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ORIGINAL ARTICLE

Phylogenetic analysis of goat warble fly (*Przhevalskiana silenus*) based on mitochondrial COI gene

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Abstract The larvae of the genus *Przhevalskiana* (Diptera: Oestridae) are the causative agents of subcutaneous myiasis in goats. Several species have been grouped under this genus based on the morphology of different larval stages, albeit with a lot of uncertainties. Thus, application of genetic tools seems to be helpful for taxonomy. During this study, the cytochrome oxidase I (COI) gene was targeted for the characterization of larval stages of goat warble fly. Fragments of 606 bp were amplified for all the specimens. Based on the COI gene analysis, all the recovered specimens were identified as larvae of *Przhevalskiana silenus*. Molecular data on the genus is relatively rare but present isolates revealed about 87–89% identity with previous isolates of *P. silenus*. According to the phylogenetic data, the present isolates branched (as a sister group) with a number of *Hypoderma* spp. including *H. bovis*, *H. diana*, *H. lineatum* and *H. sinense*. The present findings confirmed that the COI gene could be a suitable marker for genetic characterization and identification of larvae up to the species level.

Keywords *Przhevalskiana silenus* · Goat warble fly · Cytochrome oxidase I (COI) gene · Phylogeny

Introduction

Ectoparasite infestations have been considered as a major problem in animal industry. Goat warble fly infestation which was named as goat hypodermosis, is assumed to be caused by *Przhevalskiana silenus* (Brauer 1858), *P. aegagri* (Brauer 1863) and *P. crossii* (Patton 1922). The disease is distributed in tropical and subtropical countries throughout the world and also is endemic in goats in some regions of Iran. The larvae invade subcutaneous tissue and are relatively host specific (Oryan and Bahrami 2012).

Different species of *Przhevalskiana* have been characterized based on their morphological features; however there have been a long debate on their taxonomy in host. Zumpt (1965) categorized different species of *Przhevalskiana* considering the structural features of the second and third-stage larvae. Several years later, Madel (1969) and Le Riche et al. (1973) confirmed that *P. silenus*, *P. aegagri* and *P. crossii* are actually one single species (i.e. *P. silenus*). Because of this controversy, molecular tools have developed for taxonomic characterization of the parasite. Based on molecular assays, *P. aegagri* and *P. crossii* were introduced as morphotypes of *P. silenus* (Otranto et al. 2003).

Nowadays, the cytochrome oxidase (COI) gene has been successfully used as a marker to evaluate the evolutionary relations of insects originating from orders of Diptera (Lunt et al. 1996), Orthoptera (Zhang et al. 1995) and larvae of members of the family, Oestridae (Otranto et al. 2000). According to the COI and 28S rRNA genes, a high genetic homology was obtained among morphotypes of *Przhevalskiana* (Otranto and Traversa 2004); however, it seems that more investigations are needed to clarify the parasite taxonomic issue. Therefore, the present study was conducted

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to characterize the larvae of *Przhevalskiana* based on COI gene.

Materials and methods

The present study was carried on goats of local breed slaughtered in Fars Province, southwest part of Iran. The region is located between latitude of 29.1044°N and longitude of 53.0459°E. Carcasses were examined for the presence of warbles of *Przhevalskiana* spp. during a period between March, 2016 to January, 2017. Among the infected cases, a total of 25 isolates were recovered separately from the skin and subcutaneous tissues, stored in 70% ethanol and were morphologically identified (Zumpt 1965).

Internal organs of the recovered larvae were processed for molecular study and genomic DNA was extracted using a commercial kit (MBST, Iran) according to the manufacture's instructions. A conserved mitochondrial partial cytochrome c oxidase subunit 1 gene (~ 700 bp) was amplified using a specific set of primers, UEA7 (5′-TACAGTTGGAATAGACGTTGATAC-3′) and UEA10 (5′-TCCAATGCACTAATCTGCCATATTA-3′) (Zhang and Hewitt 1997; Otranto et al. 2003). The PCR cycling conditions were as follows: an initial DNA denaturation at 95 °C for 2 min, followed by 35 cycles, denaturation at 95 °C for 60 s, annealing at 57.3 °C for 30 s, 72 °C for 70 s and a final extension at 72 °C for 7 min. Sterile water was used as the negative controls. The obtained forward and reverse sequences data were used to construct a consensus DNA sequence using CLC Main Work bench 5.5 software (Tamura et al. 2007). The comparison of the sequences was made with other available sequences in NCBI using BLAST search. Data were also used for construction of the phylogenetic trees using maximum parsimony method (Tamura et al. 2013) and *Lucilia cuprina* was included as outgroup.

Results and discussion

During this study, based on the morphological characters, the recovered warbles were identified as the second stage larvae of *P. silenus*. The size and the shape of larvae, the spinulations on the fifth segment of body (Fig. 1a) and also the shape of posterior spiracles (Fig. 1b) were consistent with those reported by Zumpt (1965).

In the present study, the specific DNA fragment size of about 700 bp was amplified for all the specimens (Fig. 2). The sequences were aligned and trimmed to yield 606 bp fragments which was deposited in the GenBank with the accession no. KY595102. BLAST analysis confirmed that the specimens were isolates of *P. silenus* with 98–100% homology with the sequences deposited in the GenBank. According to the GenBank, no molecular report has been submitted on *Przhevalskiana* insect or its larval stages in Iran. The present work is therefore first one which describes the parasite in this region. The genetic data on *P.*

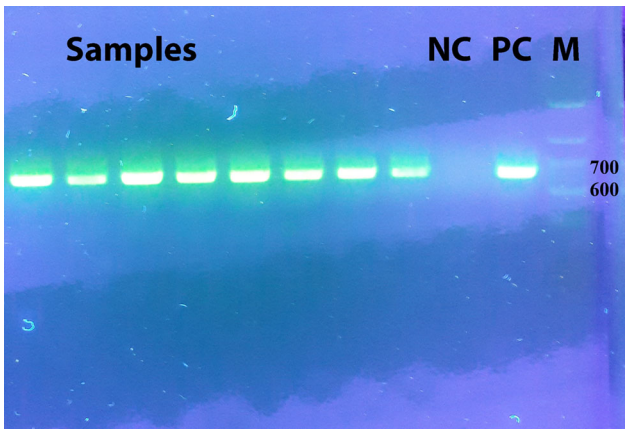

Fig. 2 PCR amplification of COI gene region from genomic DNA of *P. silenus.* Cyber-green-stained agarose gel electrophoresis showing fragments of about 700 bp (samples); *NC* negative control, *PC* positive control and *M* marker (color figure online)

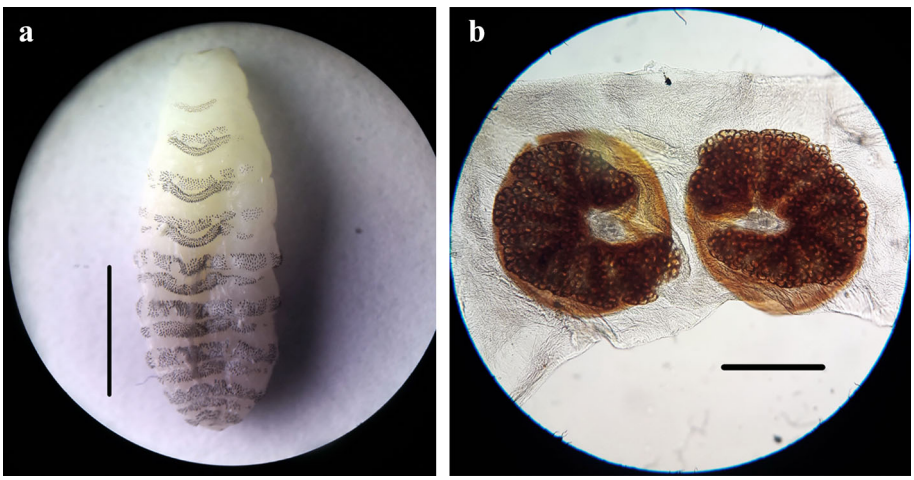

Fig. 1 Second stage larvae of *P. silenus*. **a** Dorsal view, bar = 300 μm; **b** Posterior Spiracles, bar ~ 30 μm

Table 1 Pairwise comparison of aligned COI gene sequences of *P. silenus* and other myiasis agents of Oestridae family; upper right triangles show nucleotide distance and lower left triangles represent the percent identity

Parasite name	1	2	3	4	5	6	7	8	9	10	11
Przhevalskiana silenus (current study)	–	0.12	0.12	0.17	0.17	1.07	1.07	0.16	0.19	0.20	1.07
Przhevalskiana silenus (AF497766)	89.17	–	0.00	0.19	0.19	1.1	1.1	0.17	0.20	0.21	1.1
Przhevalskiana silenus (AY332542)	88.99	99.82	–	0.20	0.20	1.09	1.09	0.17	0.20	0.21	1.09
Hypoderma bovis (KT600284)	84.9	82.95	82.77	–	0.00	1.01	1.02	0.09	0.13	0.13	1.02
Hypoderma bovis (GU984814)	84.9	82.95	82.77	100.00	–	1.01	1.02	0.09	0.13	0.13	1.02
Hypoderma bovis (EU181163)	43.03	42.34	42.51	44.41	44.41	–	0.00	1.10	1.06	1.07	0.02
Hypoderma bovis (EU181160)	43.03	42.34	42.51	44.23	44.23	99.64	–	1.11	1.06	1.08	0.01
Hypoderma diana (AF497763)	85.97	84.72	84.90	91.12	91.12	42.34	42.17	–	0.13	0.14	1.11
Hypoderma lineatum (AF497762)	83.3	82.62	82.80	88.30	88.30	43.20	43.20	87.77	–	0.08	1.06
Hypoderma sinens (AY350769)	82.45	81.38	81.38	88.12	88.12	43.03	42.86	86.88	92.38	–	1.08
Oesterus ovis (AF257118)	43.03	34.14	34.14	36.55	36.55	98.20	98.56	42.17	43.20	42.86	–

silenus is relatively rare and the past data on the COI gene in *Przhevalskiana* larvae is mainly confined to the Italian isolates (Otranto et al. 2003; Otranto and Traversa 2004); Nevertheless, the comparison of the present data with the existing sequences revealed about 87–89% similarity and a distance of about 0.12 (Table 1). Although this result suggests a relatively close genetic relationship among the present Iranian isolates with other *P. silenus* records (Otranto et al. 2003; Otranto and Traversa 2004), but it is suggested that more epidemiological studies should be conducted to assess its phylogenetic relationship with other myiasis agents in Oestridae family.

The approach of molecular biology using the mitochondrial DNA gene as a marker for taxonomic and

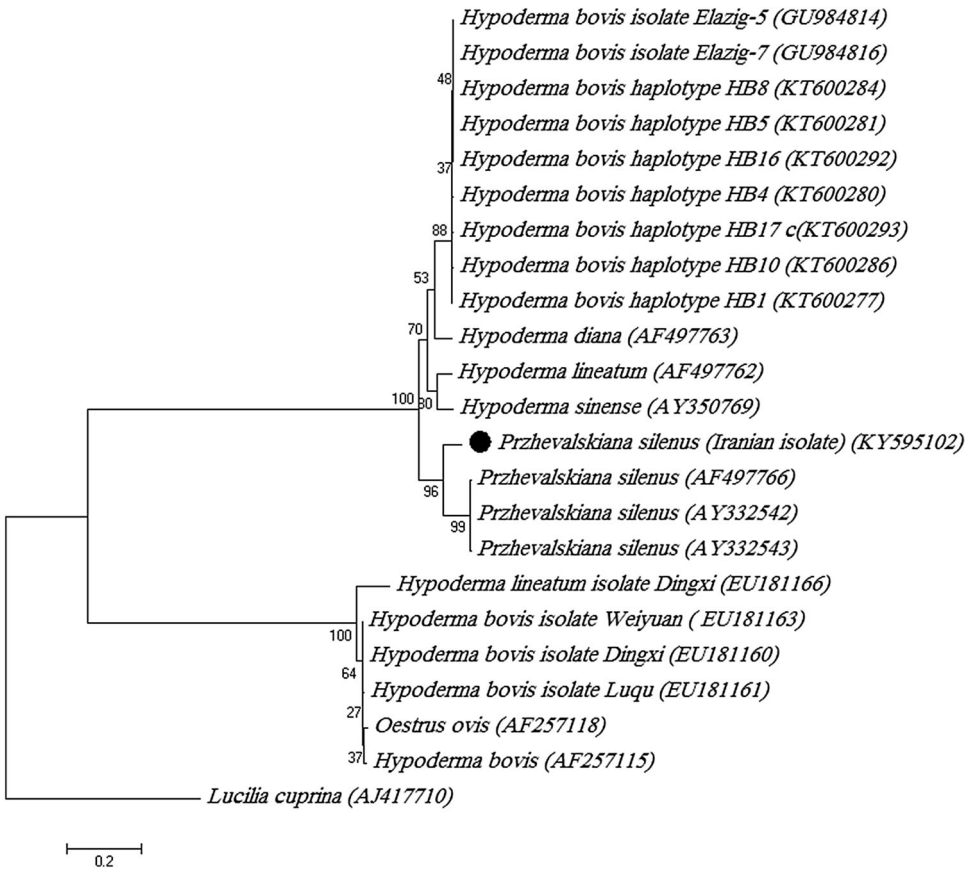

Fig. 3 Phylogenetic position of *P. silenus* isolated from goats inferred from the nucleotide sequences of partial COI gene using the maximum-likelihood method. Bootstrap values (2000 psuedoreplicates) are indicated at the nodes

population genetic studies has been considered in insects (Xiong and Kocher 1991; Lunt et al. 1996). Among insects of myiasis importance, members of the Oestridae family including *H. bovis*, *H. lineatum*, *Oestrus ovis* and *Gasterophilus intestinalis* (Otranto et al. 2000; Otranto and Puccini 2000) were more investigated. Harboring highly conserved and variable regions, different range of mutational rates and the large size have made the COI gene as appropriate target for molecular approach (Lunt et al. 1996; Zhang and Hewitt 1997).

In the present study, the phylogenetic tree (Fig. 3) confirmed that the present isolates had close association with previous records of *P. silenus* (with the accession numbers of AF497766, AY332542 and AY332543). This offers additional molecular evidence on the taxonomic pattern of *Przhevalskiana* species. In addition, we found that the isolates branched (as a sister group) with a number of *Hypoderma* spp. including *H. bovis*, *H. diana*, *H. lineatum* and *H. sinense*. In contrast, some other species of the genus *Hypoderma* were grouped in a separate clade (Table 1). However, the present isolates had either relatively close genetic relationship and also relatively high genetic distances with records of *Hypoderma* spp. These contrast findings could be explained considering that the COI region is highly variable among insects (Zhang and Hewitt 1997). Significant interspecific divergence was reported among a number of 18 myiasiscausing *Oestridae* spp. (Otranto et al. 2003). The level of interspecific variation rate between *P. silenus* and different *Hypoderma* species ranged from 13.8 to 17.8. The wide range of interspecific divergence has attributed to the host of parasite, site of infection and its biological and geographical features. Thus, the present study indicated that the mitochondrial COI gene can be targeted for the molecular identification and further resolving the taxonomical concerns for Oestrid flies.

In summary, the COI gene was investigated in the larvae of goat warble fly in Iran. Data confirmed a close association with *P. silenus*. According to phylogenetic analysis, the present isolates grouped with a number of *Hypoderma* spp. and separated from some other. It seems that the COI region is highly variable among myiasis-causing agents. The present findings also suggest that the COI gene could be used as a suitable molecular marker for identification and characterization of *Przhevalskiana* species.

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Author's contribution ER and SMR designed and supervised the experiment. RF carried out the research technically; AE and MA had roles in analyzing data and writing the manuscript. SS participated in collecting the samples and conducting the study.

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Compliance with ethical standards

Conflict of interest All authors declares that they have no conflict of interest.

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