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Morphometric and genetic characterization of *Oestrus ovis* in sheep in different climatic regions of Iran

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ABSTRACT

Oestrosis is nasal myiasis, which results from infestation with the larvae of flies that belong to the genus *Oestrus*. Nasal discharge and sneezing are the most common clinical signs in infected animals. Myiasis larvae were collected from sheep in different climatic regions of Iran. Morphological identification of the larvae was made based on the diagnostic keys. The species was confirmed by PCR amplifying the partial fragment (610 bp) of the mtCO1 gene. Genetic distance was assessed in CO1 sequences, and a phylogenetic tree was drawn. Sequencing showed no difference in the partial CO1 gene among the Iranian isolates, and this gene had a high similarity with the sequences of *O. ovis* isolates from Iraq, Bosnia and Herzegovina, and Croatia. The present study provided the first molecular dataset for *O. ovis* species, which is crucial for the phylogenetic relationships assessment and the molecular identification of these parasites.

Keywords

Oestrus ovis, Sheep, CO1, PCR, Phylogeny, Sequence

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Abbreviations

CO1: Cytochrome oxidase I
PCR: Polymerase chain reaction
mtCO1: mitochondrial cytochrome oxidase subunit I
MBST: Molecular Biological System Transfer

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Introduction

Oestrosis is nasal myiasis, resulting from infestation with the larvae of flies belonging to the genus *Oestrus* (Diptera: Oestridae). It is regarded as a severe parasitosis in goats and sheep and sometimes in other animal species [1]. Nasal discharge and sneezing are the most common clinical signs in animals [2]. Parasitic larvae seriously affect the host's well-being and cause the loss of milk, meat, and wool [3]. The sheep bot fly (*Oestrus* ovis L.) causes severe cavitary myiasis in all areas worldwide where goats and sheep are reared [4]. O. *ovis* can be diagnosed by the morphological characterization of the larvae (e.g., the slits of the posterior spiracles located on the posterior spiracular plates), clinical presentation, and infrequent adult fly identification [5]. A few years ago, molecular techniques were extensively used to achieve insights into the systematics and taxonomy of various insects. Particularly, hypervariable regions within ribosomal and mitochondrial DNA are robust genetic markers to tackle taxonomic diagnostic issues for some flies that cause myiasis and belong to the Oestridae family [6,8]. The mtCO1 gene was chosen to characterize the Oestridae larval relationships. It has previously been revealed to be necessary for various molecular phylogenetic targets due to its comparatively enormous size and the inclusion of both highly conserved and variable regions with different mutational rates [9]. The CO1 658 bp region is a standard and broadly considered animal taxa universal marker [10]. Over the past decade, the barcoding of DNA has provided an effective instrument for phylogenetic characterization using CO1 sequences and molecular identification of various insect species comprising the Oestridae family [11,14]. Therefore, the current study aimed to utilize PCR and partial mtCO1 gene sequencing as a diagnostic tool to identify and demonstrate the phylogenies of larvae in different climatic regions of Iran. The molecular detection of O. ovis is substantial to address its epidemiology and control.

Results

Morphological identification

The identification of the mature larvae of O. ovis was based on morphological characteristics. The third instar larva is up to 20 mm long, yellowish-white, and tapering anteriorly. Every fragment has an obscure lateral band on the dorsal side. They have a big black oral hook linked to the internal cephalopharyngeal skeleton. The ventral surface is lined with small spines. The results corresponded with what is mentioned in the identification key [15].

PCR amplification and sequencing of mtCOI gene

Figure 1 shows that the isolates in this study had a similar sequence alignment and they all are placed in the same consensus modification group. The 610 bp fragment of mtCO1 from all isolates was successfully amplified (Figure 1). The 610 bp CO1 region covered by the consensus sequences from all reads was effectively acquired with high-quality chromatogram scores. The sequence of the amplified fragments revealed a genome arrangement typical of the O. ovis sequence in GenBank. Phylogenetic tree elucidated phylogenetic relationships between other members of the family Oestridae published in GenBank and O. ovis larvae amplicons as shown in Figure 3. The mean reported interspecific similarity value of CO1s in the Oestrinae subfamily was 86.7% [16]. However, the sequence indicated a 99% identity using the CO1 sequences of O. ovis available at GenBank and < 91% identity using the CO1 sequences of other Oestrus species (Oestrus sp.) [17]. Furthermore, this sequence identity using the CO1 sequences of other Oestrid species was < 90%. These findings established that the examined larvae belonged to O. ovis.

Sequencing results of Iranian O. ovis isolates from six sheep sinuses in this study showed no difference in partial CO1 genes among six Iranian isolates, and they had identical gene sequences (Figure 3). The CO1 gene sequences from the other mitochondrial genomes available in GenBank were chosen and aligned. The obtained sequences in this study (MZ972997 - MZ973002) were compared with GenBank sequences.

Figure 2 demonstrates the comparison of the isolated sequences in this study with sequences in the gene bank, which shows the differences and similarities in the alignment process. Based on multiple nucleotide sequence alignments (Figure 2) and sequence identity matrix algorithms, the percent identities among O. ovis isolates in this study were 100% with O. ovis from Iraq (MW555828), Bosnia and Herzegovina (MG755264), and Croatia (MN845130), and 99% with O. ovis from Iran (KX268655), Spain (MW145179), and Brazil (KR820703). The nucleotide differences with other isolates are exhibited in Figure 3. As a result, a phylogenetic tree was inferred with CO1 gene sequences from this study and previously published correlated species of mitochondrial sequences. The phylogenetic reconstruction showed two main branches (A and B) and a sub-branch (A1) that contained our isolate (MZ972997 - MZ973002) and homolog sequences with high similarity from Iraq, Bosnia and Herzegovina, and Croatia (Figure 3).

About 100% similarity of Iranian sheep isolates with isolates from Iraq, Bosnia and Herzegovina, and

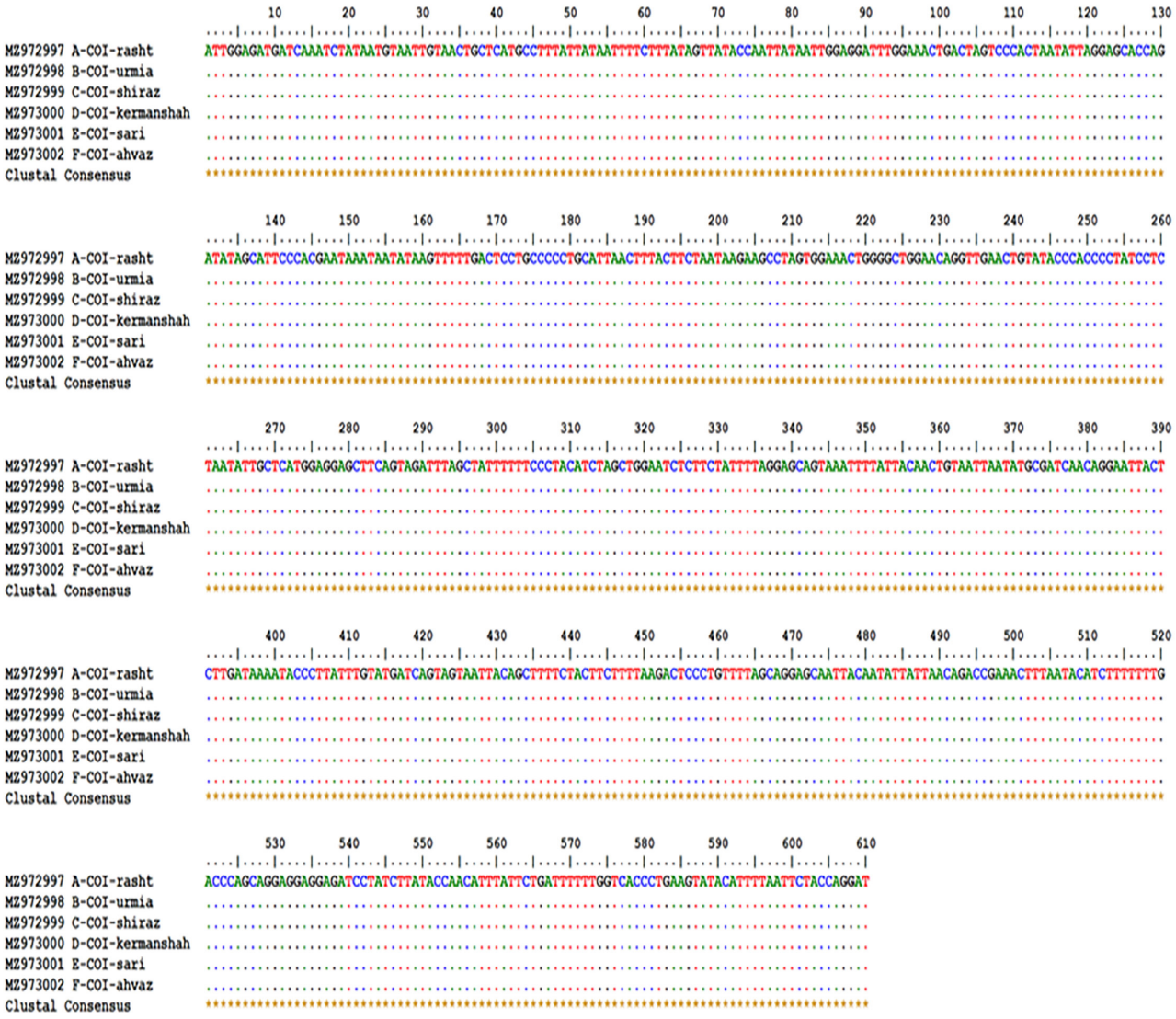

Figure 1.
Nucleotide alignment of the Oestrus ovis partial COI sequences of Iranian isolates

Croatia, shows a very close relationship, and homology between the nucleotide sequences of O. ovis isolates from sheep sinuses mtCO1 gene in these geographical areas. Figure 4 shows the alignment process between the isolates of this study and the isolates of the same genus and species identified from the GenBank.

Discussion

This study analyzed the genetic diversity of O. ovis based on mtCO1 gene nucleotide sequences isolated from the sinuses of Iranian sheep. The mtDNA provides more accurate identifications of the species to which larvae belong, and even closely related species can have different developmental characteristics [18, 19]. Phylogenetic analysis demonstrated that none of the sheep sequences obtained in this study had intraspecific variations and high similarity with the sequences of O. ovis isolates from sheep mtCO1 gene from Iraq, Bosnia and Herzegovina, Croatia, Brazil, and Spain (Figure 3).

The results of the nucleotide alignment of MZ972997 - MZ973002 with other sequences revealed that variation in the A chain of the phylogenetic tree between O. ovis sequences was in position 258 (T > C) for KR820703, MW145179, KX268655, and MT124626, 372 (G > A) for MT124626, 435 (T > A) for MW555828, 456 (C > A) for MT124626, and 459 (T > C) for MW145179, MN845130, MG755264, and MT124626 (Figure 4). The genes encoding proteins in the mtDNA are more diverse than the ribosomal genes [20]. Based on molecular alignment and phylogenetic tree analysis in this study, the close relationship of Iranian isolates with Iraq, Bosnia and Herzegovina, and Croatia from the same hosts was maybe correlated with the same geographical conditions and the high rate of trade between countries [21, 22]. Furthermore, the results of this study showed that all

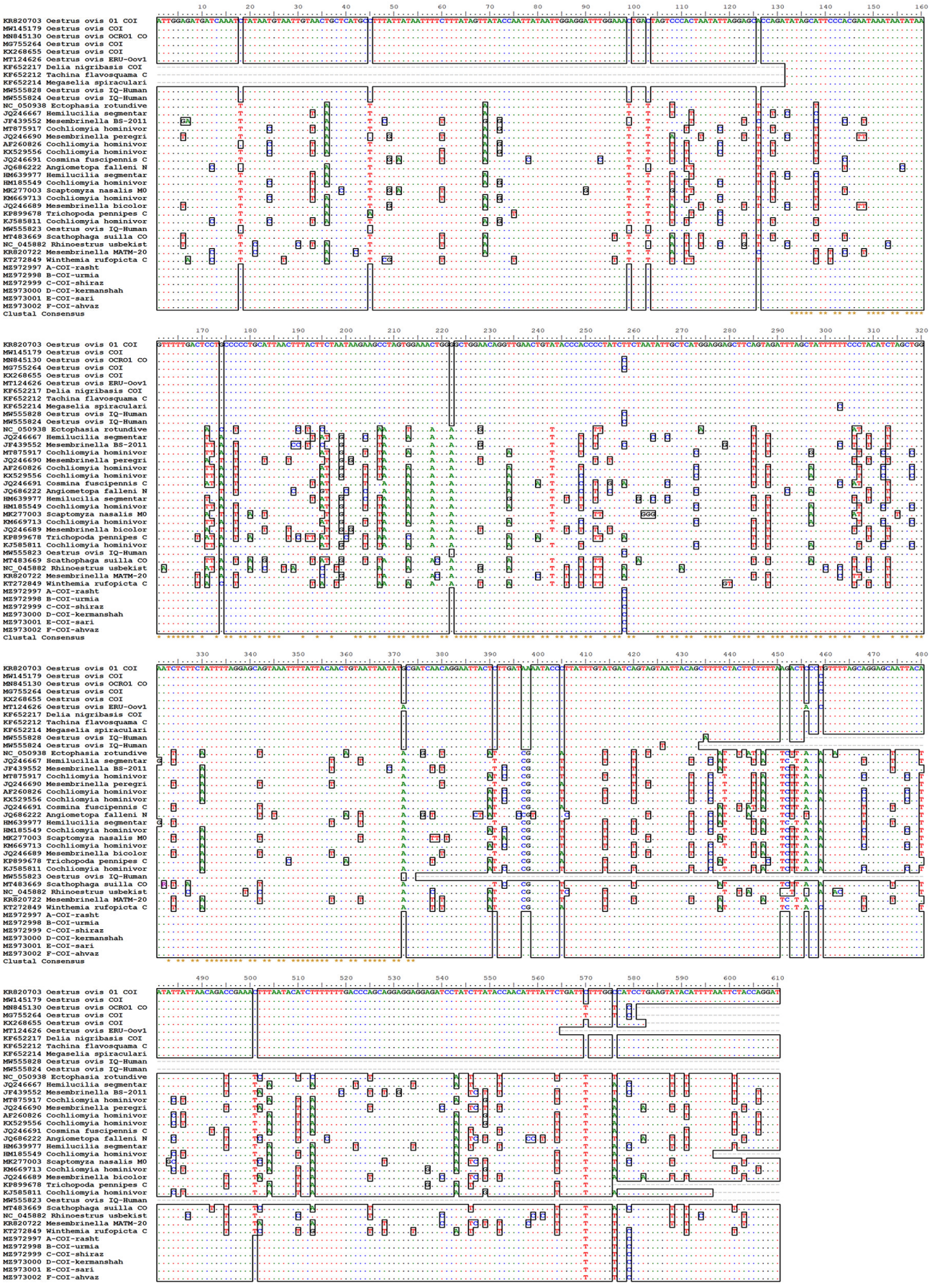

Figure 2.
Nucleotide alignment of the Oestrus ovis partial CO1 sequences of Iranian isolates and GenBank

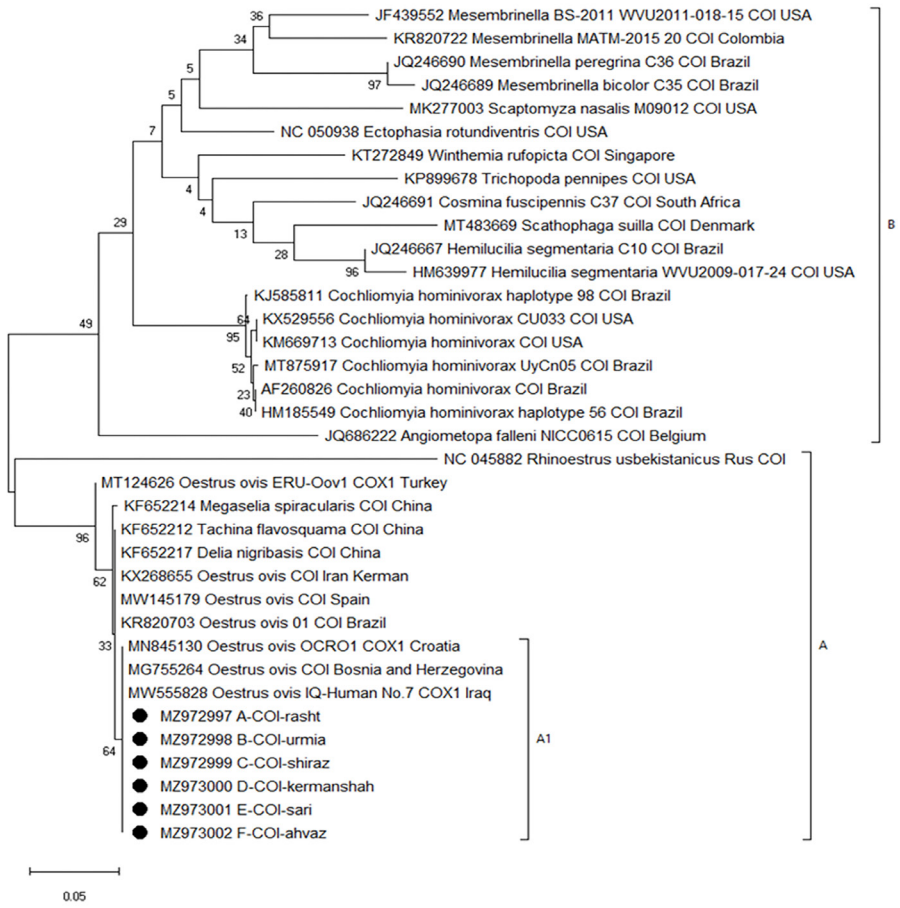

Figure 3.
Neighbor-Joining phylogenetic tree of Oestrus ovis partial CO1 sequences

O. ovis obtained in this research and from GenBank were grouped into a monophyletic group according to the results of the previous work [23, 25]. Molecular studies are being used to investigate taxonomy and perform substantial status for identifying Oestridae larvae that lead to obligate myiasis [26].

The physical traits of the larvae and morphological identification are unreliable [27]. Therefore, the molecular identification of myiasis-causing fly species may be considered a credible substitutional to morphological identification as it is challenging to recognize larvae to the genus level [27, 29]. In this study, the high similarity between the isolates from Iran and other countries isolated from the same host and interestingly between other species from other hosts were observed. The results revealed that the genetic variability of O. ovis isolated from different geographical areas of Iran was not clear based on a 610 bp fragment of the mtCO1 gene. Although a low rate of intraspecific variation existed in O. ovis, especially in different hosts, more O. ovis isolates should be collected for further investigations, and complete gene sequences from isolates would be required. The differences between O. ovis isolates may result from variations in the animal breed, geographical area, and climate [22, 24, 25, 31, 32].

Bosly, in 2018, reported a molecular identification of O. ovis larvae, which was recognized as a reliable replacement for morphological identification [25]. This research gives the initial molecular depiction data on O. ovis larvae in sheep in Iran using the barcode CO1 sequences. Our results showed a close association between O. ovis from different countries, even though limited sequences were included in the dataset. The barcode CO1 sequence successfully analyses the species of the subfamilies in Oestriade, and this result shows the usefulness of the CO1 as a suitable diagnostic molecular marker for identifying and characterizing bot fly species.

Materials & Methods

Collection and identification of larvae

O. ovis larvae were collected from the sinuses of infected sheep in the slaughterhouse of three climatic regions of Iran, including the coastal areas of the Caspian Sea, mountain plateau areas, and flat plains around the Persian Gulf. Two provinces were selected from each region, and ten samples were collected from each of these provinces. Collected larvae were conserved in 70% C2H5OH for morphometric identification and molecular survey. The larvae were identified based on their morphological traits and the three slits at the posterior larvae's spiracle (Figure 5) [15, 33].

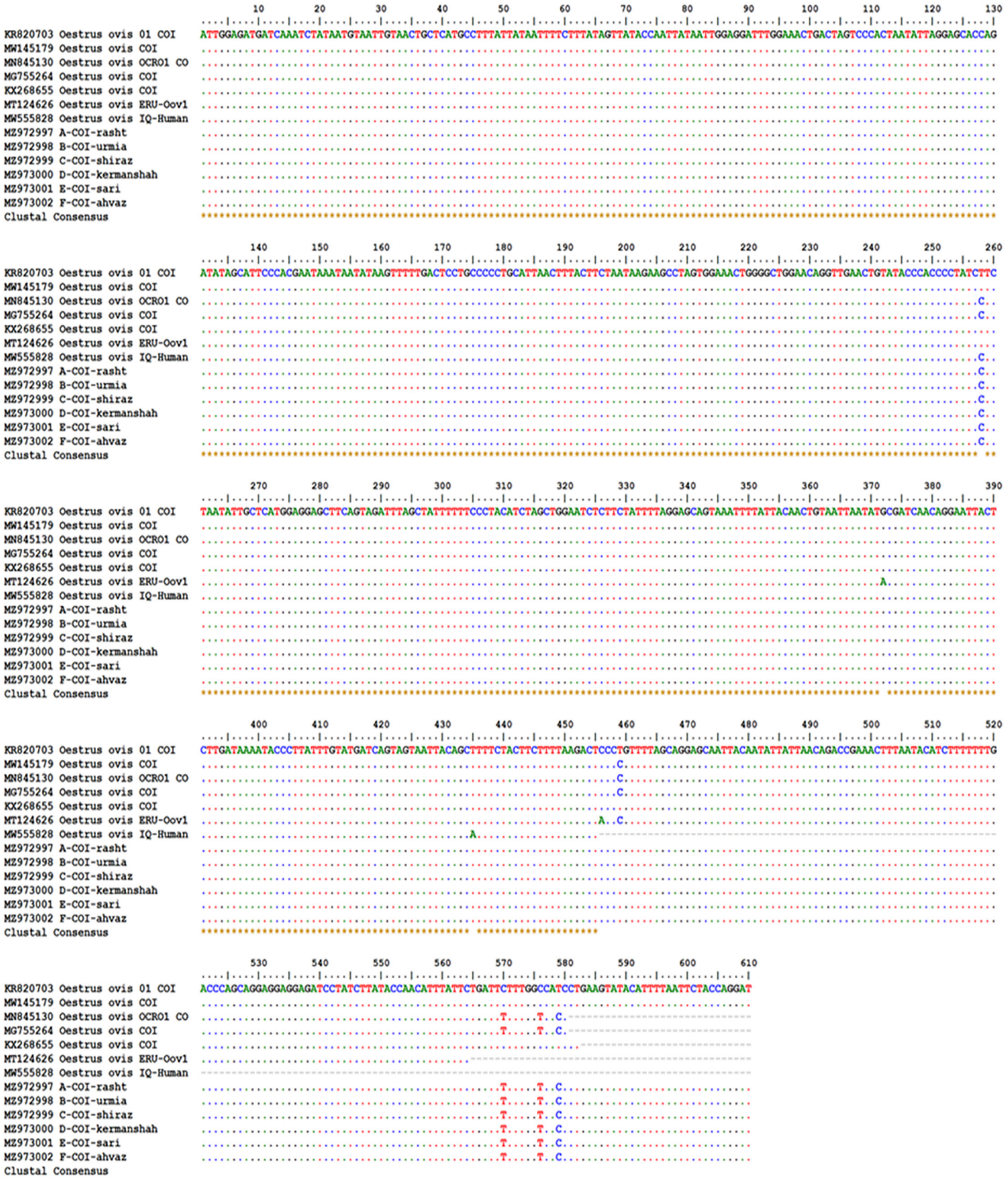

Figure 4.
Nucleotide alignment of the Oestrus ovis partial CO1 sequences of Iranian isolates and Oestrus ovis partial CO1 of GenBank

Morphological identification and microscopic examination

The mature larvae of O. ovis were identified based on the morphological characteristics of the larval stages (Figure 6). The third instar larva is 20 mm long and yellow when young, changing to light brown later, and in the maturing stage finally, bands are appear on the dorsal of the third-stage larva.It shows wide transverse blackish bands dorsally. The second segment has a variable number of small teeth on the dorsal side with the rest of the segments being bare. Ventrally, the segments bear rows of strong spines (Figures 6A and B). The findings corresponded with what was mentioned in the identification key [15].

DNA extraction

DNA was extracted from larvae using a DNA isolation kit (MBST, Tehran, Iran) according to the manufacturer's instructions.

Figure 5.
Posterior spiracles of Oestrus ovis

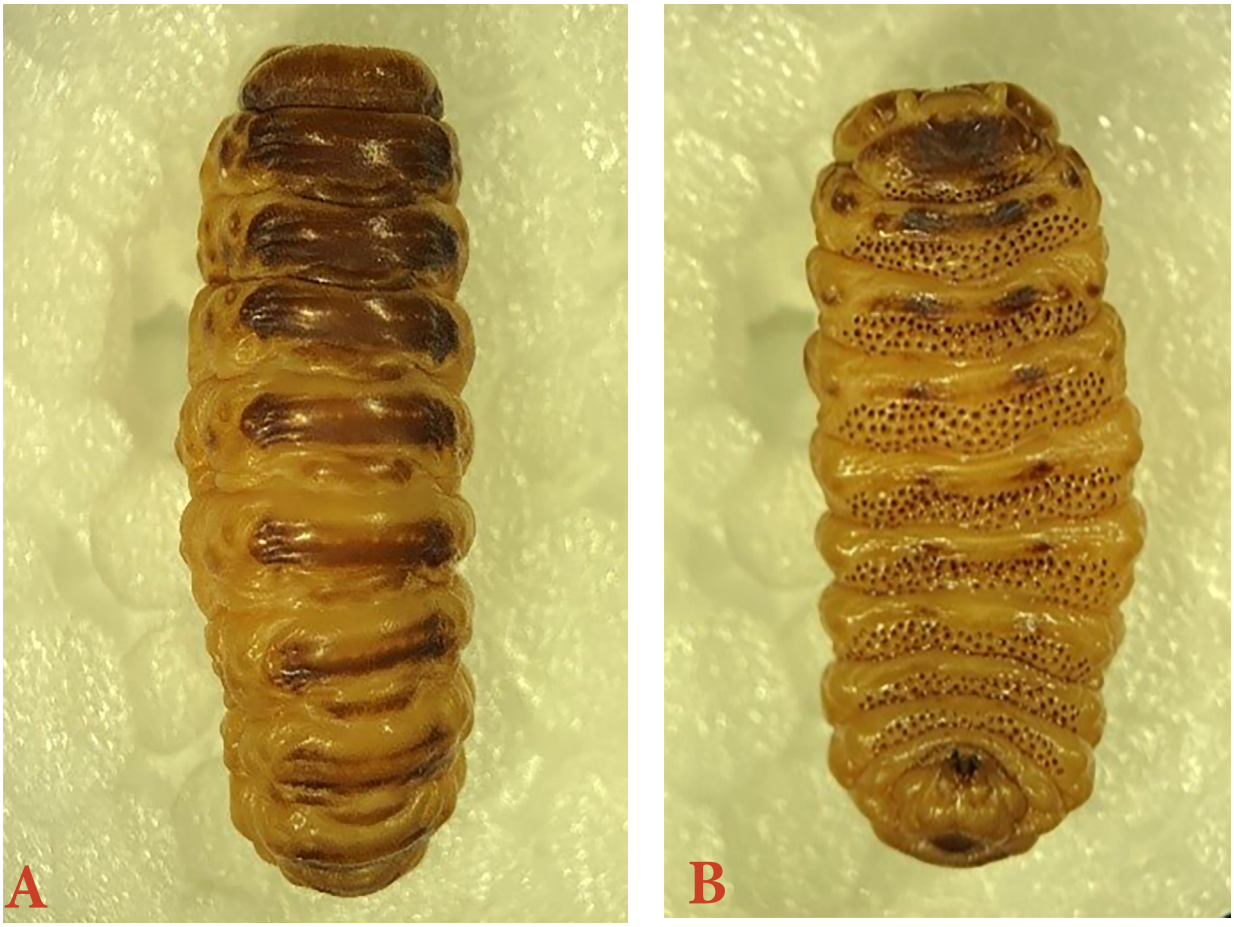

Figure 6.
Mature third instar larva of the sheep bot fly Oestrus ovis; A: Dorsal and B: Ventral view

PCR procedures and sequencing

Extracted DNA from each sample was used for amplifying a 610 bp fragment of the mtCOI gene barcoding region using the FFCOI forward (5'-GGAGCATTAATYG-GRGAYG-3') (34) and R-HCO reverse (5'- TAAACTTCAGGGTGACCAAAAAT-CA-3') primers (35). The PCR reaction was a total of 25 µl containing 1 µl of DNA template, 0.25 mM dNTPs, 1x enzyme buffer, 0.25 mM MgCl2, 2.5 µM primers (FFCOI / R-HCO), and 0.5 U of Taq DNA polymerase. The PCR conditions were 40 cycles of initial denaturation at 94°C for 30 sec, followed by denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec. At the final extension step, the reaction terminated at 72°C for 10 min. During PCR, control reactions were performed without adding DNA to the reaction to exclude contamination. To assess the amplified fragment, the amplicons from the PCR were electrophoresed on 1.5% agarose gel and were compared with a marker of standard molecular weight. All amplicons were sequenced using an ABI-3730XL capillary machine (Macrogen Inc., South Korea). O. ovis COI nucleotide sequences were trimmed for any error in nucleotides and the primer sequences were removed using DNA SISMax 3.0 software and then, were submitted to GenBank (Table 1). Based on nucleotide sequences, Bioedit 7.0 software was used for multiple sequence alignments with the Clustal W method. Using the Neighbor-Joining method, phylogenetic trees, and statistical analyses were conducted. Moreover, the evolutionary distances were computed utilizing the p-distance method with the MEGA X software. The analysis involved 36 nucleotide sequences with several 1000 bootstrap replications. Sequences were compared with the identities of isolates confirmed using the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were retrieved from GenBank and were utilized to paradigm a phylogenetic tree.

Table 1.
Oestrus ovis partial CO1 sequences originated within different geographical regions used in this study

Isolate	Country-Provenience	Host	Nucleotide accession number
A-CO1	Iran- Rasht	Sheep	MZ972997
B-CO1	Iran-Urmia	Sheep	MZ972998
C-CO1	Iran-Shiraz	Sheep	MZ972999
D-CO1	Iran-Kermanshah	Sheep	MZ973000
E-CO1	Iran-Sari	Sheep	MZ973001
F-CO1	Iran-Ahvaz	Sheep	MZ973002

Authors' Contributions

M.T. designed the study. B.E. helped for designing the study and writing of manuscript. S.R. sampling and laboratory examination. All authors reviewed the manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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