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Molecular evidence of *Macracanthorhynchus hirudinaceus* (Pallas, 1781) in cockchafers in rural areas of Elazig, Türkiye

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Research Paper

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Abstract

The primary definitive host of the giant acanthocephalan, also known as the giant thorny-headed worm *Macracanthorhynchus hirudinaceus* (Pallas, 1781), is *Sus scrofa*. The definitive host ingests the parasite by consuming infected scarabaeoid or hydrophilid beetles. This study aimed to ascertain the presence of *M. hirudinaceus* in the intermediate hosts through molecular analysis. The cockchafers were collected from Elazig province of Türkiye. A total of 30 pools, comprising 10 pools for each of three districts were obtained from cockchafers collected from 10 areas. The gDNA was isolated and PCR was conducted using specific primers which amplify the mitochondrial cytochrome c oxidase subunit 1 (mt-CO1) gene of *M. hirudinaceus*. Then, the PCR-positive samples were sequenced, and phylogenetic and haplotype analyses were performed. A total of 300 cockchafer adults and/or larvae were collected for this study from different regions of three districts (Sivrice, Baskil, and Keban) in Elazig province of Türkiye. No PCR band was observed in any of the samples in Sivrice (0%). However, a total of 16 samples (5.3%), 10 from the Baskil (10%) and 6 from Keban (6%), showed a PCR band of 491 bp. All sequences were confirmed as *M. hirudinaceus*. Two distinct haplotypes were detected at two points. Of the total number of sequences, twelve were found to consist of a single haplotype. One of the two haplotypes was comprised of 10 isolates, while the other included six isolates. This study is one of the limited studies on the molecular identification and haplotyping of *M. hirudinaceus* in cockchafers.

Introduction

The wild boar (*Sus scrofa*) is distributed across Europe, Asia, and North Africa (Wilson 2005). It is estimated that the wild boar population in Türkiye has increased at a steady rate, particularly over the past decade (Ucarli 2011). The giant acanthocephalan, also known as the giant thorny-headed worm *Macracanthorhynchus hirudinaceus* (Pallas, 1781), is a parasite that can affect a wide range of hosts, including canids, pigs, birds, and humans (de Estrada 1997).

The definitive hosts are primarily domestic pigs and wild boars (*Sus scrofa*). The female worms typically release eggs containing larval acanthor in feces, which are ingested by the intermediate hosts. The definitive host is infected by ingestion of insects or their parts containing an infectious cystacanth, which develops into adult male and female Acanthocephalans (Mehlhorn 2016). *M. hirudinaceus* can also be transmitted to humans by accidental consumption of intermediate scarabaeoid or hydrophilid beetle hosts. In many parts of the world, the consumption of wild boar meat can lead to direct human contact with this animal and thus to the transmission of diseases between humans and animals (Masuda *et al.* 2005). There are cases in Türkiye where wild boars are hunted and then illegally offered for human consumption (Akkoc *et al.* 2009).

The attachment of *M. hirudinaceus* to the intestinal wall of the host is achieved through the use of its proboscis, which can result in the development of inflammation and granulomas at the site of attachment. Severe infections may result in catarrhal enteritis or ulcerative necrosis, accompanied by inflammation of the submucosa (Sarkari *et al.* 2016). The length of female parasites can reach up to 40 cm, while males are typically up to 10 cm in length (Mowlavi *et al.* 2006). In cases of severe infection, perforation of the intestinal wall can result in fatal peritonitis (Gassó *et al.* 2016; Taylor *et al.* 2015). *M. hirudinaceus* is a significant parasite affecting the health and productivity of free-ranging pigs (Barbosa *et al.* 2017; Brianti *et al.* 2007). Over the past two decades, there has been a growing recognition of this disease as a zoonotic parasitic illness associated with unsanitary practices (de Estrada 1997).

The wild boar is an omnivore, consuming a diet that includes both plant and animal matter. The diet of the wild boar encompasses a diverse range of species, including smaller animals such as snails, worms, and larvae, as well as larger animals such as hedgehogs and rabbits. The wild boar's preferred plant material includes roots, bulbs, mushrooms, and various forest fruits,

particularly acorns. However, wild boars frequently traverse areas adjacent to human settlements and agricultural operations. In addition to social and economic concerns regarding the damage wild boars may cause to crops, they can also play a significant role in the transmission of various zoonotic helminth species. This is due to their role as reservoir hosts, which allows them to maintain helminths within the parasite's sylvatic cycles, independent of domestic cycles. In rural communities, wild boars are in close contact with farmers and may represent a potential risk for the transmission of zoonotic diseases (Mansouri *et al.* 2016).

Despite the considerable wild boar population and extensive distribution in Türkiye, research on parasites in this species remains inadequate. In a study conducted by Merdivenci (Merdivenci 1964), three *M. hirudinaceus* were identified within the small intestine of a domestic pig that had been slaughtered in Istanbul. In a study conducted by Senlik *et al.* (2011), 27 wild boars were examined in the Bursa province of Türkiye, with 19% of the samples testing positive for *M. hirudinaceus*. Celik *et al.* (2024) conducted necropsies on a total of 25 wild boars in rural areas of Elazig province in Türkiye. Their findings indicated that adult *M. hirudinaceus* was present in 21 of the animals, with between one and five adult parasites collected from each infected animal. The researchers extracted genomic DNA from all *M. hirudinaceus* isolates, amplified the mt-CO1 gene region (489 bp) by PCR, and confirmed all isolates as *M. hirudinaceus* by sequence analysis.

The infection of wild boar with *M. hirudinaceus* has been documented in multiple studies; however, the infection status of intermediate hosts remains incompletely understood. The majority of studies investigating infection of intermediate hosts with larvae have been conducted experimentally. It has been demonstrated that the may beetle species *Melolontha melolontha* and *Melolontha hippocastani* have the potential to act as intermediate hosts for *M. hirudinaceus*, as well as for more than 30 other species of Coleoptera. The discovery of *M. hirudinaceus* in almost all of the wild boars necropsied in our field studies indicates that the molecular determination of the prevalence of this parasite, which is also zoonotic, in the intermediate hosts will contribute to the control studies to be carried out on the parasite by determining the real status of this parasite and its intermediate hosts in our region. Nagy et al. (2015) employed a sampling methodology whereby 50 holes (1×1×0.5 m) were dug in southwest Hungary between February and October 2013. This resulted in the collection of 273 larvae. A microscopic investigation was conducted to ascertain the prevalence of *M. hirudinaceus* larvae in the collected specimens. The results indicated a prevalence of 44.8%.

The infection of intermediate hosts with acanthocephalans has been a topic of extensive study (Kennedy 2006; Schmidt 1985). These studies have revealed a considerable degree of variation in the prevalence of certain species. Only a limited number of publications have addressed the prevalence of larval infection by *M. hirudinaceus* in members of the subfamily Melolonthinae. These studies have indicated that the prevalence may reach 60% (Pavlović *et al.* 2010). The larval development of *M. hirudinaceus* in intermediate host species has previously been investigated through experimental infection, which has confirmed that predominantly Scarabaeidae are intermediate hosts. Furthermore, experimental studies have corroborated the hypothesis that Scarabaeidae (subfamilies Melolonthinae and Dynastinae) may play a pivotal role in the acanthocephalous life cycle (Moore 1942). Conversely, there is a paucity of data concerning natural larval infection by *M. hirudinaceus.* Accordingly, this study aimed to ascertain the presence of *M. hirudinaceus* in the intermediate host cockchafers through molecular analysis.

Material and methods

Sample collection

Ten distinct areas were identified within the rural zones of each district, and the soil was excavated to a depth of one meter by one meter and searched for insects. The larvae and adults of the insects obtained from the soil were transported to the laboratory where they were identified to the genus level (Rana et al. 2022). As a result, larvae and/or adults identified as cockchafer were used in the study, and insects that were considered to belong to other genera were excluded. They were then washed a minimum of three times with 1X PBS (pH=7.4) to remove residual soil and stored at -20°C until genomic DNA (gDNA) isolation.

Genomic DNA isolation and PCR

A total of 30 pools, comprising 10 pools for each of the three districts within Elazig province, were obtained from larvae collected from 10 areas. In each pool, 20 ml of 1X PBS (pH=7.4) was added to the insects, which were placed in a beaker with a maximum of 10 adults and/or larvae. The mixture was then mixed with a magnetic stirrer until homogenized. Subsequently, the mixture was subjected to centrifugation at 5,000 rpm for 10 minutes, after which the supernatant was collected and employed for gDNA isolation. A commercial kit (DiaRex, DIAGEN, Türkiye) was utilized, and gDNA was obtained by the prescribed procedure. PCR was conducted using the genomic DNA obtained from each pool with the specific primers Mh-Forward, 5'-TAACAGTTCCGGTGTTTGGCA-3' and Mh-Reverse, 5'-TCGACACACAATAACCCCGGTC-3', which amplify the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene fragment of *M. hirudinaceus*. To the PCR mixture, which had been prepared in a total volume of 50 µl, the following components were added: 5 µl 10X PCR buffer, 2 µl 25 mM $MgCl_2$, 250 µM each of deoxynucleotides, 1.25 U Taq DNA polymerase, 20 pmol each of primer pairs, 31.8 µl distilled water, and approximately 200 ng of template gDNA. Following the pre-denaturation step at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 50 seconds, annealing at 52°C for 50 seconds, extension at 72°C for 50 seconds, and PCR at 72°C for 10 minutes as the final extension were performed. The PCR products were run in 1.5% agarose gel (containing 10 mg/ml ethidium bromide) at 100 volts for 30 minutes, after which the gel was analysed for the presence of bands under an UV transilluminator. The molecular weight of the bands was determined by the use of a 100 bp marker.

Sequence analysis, alignment, and phylogeny

A unidirectional DNA sequence analysis of PCR-positive samples was conducted at BM Labosis, Ankara, Türkiye. The sequences were displayed using the FinchTV 1.4.0 program (Geospiza Inc., Seattle, Washington, USA) (http://www.geospiza.com). The raw sequence data were subjected to a BLAST search to facilitate comparison with previously published sequences. Any regions deemed to be unreadable were trimmed from both ends. Subsequently, the sequence data were imported into the CLC Sequence Viewer 8 programme. The alignment was performed by comparing the sequence with published reference and outgroup sequences obtained from the NCBI-PubMed database. Subsequently, the sequence data were transferred to the MEGA X programme. The ClustalW program was employed to generate a range of output formats suitable for sequence alignment and subsequent analysis, including PHYLIP, NEXUS, and FASTA. Subsequently, a phylogenetic tree was constructed using the maximum likelihood

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method, as described by Kumar *et al.* (2018). Sequences visualized in DnaSP6 (Rozas *et al.* 2017) with FASTA extension were subjected to haplotype analysis. Haplotype (h), haplotype diversity (Hd), nucleotide diversity (π), and neutrality indices including Tajima's D (Tajima 1989) and Fu's F (Fu 1997) indices of population diversity were determined. Subsequently, the NEXUS extension was obtained from the same program, and the haplotype network was constructed using the Minimum Spanning Network in PopART-1.7 (Leigh *et al.* 2015).

Results

A total of 300 cockchafer adults and/or larvae were collected for this study from different regions of three districts (Sivrice, Baskil, and Keban) in Elazig province. Following PCR using the gDNAs obtained from the pools from the Sivrice district (0 %), no band was observed in any of the samples. Conversely, a total of 16 samples (5.3%) – 10 from the Baskil district (10%) and 6 from the Keban district (6%) – showed a band of 491 bp. Subsequently, all of the aforementioned samples were subjected to sequence analysis. Subsequently, the unread ends of the data set were trimmed, and all sequences were aligned to a length of 450 bp. A BLAST analysis confirmed the sequences as belonging to *M. hirudinaceus*, and the sequences were subsequently submitted to the NCBI database (isolate codes are KMh01-KMh10 and BMh01-BMh06 and accession numbers are PQ517172-PQ517187).

Phylogeny and haplotype analyses

A total of 16 sequences, comprising 450 bp of the mt-CO1 gene region of *M. hirudinaceus*, were subjected to haplotype analysis, resulting in the detection of two distinct haplotypes at two points. Of the total number of sequences, 12 were found to consist of a single haplotype. One of the two haplotypes was comprised of 10 isolates, while the other included six isolates. The haplotype groups, sequences within these groups, and the corresponding accession numbers are presented. The sequences were aligned using the reference sequence from the NCBI database, accession number NC_019808. The sequences within the first haplotype exhibited 98.22% similarity with the selected reference sequence, while the second haplotype demonstrated 98.44% similarity. As a consequence of the alignment process, point mutations were identified, and the nucleotide changes were mapped according to the reference sequence. Accordingly, a total of nine distinct mutations were identified between the two haplotypes. The aforementioned mutations were observed at the following nucleotides: 23, 39, 47, 104, 104, 128, 278, 305, 407, and 437.

The 31 sequences of the mt-CO1 gene region of *M. hirudinaceus* already published in GenBank were downloaded, and nine sequences (one isolate from Japan, two from Germany, and six from Italy) were included in the analysis. As a result of the haplotype analysis, a total of nine haplotypes with 57 point mutations were detected. Six of these were single haplotypes. The major haplotype consisted of 10 isolates, while the minor haplotype consisted of six isolates (Table 1 and Figure 1). Fifty-seven polymorphic regions were detected in mt-CO1 sequences, of which 11 were parsimony informative. The gene region showed lower haplotype diversity and higher nucleotide diversity as shown in Table 2. Tajima's D value, a statistic used to indicate whether a population has undergone expansion and/or purification by selection, was positive for the 16 sequences obtained in this study and negative when the sequences used as reference were added.

Table 1. Haplotype groups, sequences, and accession numbers

	Haplotype name	Number of isolate	Isolate codes (accession number)
This study	Hap_1	10	KMh01(PQ517172) KMh02(PQ517173) KMh03(PQ517174) KMh04(PQ517175) KMh05(PQ517176) KMh06(PQ517177) KMh07(PQ517178) KMh08(PQ517179) KMh09(PQ517180) KMh10(PQ517181)
	Hap_2	6	BMh01(PQ517182) BMh02(PQ517183) BMh03(PQ517184) BMh04(PQ517185) BMh05(PQ517186) BMh06(PQ517187)
Published sequences	Hap_3	1	MZ683370
	Hap_4	1	MZ683372
	Hap_5	1	MZ683373
	Hap_6	1	MZ683374
	Hap_7	1	MZ683375
	Hap_8	3	OR168977 NC_019808 FR856886
	Hap_9	1	LC350021

The observed Fu's Fs value was positive for the 16 sequences in this study and negative for the pooled analysis, indicating the presence of rare haplotypes expected from hitchhiking or recent population growth (Table 2). As a result, 66.6% (6/9) of these haplotype groups were single haplotypes, supporting the above findings. Point mutations among all haplotypes were determined according to the reference sequence in Hap_8 with accession number NC_019808 (Table 3). Accordingly, 57 point mutations were detected among the haplotypes. The phylogenetic tree was constructed using Italian isolates of *M. hirudinaceus* with accession codes MZ683370, MZ683372, MZ683373, MZ683374, MZ683375, and OR168977 and Japan isolate with accession code LC350021, as well as German isolates with accession code NC_019808, FR856886, and the *Oncicola luehei* sequence with accession code NC_016754. The phylogenetic tree was constructed using the TN93 +G+I model in the MEGA X programme with 1,000 bootstrap replicates (Figure 2).

Discussion

There have been several reports of *M. hirudinaceus* (Pallas, 1781) infection in wild boars in Türkiye and various other locations worldwide (Amayour *et al.* 2017; Celik *et al.* 2024; Migliore *et al.* 2021; Papini *et al.* 2018; Senlik *et al.* 2011). However, the available data on the infection status of intermediate hosts is insufficient. The larval infection of intermediate hosts has been the subject of experimental investigation (Moore 1942; Stilesi 1891) or, on rare occasions, in their natural environment (Nagy *et al.* 2015; Pavlović *et al.* 2010). Despite the proposal of over 30 Coleoptera species as

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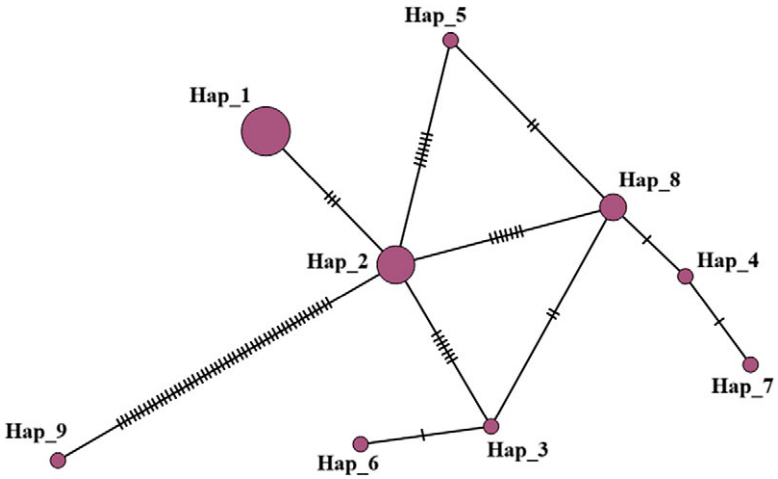

Figure 1. Haplotype network shaped for the mt-CO1 gene region (450 bp) of *Macracanthorhynchus hirudinaceus* isolates. The size of the circles is related to the haplotype frequency. Small circles indicate additional mutational areas. The numbers in the figure indicates the number of mutations.

Table 2. Diversity and neutrality indices obtained using nucleotide data of the mt-CO1 genes of *Macracanthorhynchus hirudinaceus*

	n	H	hd ± SD	πd ± SD	Tajima's D	p Value	Fu's Fs	p Value	FLD	p Value	FLF	p Value
This study	16	2	0,500 ± 0,074	0,00333 ± 0,00049	1,91076	0.10 > P > 0.05	3,877	0,101	1,04435	P > 0.10	1,45615	0.10 > P > 0.05
Published Sequences	25	9	0,790 ± 0,063	0,01822 ± 0,00720	–1,80582	0.10 > P > 0.05	2,619	0,082	–3,47964	**P < 0.02	–3,46566	**P < 0.02

n: Number of isolates; *H*: number of haplotypes; *h*d: haplotype diversity; πd: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F test statistic. Statistical significance: **, P < 0.02

potential intermediate hosts for *M. hirudinaceus*, cockroach species such as *Melolontha melolontha* and *M. hippocastani* have been identified as the most probable candidates (Kennedy 2006). The presence of *M. hirudinaceus* larvae in species of the subfamily Melolonthinae is only documented in a limited number of cases. However, the prevalence of infection in these studies has been found to reach as high as 60% (Pavlović *et al.* 2010). Nevertheless, there is still a paucity of data concerning the natural larval infection by *M. hirudinaceus.*

The idea for this study was developed as a result of the detection and molecular confirmation of adult *M. hirudinaceus* in the intestines of 21 of 25 wild boars necropsied in rural areas of Elazig province of Türkiye between January 2022 and December 2023 (Celik *et al.* 2024). Then, cockchafer larvae and adults were collected and pooled for gDNA isolation, followed by PCR and sequence analysis. Finally, 30 pools of cockchafer larvae and/or adults revealed that 16 pools were positive for *M. hirudinaceus.* This may be due to the clustering of wild boars in the study area. As a matter of fact, the areas where samples were collected consist of small hills, far from the city centre, and scattered houses with different numbers of gardens. Such areas are ideal places where wild boars cluster to find food.

Indeed, there are reports that feeding areas in wildlife contribute to the spread of parasites (Acevedo *et al.* 2006; Ruiz-Fons *et al.* 2008; Sorensen *et al.* 2013). Temperature, soil composition, and land use are factors that play an important role in the reproductive biology and reproduction of intermediate hosts (Svetska 2006). The soft soil structure of the land where cockchafer larvae were collected and the presence of cultivation areas indicate that the density of intermediate hosts may be related to the land. The density of wild boars in the area will also increase the number of eggs that can infect the intermediate host. This will contribute to an increase in the prevalence of parasite infection in definitive and/or intermediate hosts.

There is very limited data on the molecular analysis of *M. hirudinaceus* isolates and especially on the status of haplotypes. Celik *et al.* (2024) identified a total of four haplotypes, one of which was the main haplotype, in the haplotype analysis of mt-CO1 sequences obtained from adult *M. hirudinaceus* isolates. They stated that three haplotypes differed from the main haplotype by one to five mutation steps and constituted 71.42% (15/21) of all samples. In the present study, a total of 16 sequences belonging to the sequences of the mt-CO1 gene region of *M. hirudinaceus* isolated from intermediate hosts were subjected to haplotype analysis, and two different haplotypes were detected at two points. Twelve of the total number of sequences were found to consist of a single haplotype. One of the two haplotypes consisted of 10 isolates, while the other one consisted of six isolates. The evolution of parasitism in nematodes has occurred independently, starting from distinct ancestral gene sets and physiologies. However, common selective pressures of adaptation to host gut, blood, or tissue environments, the need to avoid host immune systems, and the acquisition of complex life cycles to enable transmission may have led to genetic differentiation (Coghlan *et al.* 2019). Therefore, it was thought that there were more haplotypic differences in adult parasites.

This study is one of the limited studies on the molecular identification and haplotyping of *M. hirudinaceus* in intermediate host cockchafers. In a region where the adult parasite is widespread in wild boars, it is also important in terms of molecularly revealing the situation in the cockchafers. The findings obtained in this study show that the presence of *M. hirudinaceus*, a zoonotic parasite, in

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Table 3. Point mutations identified according to reference sequence (NC_019808)

Nucleotide position (n)	14	15	17	23	29	32	38	39	47	53	74	77	86	94	95	104	125	128	152	155	158	173	191	200	209	215	218	224	227	230	248	257
NC_019808 (Ref. seq.)	G	T	G	A	A	T	C	T	C	T	T	A	G	C	G	C	C	C	C	G	T	T	T	A	G	T	G	G	C	G	A	T
Hap_1				C				C	T							T		T														
Hap_2									T							T		T														
Hap_3																		T														
Hap_4																																
Hap_5									T																							
Hap_6																		T														C
Hap_7										C																						
Hap_8																																
Hap_9	A	A	A		G	C	T		T		C	T	T	T	A	T	T	A	T	A	A	C	C	G	A	C	A	A	T	A	G	

Nucleotide position (n)	263	266	267	278	281	302	305	311	314	344	359	371	374	377	383	389	390	407	413	416	417	419	422	437	449
NC_019808 (Ref.seq.)	G	G	C	T	C	A	C	T	T	A	A	G	T	G	A	G	C	T	A	T	G	G	A	T	T
Hap_1							T											C						C	
Hap_2				C			T											C						C	
Hap_3																	T							
Hap_4					T																				
Hap_5													C												
Hap_6																	T								
Hap_7					T																				
Hap_8																									
Hap_9	A	A	T		T	G	T	C	C	T	G	C		A	G	A	T	C	G	C	A	A	T		C

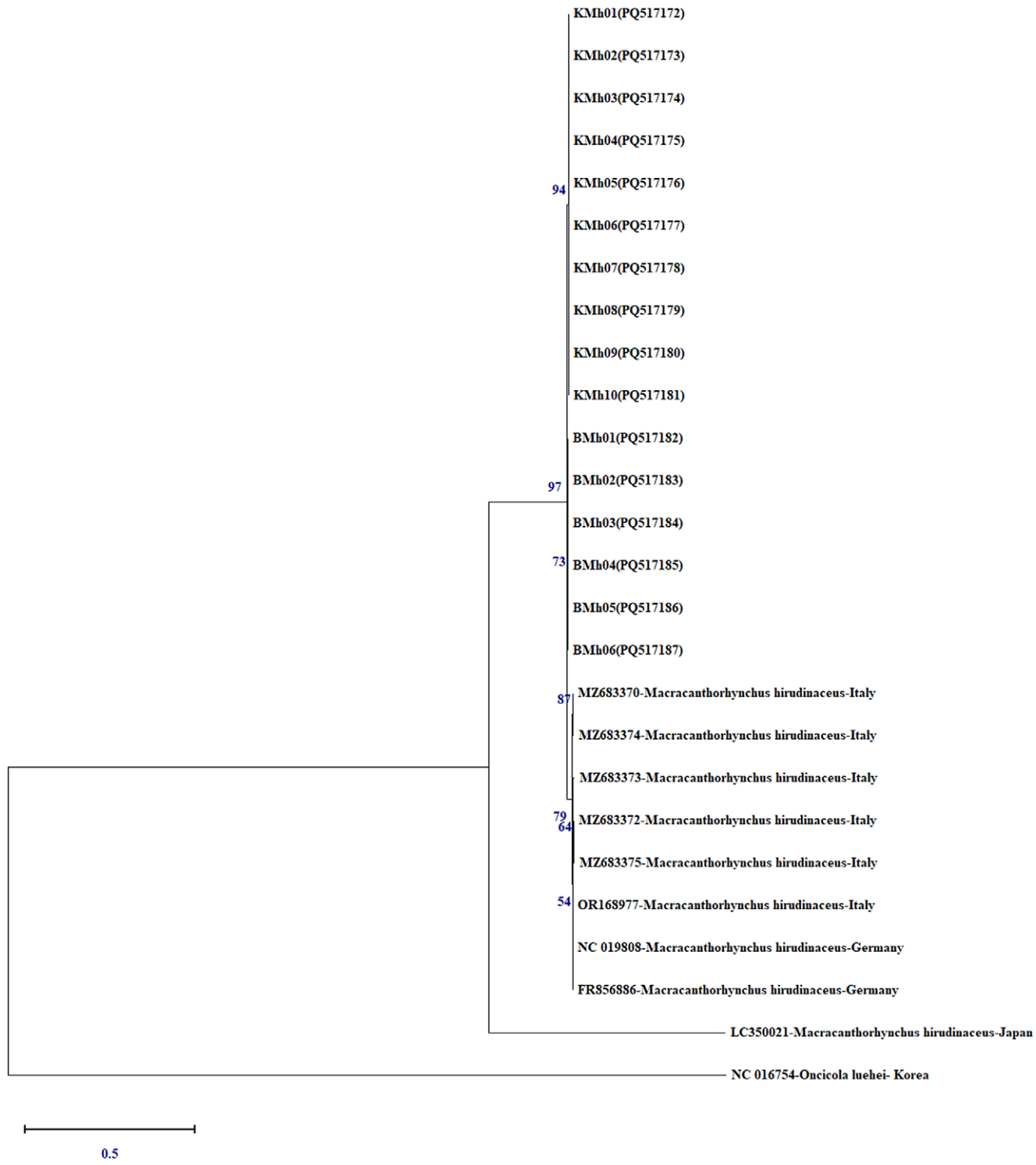

Figure 2. Phylogenetic tree of *Macracanthorhynchus hirudinaceus* sequences. The tree was constructed using the mt-CO1 gene (450 bp) and reference sequences (MZ683370, MZ683372, MZ683373, MZ683374, MZ683375 and OR168977, LC3500219, NC_019808, FR856886) and the sequence *Oncicola luehei* (NC_016754) as an out group. It was constructed using the TN93+G+I model with the Maximum Likelihood method in the MEGA X programme, and its reliability was ensured by 1,000 replicate tests.

cockchafer in rural areas close to living areas is a situation that threatens public health.

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Author contribution. FC, AST, and MU: Data acquisition, material examination, analysis, and manuscript preparation; FC, AST, and SS: Conceptualization and data analysis; SS: Critical manuscript analysis.

Data availability. All the parasite specimens are deposited at the Molecular Parasitology Laboratory, Firat University Veterinary Faculty, Elazig, Türkiye,

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and can be verified after sending a request to the corresponding author. DNA sequence data was deposited in GenBank.

Competing interest. The authors declare no competing interests.

Ethical standard. Not applicable.

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