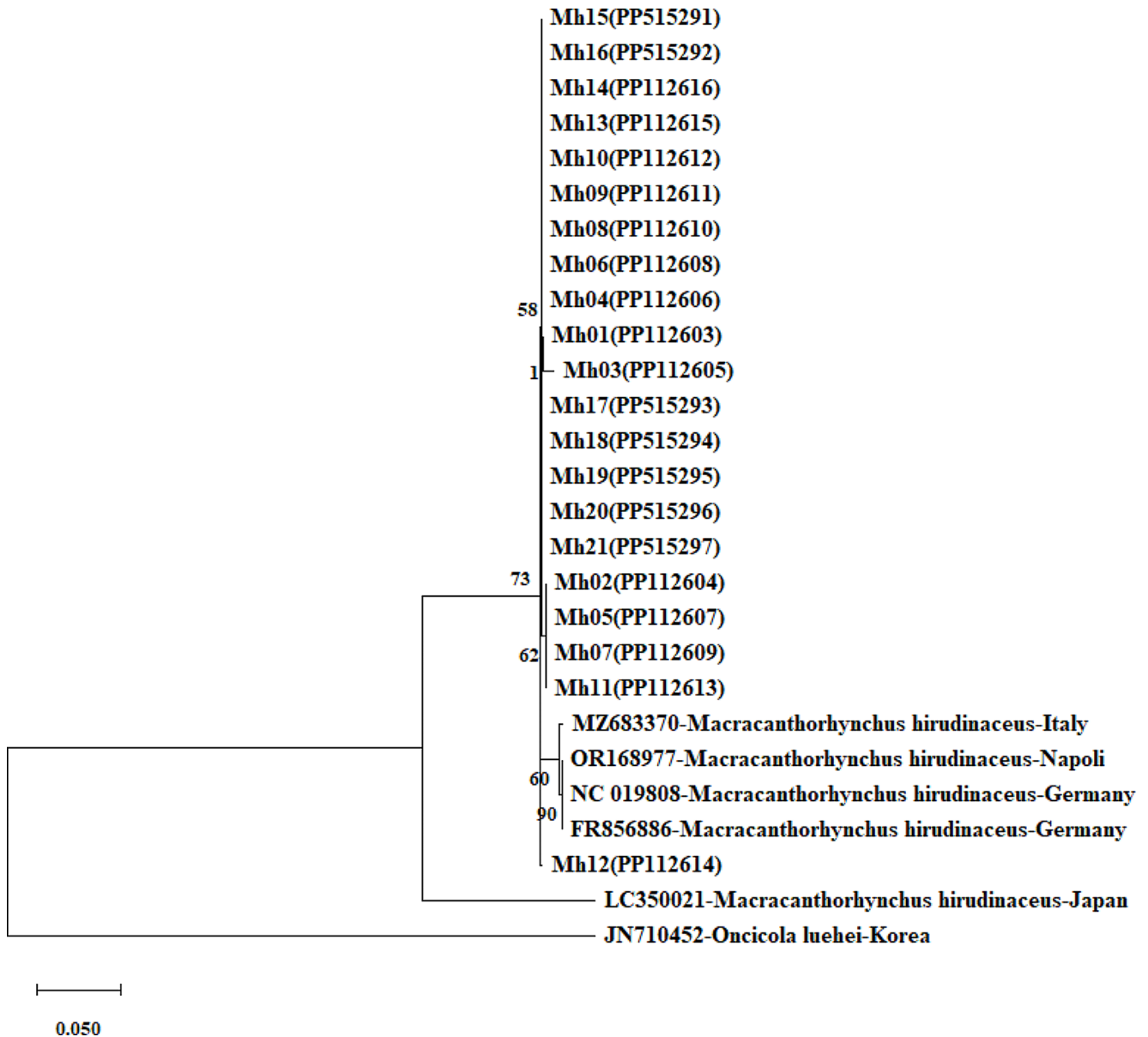
## Discussion

Wild boars eat small animals such as snails and worms, as well as larger animals such as hedgehogs and rabbits. They can become infected with *M. hirudinaceus* by ingesting cystacanths, especially when they eat scarabaeoid or hydrophilid insects, and can act as a reservoir for this parasite that can be transmitted to humans. Thus, wild boars in rural communities may pose a risk for disease transmission [1]. Merdivenci (1964) found three *M. hirudinaceus* in the small intestine of a domestic pig in Istanbul [16]. Senlik et al. (2011) examined 27 wild boars in Bursa province of Türkiye and found that 19% were positive for *M. hirudinaceus* [17]. The study found a high prevalence in various regions, including Italy (9.4%) [22], Sicily (11.1%) [23], Spain (12.1%) [24], Morocco (12.1%) [25], Argentina (13.5%) [26]. As can be seen, the prevalence of *M. hirudinaceus* was found to be below 20% in studies conducted both in Türkiye and in other countries. However, interestingly, *M. hirudinaceus* was found in 21 of 25 wild boars necropsied in our study. This may be related to the increasing wild boar population in the region (as stated by the villagers) and the survival of dung beetle larvae in the soil due to the warmer and rainy winter season.

Migliore et al. (2021) [23] found in the intestinal histopathology of wild boar infected with *M. hirudinaceus* a variable number of small round nodules surrounded by

**Table 3** Genetic divergence between the haplotypes identified in this study and previously published sequences using pairwise analysis

	Hap01	Hap02	Hap03	Hap04
Hap01				
Hap02	0,00220			
Hap03	0,00670	0,00895		
Hap04	0,00220	0,00220	0,00670	
OR168977- <i>M.hirudinaceus</i> -Italy	0,01339	0,015669	0,02039	0,013399
MZ683370- <i>M. hirudinaceus</i> -Italy	0,01339	0,015669	0,02039	0,013399
NC_019808- <i>M.hirudinaceus</i> -Italy	0,01339	0,015669	0,02039	0,013399
FR856886- <i>M. hirudinaceus</i> -Italy	0,01339	0,015669	0,02039	0,013399
LC350021- <i>M. hirudinaceus</i> -Japan	0,12184	0,124740	0,131850	0,121842
JN710452- <i>Oncicola luehei</i> -Korea	0,27090	0,275386	0,288879	0,270903

**Fig. 3** Phylogenetic relationships of *Macracanthorhynchus hirudinaceus* (Pallas, 1781), (Acanthocephala: Oligacanthorhynchidae) from the wild boar *Sus scrofa* Linnaeus, 1758 province, Türkiye, and other *M. hirudinaceus*, as inferred from mitochondrial cytochrome c oxidase

subunit 1 (CO1) gene sequences analyzed using Maximum-Likelihood (ML) method. Nodal support is indicated above internodes ML (bootstrap value). The tree is drawn to ML scale, with branch lengths measured in the number of substitutions per site (below the branches)

a hyperemic haemorrhagic halo in the serosa of the small intestine. On the mucosal surface of the small intestine, the nodules showed a slight, centrally located swelling, in most of which the parasite was attached so that the apical part was completely embedded in the nodule. At the site of attachment, the worm penetrated the mucosa, submucosa and part of the muscularis mucosa. The intestinal villi and epithelium were destroyed in this area, as were the underlying mucosa and submucosa. In addition, partial perforation involving all layers of the intestinal wall except the serosa was observed at the site of parasite attachment [23]. In our study, the worm was observed to invade the submucosal and muscularis mucosal layers. It was observed that the intestinal structure at the site of parasite attachment was disrupted and the villi and epithelium of the intestinal mucosa were completely destroyed. However, no perforation of the intestine was observed in our study. Migliore et al. (2021) [23] found that the total body length of the worms ranged from 4.9 to 9.1 cm in males and from 6.1 to 37.4 cm in females. In a few cases, juvenile acanthocephalans (measuring 110–130 mm) were observed. The highest density of *M. hirudinaceus* infection per sample was 19 worms. We did not measure the adult parasites were obtained, but roughly all of them were adult parasites. We found a maximum of 5 adult parasites in one wild boar. Similarly, Sarkari et al. (2016) [7] found a total of 24 adult parasites in 13 infected boars, indicating an average of 2 parasites per animal.

We need more information about the parasite to know if it is causing more problems in wild boars in Türkiye. This is the preliminary study in Türkiye to use the mt-CO1 sequences, which is the most reliable gene region for population studies. The differences between the *M. hirudinaceus* sequences from this study and those from Europe and Japan show that this parasite is in Türkiye's wild boar population. To find out where this isolate came from and how it spread, we need to study it more. This could include looking at genes that change quickly, like the mt-CO1 gene. To do this, we need to get the mt-CO1 sequence of lot of *M. hirudinaceus* isolates from nearby cities or countries. This will help us see how unique this current isolate is compared to others. The study presents the first molecular analysis of *M. hirudinaceus* sequences in a wild boar population from Türkiye. Four haplotypes were identified differ from other haplotypes by one to five mutations. These haplotypes cover 71.42% of all samples.

## Conclusion

This preliminary investigation, which employed histopathological and molecular approaches to characterise *M. hirudinaceus*, a zoonotic acanthocephalan species, in a wild boar

population in Türkiye, indicates an increased prevalence of this parasitic species in Türkiye compared to the limited number of previous studies. Given the paucity of epidemiological and genetic data on this zoonotic parasite in Türkiye, efforts to generate molecular data to identify relationships between different regional and endemic populations should be continued. Conversely, it is of paramount importance to devise efficacious strategies to forestall the transmission of this zoonotic parasite to humans.

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**Author Contributions** FC, SGK, and SC: Data acquisition, material examination, analysis, and manuscript preparation; FC, HKK, and SS: Conceptualization and data analysis; SS: Critical manuscript analysis.

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**Data Availability** All the parasite specimens are deposited at the Molecular Parasitology Laboratory, Firat University Veterinary Faculty, Elazığ, Türkiye, and can be verified after sending a request to the corresponding author.

## Declarations

**Ethical Approval** Ethics committee approval was obtained from the local ethics committee of Firat University Animal Experiments (dated 05.12.2023 and numbered 2023/20–02) and the General Directorate of Nature Conservation and National Parks of the Ministry of Agriculture and Forestry of Türkiye.

**Competing Interests** The authors declare no competing interests.

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