First Report of *Spirocerca Lupi* Larva in Dung Beetles (*Scarabaeus Armeniacus*) in Iran: A Morphological and Molecular Identification

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Abstract

Background: Dung beetles are detritivores insects. They naturally use feces of vertebrates as foods and reproduction beds. This leads to frequent contacts between dung beetles and parasitic helminths. The current study was carried out to assess infections of dung beetles with larval stages of helminths in rural areas of Taleqan County, Alborz Province, Iran. In total, 200 dung beetles were randomly collected in June 2017 from the highlands of Taleqan County. Beetles were dissected in normal saline and carefully studied using stereomicroscopy. Morphological characteristics of the recovered larvae were drawn using camera lucida equipped microscope at 400× magnification. Furthermore, genomic DNAs of the recovered larvae were extracted and PCR amplifications of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) genes were carried out and the amplicons were sequenced.

Results: All collected dung beetles were identified as *Scarabaeus armeniacus* from Scarabaeidae family (55.5% were male and 44.5% female). Three females of the beetles were infected with nematode larva, morphologically identified as the third-stage of *Spirocerca lupi* larvae. The average length and width of the larvae were 2.95 (2.81–3.15; CI 95%) and 0.12 (0.1–0.15; CI 95%) mm, respectively. The phylogenetic analysis showed that *S. lupi* belonged to a clade within Spirocercidae family, well separated from Onchocercidae family.

Conclusions: In the current study, *S. armeniacus* was introduced as a potentially biological vector for the transmission of *S. lupi* to vertebrates in the region. To the best of the authors' knowledge, this is the first reported on larval stages of *S. lupi* in *S. armeniacus*.

Background

Dung beetles are diverse detritivores insects, which use feces of vertebrates for feed and reproduction from Cenozoic Era [1]. Several coprophagous species in Scarabaeidae (Scarabaeinae and Aphodiinae subfamilies) and Geotrupidae (Geotrupinae subfamily) families play significant roles in transmission of vertebrate parasites. These arthropods have intense contacts with animal and human feces, constant accessing to helminth eggs [2]. Therefore, dung beetles play important roles as intermediate hosts for helminths, including *Gongylonema sp.*, Acanthocephalans and *Sprirocerca* [3–5]. Spirocercosis is a canine disease caused by *Spirocerca lupi* (*S. lupi*) and *Spirocerca vulpis* (*S. vulpis*) nematodes which potentially results in fatalities in domestic dogs (Carnivora: Canidae). The *S. lupi* is a cosmopolitan parasite, more commonly found in warmer tropical and subtropical regions of the world [6–8]. A majority of reports are from Brazil, the southern United States, Kenya, India, Israel and South Africa [9–14]. Usually, the worms are coiled in a spiral pink form, with males are up to 54 and females up to 80 mm in length [6, 7]. This nematode includes an indirect lifecycle, and various species of coprophagous dung beetles (Coleoptera: Scarabaeidae) serve as intermediate hosts after ingesting embryonated eggs of *S. lupi* through feces of canids. Infection transmissions to humans and animals are similar in principles, which can be resulted from the ingestion of infected arthropods by humans or grazing in grasslands by

animals. However, a wide variety of paratenic hosts, including mammals, birds and reptiles, are known [6, 7].

Recently, studies have shown that mitochondrial DNA (mtDNA) regions are useful in analysis of population genetics, biology, epidemiology and diagnosis of parasitic nematodes of human and veterinary significances [15, 16]. Significant variations in the nematode mitochondrial genome are reported, compared to other animal species [17]. In fact, DNA sequencing of informative regions within the mtDNA gene encoding cytochrome c oxidase subunit 1 (*cox*1) has led to basic and applied potential studies of Spirurida nematodes [18–21]. In studies on sequence variability and population genetics of parasitic nematodes and arthropods and their diagnostic approaches, the *cox*1 gene has been described as an appropriate choice [15–17, 22, 23]. Hence, *cox*1 was considered for the molecular identification of this parasite and its molecular characterization in the current study. Rich literatures of clinical, diagnostic and epidemiological elements of dog spirocercosis are available. To date, a few studies have been carried out on the host-parasite interactions of dung beetles and *S. lupi* [24–26]. Therefore, the current study was carried out for the first time to assess infections of dung beetles with larval stages of helminths in rural areas of Taleqan County, Alborz Province, Iran, using morphological and molecular analyses. Molecular characteristics were characterized by sequencing of the *cox*1 gene to detail larval stages of *S. lupi* in *Scarabaeus armeniacus* (*S. armeniacus*) and assess its taxonomic position within the genus.

Results

Species identification of the dung beetles

Of the dung beetles, 55.5% (111/200) were males and 44.5% (89/200) females, identified as *S. armeniacus* from Scarabaeidae family using available entomology keys. The posterior margin of metafemore in this species was marginate. Pronotum was characterized using sparse distributed fine puncture with usual sizes of 11–25 mm (Fig. 1).

Morphological Findings Of The Recovered Larvae

Larvae were recovered from three female *S. armeniacus* beetles, based on the published description of infective third-stage (L3) larvae of *S. lupi*. One of the beetles included one *S. lupi* larva while the other two beetles included three and six *S. lupi* larvae. Identification of the L3 larvae of *S. lupi* was completed by referring to characteristics described in keys. The average length and width of the larvae were 2.95 (2.81–3.15 mm; CI: 95%) and 0.12 mm (0.1–0.15 mm; CI: 95%), respectively. The other measurements included distance of nerve ring [0.111 mm (0.108–0.113 mm), CI: 95%] and excretory pore from the anterior end [0.09 mm (0.07–0.11 mm, CI: 95%], respectively. The average size of buccal capsule was 0.051 × 0.013 mm. the average size of rectum and tail were 0.053 and 0.041 mm, respectively. Most of the larvae moved freely in the normal saline after the beetle dissection; some of them were still in encysted form, which were spontaneously emerged. Although some cysts were found attached to the tracheal tubes,

most of them were free in the normal saline. In morphological characteristics of *S. lupi* larvae, thickness of the larvae was uniform, with transverse ridges. Two cephalic horns were recognized at the interior end of the terminal mouth, leading to a strong, sclerotic cylindrical buccal capsule. The tail was formed with five closely sets of circular spines. Camera lucida drawings of the anterior and posterior ends of *S. lupi* 3rd larval stage are illustrated in Fig. 2.

Molecular Findings Of The Recovered Larvae

Specimens of *S. lupi* were successfully amplified with nearly 680-bp amplicons for the partial *cox*1 genes. In this study, a unique nucleotide sequence of the partial *cox*1 gene from *S. lupi* was annotated in GenBank database (accession number MT522373). Phylogenetic analysis based on the *cox*1 gene illustrated that *S. lupi* belonged to a clade within the Spirocercidae family and was well separated from Onchocercidae family (Fig. 3). In this study, the sequence alignment revealed high levels of sequence homologies between *S. lupi* isolates and those globally documented in GenBank (Fig. 4). Results showed that the current nucleotide sequence of *S. lupi* included 100% identity with the recovered isolates of dogs from South Africa (EF394612.1), Israel (EF394606.1), Austria (EF394605.1) and Iran (EF394608.1) (Fig. 4). Moreover, pairwise distance showed intraspecies genetic variations of 0–0.4% between the current isolate of *S. lupi*, and those available in GenBank.

Discussion

Regarding prospective roles of dung beetles in transmission of certain helminths such as acanthocephalans and Spirurid nematodes to humans and animals, faunistic investigation of their parasites seems to include great veterinary and medical significances [1]. The current study firstly demonstrated roles of dung beetles (*S. armeniacus*), as intermediate hosts of *S. lupi*, in Iran. Literatures are available on spirocercosis prevalence in dogs in Iran. Oryan et al. reported 19% of *S. lupi* infection in stray dogs in the southern regions of Iran [26]. In Mirzayans et al. study, *S. lupi* was reported as one of the most common helminths in house-dogs in Tehran Province, Iran [27]. Moshfe et al. documented 17.4% of *S. lupi* in stray dogs in western areas of Iran [28]. In Sadighian study, 45.4% of the stray dogs were infected by *S. lupi* in Northern Iran [29]. Moreover, *S. lupi* infection in *Vulpes vulpes* (red fox) in Northwestern Iran was reported as 17.9% [30]. In some of these studies, high prevalence of spirocercosis is attributed to the presence of dung beetles. However, presence of intermediate hosts (e.g. amphibians, reptiles, lizards, domestic and wild birds) and small mammals (e.g. mice and rabbits) as well as different life and hunting styles are important factors in prevalence of *S. lupi* in carnivores [26, 31, 32].

To the best of the authors' knowledge, no studies have been carried out on *S. lupi* infection in dung beetles in Iran. However, dung beetle infection with *Gongylonema* spp. has been recorded in Northwestern Iran [33]. Studies have documented presence of *S. armeniacus* in Central and Northern Iran [34, 35]. Dung beetles play critical roles in ecosystem, manure and nutrient recycling. They rely on feces for food for themselves and their larvae and lay their eggs in feces. An individual dung beetle can bury feces 250

times larger than that a beetle can in one night. Dung beetles are capable of carrying weights, nearly 50 times heavier than their body weights. Many dung beetles prefer a certain type of animal feces and can fly up to 10 miles to find their desirable food [36, 37]. Several factors affect dung beetle communities, including nature of the soil substrate, flora of the specific region, rainfall and temperature [7, 23]. In Iran, various aspects of *S. lupi* life cycle is still undescribed. In the present study, beetles were collected in summer. Prevalence of spirocercosis differs over a comparatively brief period [7, 38]. In a study by Chhabra and Singh [24], prevalence of infection in beetles increased in mid-season of laboratory-infected dung beetles. In another study, detection rates of spirocercosis during cold months have significantly been higher [39]. This might be explained by the seasonality in the studied country.

Nucleotide BLAST analysis from the current study showed high sequence homologies between *S. lupi* isolates and those from other studies. The current results showed that nucleotide sequence of *S. lupi* from this study included 100% identity with recovered isolates of dogs from South Africa (EF394612.1), Israel (EF394606.1), Austria (EF394605.1) and Iran (EF394608.1) [40]. Based on the pairwise distance of the isolate from the current study, intraspecies genetic variation within *S. lupi* nucleotide sequences included 0–0.4% with an overall average of 0.1% between the current isolate of *S. lupi* and the sequences available in GenBank. Several studies have been carried out on the molecular characterization of *Spirocerca* spp. In Traversa et al. study, the *cox*1 gene of *S. lupi* collected from five countries was investigated and only a low genetic variation within the sequences was detected, demonstrating at least 99% nucleotide similarity [40]. In contrast, de Waal et al. reported significant genetic diversities in *Spirocerca* isolates collected from a dog [41]. A study by Rojas et al. on detection of spirocercosis in dogs using HRM qPCR in fecal samples showed that HRM qPCR of ITS1 included the best performance in limit of detection and absence of cross-amplification with other canine parasites [42]. Ruggeri et al. detected 18S ribosomal DNA (rDNA) of *S. lupi* gene in cerebrospinal fluid (CSF) of presumptively-affected dogs using PCR. Results revealed that PCR targeting 18S rDNA of *S. lupi* in CSF was capable of antemortem diagnosis of canine intraspinal spirocercosis [43].

Conclusion

In the current study, *S. armeniacus* has been introduced as a potentially biological vector for the transmission of *S. lupi* to vertebrates in Iran. To the best of the authors' knowledge, this is the first report on the larval stages of *S. lupi* in *S. armeniacus*. Further studies are necessary to investigate better control and prevention of the disease in dogs. Better knowledges of dynamics of the intermediate host-parasite intersections between dung beetles and *S. lupi* in Iran can establish preventive methods to limit spread of this disease in domestic dogs.

Materials And Methods

Sample collection

Totally, 200 dung beetles were randomly collected in June 2017 from the highlands of Taleqan County, Alborz Province, Iran (36.1748° N, 50.7650° E), a common passage of jackals, foxes and other canids and felids (Fig. 5) [44]. Wild boar, dog and livestock dung pitfall traps were used to capture dung beetles, previously described by Du Toit et al. [14]. Trapped dung beetles were transferred to the Helminthology Lab of the Department of Medical Parasitology and Mycology, Tehran University of Medical Sciences, Tehran, Iran, using ventilated containers.

Species identification of the dung beetles

At the time of the assessment, most of the dung beetles were still alive. Therefore, dung beetles were stored in a refrigerator with autoclaved soil for 2 h to immobilize them. Then, species and sexes of the collected beetles were identified taxonomically, using entomology keys [45].

Morphological analysis of the recovered larvae

Beetles were dissected in normal saline solution and carefully studied for the presence of parasites with special focuses on larval stages using stereomicroscope. The beetles have been reported individually as positive or negative for larval stages of helminths. For positive beetles, larvae were removed and transferred into normal saline on ice for 1 h to relax and immobilize [33]. Removed larvae were transferred into lactophenol and 70% ethanol for morphological and molecular studies. Morphological characteristics of the isolates were recorded carefully using camera lucida equipped microscope at 400× magnification. Identification was comparatively carried out based on taxonomic key references [46].

Molecular analysis of the recovered larvae

DNA extraction

Ethanol preserved larvae were washed three times with sterile distilled water by centrifugation at 5000× g for 5 min to remove ethanol. Then, larvae were subjected to five cycles of freezing in liquid nitrogen and thawing in boiling water. Approximately 300 mg of glass beads (0.5 mm in diameter) were added to the larvae and shaken intensively for 5 min. Then, genomic DNA was extracted using genomic DNA extraction kit (GeneAll Exgene, South Korea) according to the manufacturer's instructions and stored at -20 °C until use. The DNA concentration was assessed spectrometrically (NanoDrop ND-1000, Thermo Fischer Scientific, USA) at 260 nm. The 260/280 absorbance ratios of the DNA samples included 1.8–2.0, indicating no major protein contaminations.

Amplification of the *cox*1 gene and sequencing of the amplicons

Polymerase chain reaction (PCR) on *cox*1 gene was carried out using primer set of NTF (5'-TGATTGGTGGTTTTGGTAA-3') and NTR (5'-ATAAGTACGAGTATCAATATC-3') as previously described for Spirurida [40]. The PCR amplification was carried out in a final reaction mixture of 50 µL, including 25 µL of 2x red PCR master mix (Ampliqon, Denmark), 2 µL of each primer (10 pmol), 5 µL of the extracted DNA and 16 µl of sterile distilled water. A negative control (distilled water) and positive control (extracted DNA

from *S. lupi* provided by the Faculty of Veterinary Medicine, University of Tehran) were used in each set. Amplification was carried out using PeqSTAR Thermal Cycler (PeqLab, Germany) using the following cycling protocol of initial denaturation at 94 °C for 7 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 10 min. Amplified products were electrophoresed on 1.5% agarose gels and visualized using UV transilluminator. The PCR products were sequenced using Sanger method in both directions (Bioneer, South Korea).

Sequence analysis

Sequences were edited and trimmed using Chromas software v.2.6.1 (Chromas, Australia). Analysis of the sequencing data was carried out using BLAST program and NCBI databases (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were carried out using Clustal W method and BioEdit software v.7.1 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and results were compared to sequence results from GenBank database.

Phylogenetic analysis

Sequences were edited and compared to entries from NCBI GenBank for further analysis. The best-fit model of nucleotide substitution was statistically selected by the MEGA software v.6.0 (Pennsylvania State University, USA) [47]. The phylogenetic tree of *Spirocerca* spp. was constructed using the Maximum Likelihood (ML) method in agreement with Hasegawa-Kishino-Yano model with uniform rates for transitions and transversions. Bootstraps of 1,000 replicates were used for the assessment of topology reliability of the trees.

Abbreviations

L3: third stage of larvae; *cox*1: cytochrome c oxidase subunit 1; mtDNA: mitochondrial DNA; PCR: Polymerase chain reactions; BLAST: basic local alignment search tool; ML method: Maximum Likelihood method; HRM: High Resolution Melting Analysis; qPCR: quantitative PCR; rDNA: ribosomal DNA; CSF: cerebrospinal fluid.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The original datasets are available upon request to the corresponding author. The generated sequence of *S. lupicox*1 gene was deposited in the GenBank database under the accession number MT522373.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

SM and GM conceived and designed the study. AM and MA collected the dung beetles. NT performed the entomological identification. HA dissected and examined the collected dung beetles. IM performed the morphological identification of the recovered larvae. SM, AT and MJAA drafting the manuscript and performed all DNA extraction, PCR and phylogenetic analyses. All authors read and approved the final manuscript.

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Figures

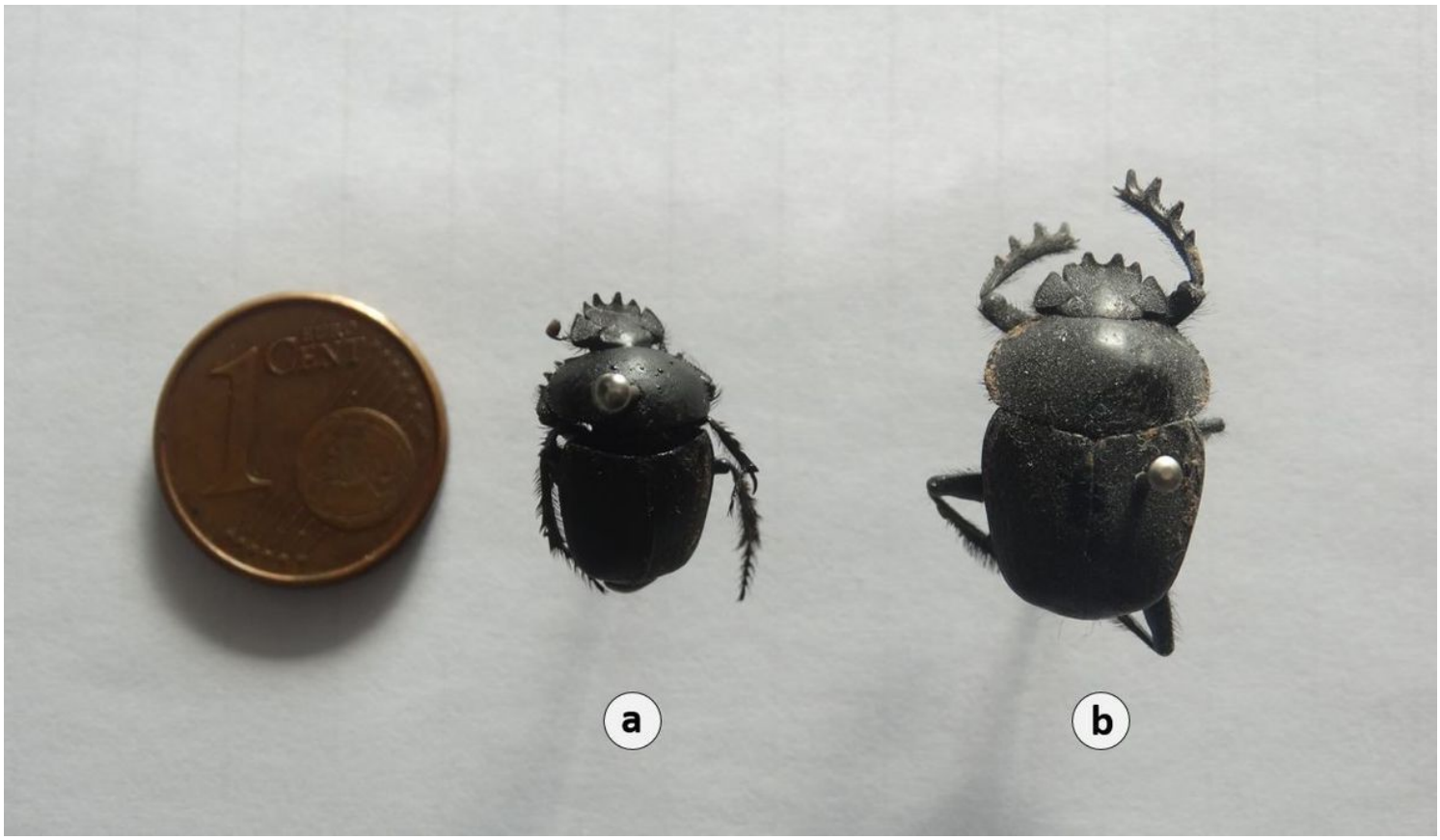

Figure 1

Scarabaeus armeniacus from family scarabidae. (a) Male (b) Female

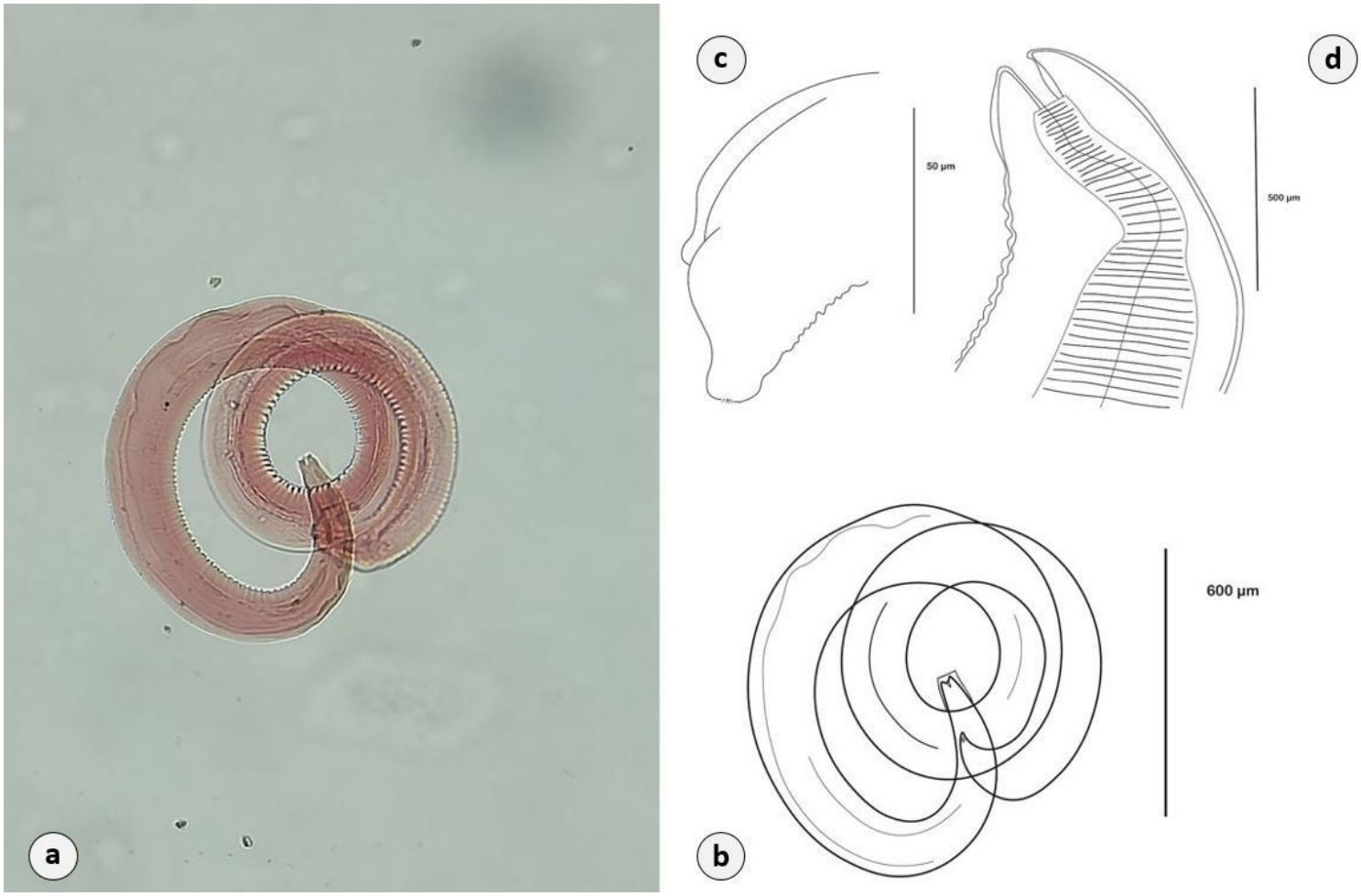

Figure 2

3rd Larval stage of Sprirocerca lupi recovered from the Scarabaeus armeniacus beetles (a), Camera lucida drawings of the larval stage (b), and its anterior (c) and posterior (d) ends.

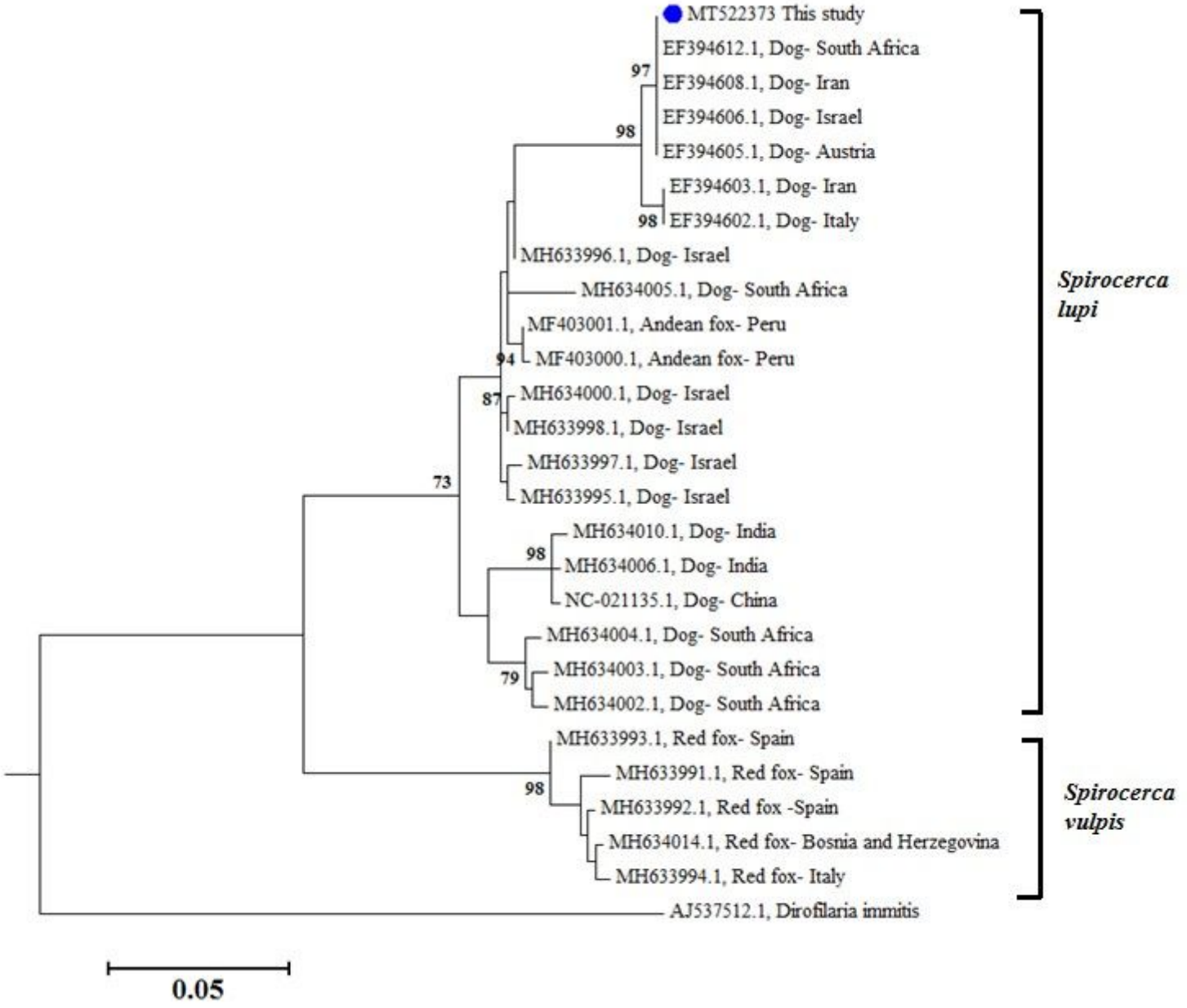

Figure 3

Phylogenetic tree obtained via the Maximum Likelihood (ML) method and Hasegawa-Kishino-Yano model based on the cox1 gene (The blue circle indicates the sequence derived from this study). The numbers above branches correspond to bootstrap values based on 1,000 replicates. Branches without numbers include values of less than 70%. Dirofilaria immitis served as an outgroup. Scale bar represented 0.05 changes per nucleotide

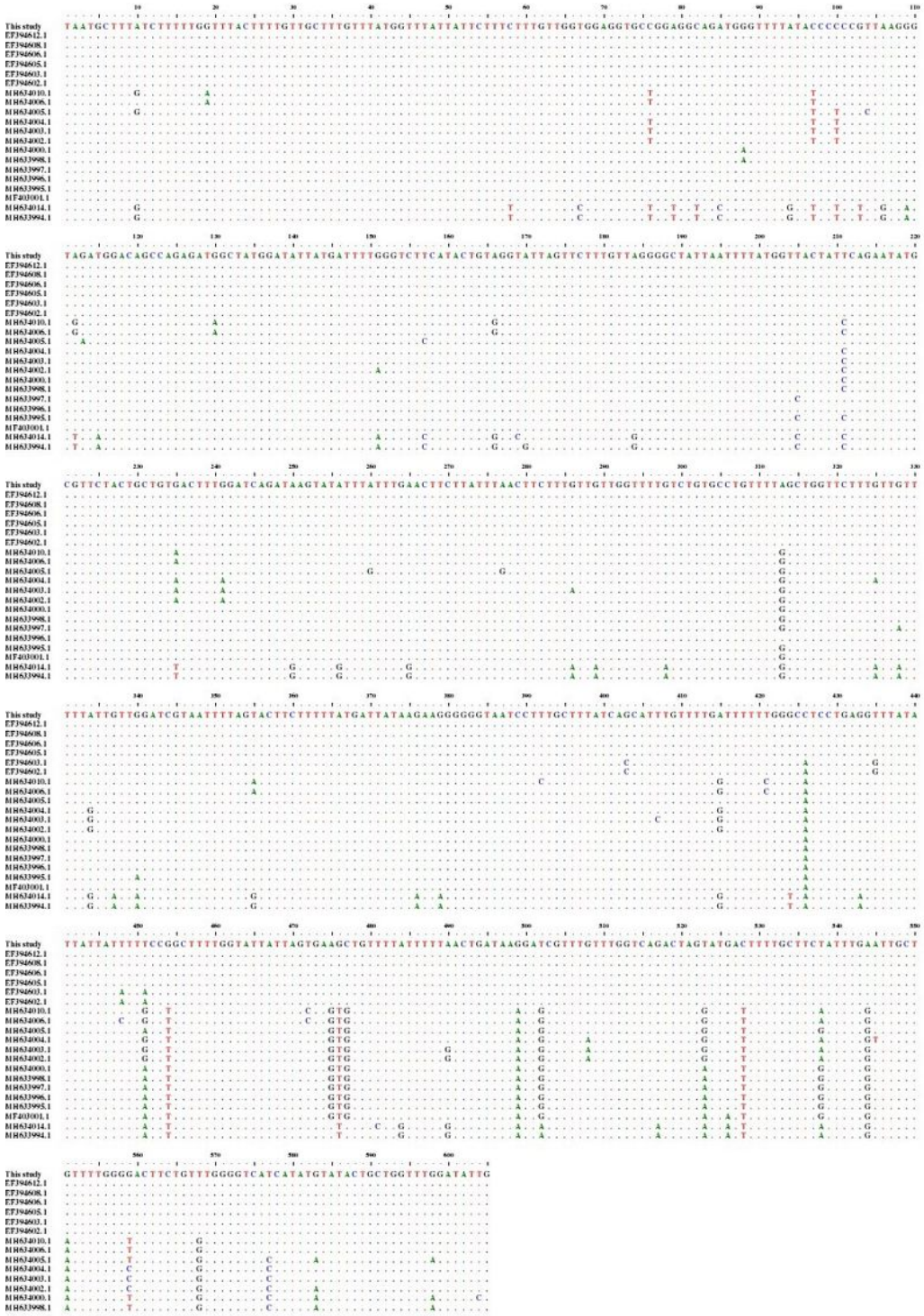

Figure 4

Sequence alignment of Spirocerca spp. isolate obtained in the current study based on cox1 gene by Clustal W method via Bioedit software version 7.1

Figure 5

Map of Iran, Taleqan County in Alborz Province Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.