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Molecular Characterization of Mitochondrial Cytochrome *c* oxidase subunit 1 *(Cox1)* gene from *Trichostrongylus* Species (Nematoda: Trichostrongylidae) in Northern Iran

Kuzey İran'daki Trichostrongylus Türünden (Nematoda: Trichostrongylidae) Mitokondriyal Cytochrome c oxidase subunit 1 (Cox1) Geninin Moleküler Karakterizasyonu

Meysam Sharifdini[1], Elham Hajialilo[2,3], Hedayat Hosseinnezhad[1], Mohammad Ali Mohammadi[4]

[1]Guilan University of Medical Sciences, Department of Medical Parasitology and Mycology, Rasht, Iran
[2]Qazvin University of Medical Sciences, Medical Microbiology Research Center, Qazvin, Iran
[3]Qazvin University of Medical Sciences, Department of Parasitology and Mycology, Qazvin, Iran
[4]Kerman University of Medical Sciences, Research Center for Hydatid Disease in Iran, Kerman, Iran

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ABSTRACT

Objective: The objective of the present study was to identify *Trichostrongylus* species by molecular analysis and also phylogenetic relationships of *Trichostrongylus* species by mitochondrial Cytochrome c oxidase subunit 1 (*Cox1*) gene in Guilan province, northern Iran.

Methods: Abomasum and duodenum contents of 144 livestock were collected from sheep, goats, and cattle in Guilan province. Morphological survey was performed for initial screening. Total DNA was extracted, and the partial region of *Cox1* gene was amplified and sequenced. Genetic diversity was calculated and phylogenetic analysis of the data on nucleotide sequence was conducted by MEGA7 software.

Results: Three species of *Trichostrongylus* including *T. colubriformis, T. vitrinus, and T. axei* were identified by morphological characteristics. The genetic divergence within the species in the present study was observed for *T. axei* (0-2.5%), *T. colubriformis* (0.77%), and *T. vitrinus* (0%). The mean inter-species difference between the three species of *Trichostrongylus* obtained in this study was 14.4-15.4%.

Conclusion: The *Cox1* sequences of the members of *Trichostrongylus* spp. were highly variable and this could be used as a valuable measure to achieve a proper assessment on biodiversity. Sequence data generation from other species of *Trichostrongylus* will be needed to reconstruct the phylogenetic relationships of this genus of nematodes.

Keywords: *Trichostrongylus* spp., ruminants, *Cox1*, phylogenetic analysis, Iran

ÖZ

Amaç: Bu çalışmanın amacı, İran'ın kuzeyindeki Guilan eyaletindeki *Trichostrongylus* türlerinin moleküler analiz ve ayrıca mitokondriyal Cytochrome c oxidase subunit 1 (*Cox1*) geni ile *Trichostrongylus* türlerinin filogenetik ilişkilerinin belirlenmesidir.

Yöntemler: Guilan ilindeki koyun, keçi ve sığırlardan 144 hayvanın abomasum ve duodenum içerikleri toplanmıştır. İlk tarama için morfolojik inceleme yapıldı. Toplam DNA ekstrakte edildi ve *Cox1* geninin kısmi bölgesi amplifiye edildi ve sekanslandı. Genetik çeşitlilik hesaplandı ve nükleotid dizisine ilişkin verilerin filogenetik analizi MEGA7 yazılımı ile yapıldı.

Bulgular: *T. colubriformis, T. vitrinus* ve *T. axei* dahil olmak üzere üç *Trichostrongylus* türü morfolojik özelliklerle tanımlanmıştır. Bu çalışmada türler içindeki genetik farklılık *T. axei* (%0-2,5), *T. colubriformis* (%0,77) ve *T. vitrinus* (%0) için gözlenmiştir. Bu çalışmada elde edilen üç *Trichostrongylus* türü arasındaki ortalama türler arası fark %14,4-15,4 olmuştur.

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Address for Correspondence/Yazar Adresi: Elham Hajialilo, Qazvin University of Medical Sciences, Department of Parasitology and Mycology, Qazvin, Iran
Phone/Tel: +989127860343 **E-mail/E-Posta:** e.hajialilo@gmail.com **ORCID ID:** orcid.org/0000-0003-2159-4066

Türkiye PARAZİTOLOJİ Dergisi

Sonuç: *Trichostrongylus* spp. üyelerinin *Cox1* dizileri. oldukça değişkendi ve bu, biyoçeşitlilik hakkında uygun bir değerlendirme elde etmek için değerli bir ölçü olarak kullanılabilirdi. Bu nematod cinsinin filogenetik ilişkilerini yeniden yapılandırmak için diğer *Trichostrongylus* türlerinden dizi verisi oluşturulması gerekecektir.
Anahtar Kelimeler: *Trichostrongylus* spp., ruminantlar, *Cox1*, filogenetik analiz, İran

INTRODUCTION

Trichostrongylus nematodes are highly prevalent and considered as gastrointestinal parasitic pathogens among ruminants with worldwide distribution (1,2). Clinical symptoms of humans are mild although in some patients gastrointestinal signs and eosinophilia may occur (3,4). These nematodes are major health challenges, causing reduced animal products (5,6). Several species of the parasite have been reported from herbivores with twelve valid species of species identified in humans (2,7,8).

Also, the frequency of *Trichostrongylus* spp. in human and animal hosts has been repeatedly reported in Iran (9-12). Ruminant infection was reported from various parts of Iran, with human infections found in Khuzestan, Isfahan, Tehran, Hormozgan, Kermanshah, Mazandaran, Guilan, Sistan & Baluchestan, and West Azerbaijan provinces (2,13-16). According to the morphological features reported in previous studies from Iran, several species of nematodes have been identified in human including *T. orientalis, T. vitrinus, T. axei, T. colubriformis, T. probolurus*, *T. skrjabini*, *T. capricola*, and *T. lerouxi* (9,10,15). In recent years, some studies clarified the human infections with *T. vitrinus, T. axei, T. colubriformis*, and *T. longispicularis* species by polymerase chain reaction (PCR) amplification of ITS2-rDNA region in endemic areas of northern Iran with *T. colubriformis* considered as the predominant species (2,8,13,17). Infection with various species of *Trichostrongylus* including *T. colubriformis* (11,12,15,18,19), *T. vitrinus* (11,12,15,18,19), *T. axei* (15), *T. capricola* (11,15), *T. probolurus* (11,12,15,18,19), *T. longispicularis* (11), *T. orientalis* (15), *T. lerouxi* (20), *T. skrjabini* (15), and *T. hamatus* (18) were reported in different herbivores such as sheep (11,12,15), goats (11,15), cattle (11,15), buffalos (11,15), and camels (18,19) in most parts of Iran. The predominant species of *Trichostrongylus* among different herbivores are *T. colubriformis*, *T. vitrinus*, and *T. axei* found in most parts of the country (15).

There is a tremendous diversity of the nematodes in the country (11,21) however, the molecular approaches, currently available and easily applicable, could accurately identify these species. Molecular studies based on ITS and 28S regions of ribosomal DNA were applied for genetic variation and phylogenetic analysis of super family Trichostrongyloidea (8,22-25). Although, numerous number of studies have focused on ITS2 for analysis of the Trichostrongylidae family in genetic variation, species detection, and phylogenetic relationships (2,8,21), yet mitochondrial (mt) genomes have the potential to present valuable information. Mt genomes are conserved and present large amounts of sequence data in the organisms, therefore mtDNA are used for evolutionary analyses, taxonomy, population genetics, and systematics studies (26-28). There are few studies that have investigated the mitochondrial gene of the Trichostrongylidae family, in which the mtDNA of *Marshallagia marshalli*, *Haemonchus placei, Haemonchus contortus, T. vitrinus*, *T. axei*, *Ostertagia trifucata*, and *Teladorsagia circumcincta* species were evaluated for phylogenetic relationship and species identification (26,29-34). The study explained to mitochondrial DNA diversity of *O. ostertagi* are five to ten times greater than typical estimates reported for species in other taxa (35). The population genetic structure and diversity of *H. contortus* by ribosomal and mitochondrial gene in Bangladesh showed low genetic differentiation but high gene flow among different populations of the parasite (36). Taxonomy studies of the nematodes based on sequences of coding mitochondrial genes are more accurate than non-coding ribosomal genes. While mitochondrial genomes are considered as suitable markers for population evolution studies (32,33), the studies targeting the mtDNA for identification of Trichostrongylidae family are very limited worldwide with even no single report on mitochondrial gene of the nematodes from Iran. Therefore, the present study focused on molecular phylogenetic analysis based on *Cox1* gene of *Trichostrongylus* species in northern Iran.

METHODS

Sample Collection and Morphological Identification

In current study, a total of 144 abomasum and duodenum specimens from livestock, including 72 cattle, 59 sheep, and 13 goats were collected from the abattoir of Talesh district in Guilan province, northern Iran during July to September 2018 (37).

The Trichostrongylidae family members were isolated by washing the abomasum and duodenum contents followed by passing through the 20, 40, and 100 mesh screens. The helminthes captured on mesh screens were examined under stereomicroscope. Morphological features were evaluated after cleaning the worms with normal saline and lactophenol. The samples were preserved in 70% ethanol at room temperature until used (38).

DNA Extraction and PCR Amplification

Male parasites were isolated for DNA extraction. Selecting of male worms for molecular analysis was performed randomly. Total genomic DNA was extracted from one male worm of each species of trichostrongyloid nematodes collected from all study animals, using a commercial DNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instructions. The partial region of the *Cox1* gene with approximately 700 bp was amplified using the LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) sequences as forward and reverse primers (39). The thermal PCR profiles included an initial denaturation step at 95 °C for 6 minutes followed by 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 50 °C for 45 seconds, an initial extension step at 72 °C for 60 seconds, and a final extension step at 72 °C for 10 minutes.

Sequencing and Phylogenetic Analysis

The PCR products were sequenced using an ABI 3130xl platform (Applied Biosystems, Foster City, California, USA). The sequences identified by the ABI system were edited and analyzed by BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

The sequences were compared with the sequences deposited in the GenBank database by BLAST program (http://www.ncbi.nlm.nih.gov/). The sequences of the three species of genus *Trichostrongylus* derived from the domestic ruminants and deposited in the GenBank database were marked by the following Accession Numbers: MW051252-MW051254 for *T. axei*; MW051250 and MW051251 for *T. colubriformis*, and MW051255 and MW051256 for *T. vitrinus*.

Multiple sequence alignments were conducted by ClustalW incorporated in the BioEdit software. Phylogenetic tree was constructed by the MEGA7 software (Molecular and Evolution Genetic Analysis v7). The maximum likelihood method and the Tamura 3-parameter model were used for phylogenetic tree reconstruction. Bootstrap value was done based on 1000 replications in the topology of the tree. Genetic analysis of haplotypes was performed for Haplotype diversity, number of variable sites and number of haplotypes using DNAsp software (40).

RESULTS

All of the study male worms were identified based on the morphological characteristics of male copulatory spicules and gubernaculum (Figure 1). *Trichostrongylus axei* was isolated from the cattle, sheep, and goats while *T. colubriformis* and *T. vitrinus* were only detected among the sheep and goats. The isolates were successfully amplified for *Cox1* gene with specific band. The sequence results confirmed three species of *T. colubriformis*, *T. vitrinus*, and *T. axei* among the specimens. A dendrogram, based on the phylogenetic analysis, showed that the species were placed along, with the same species obtained from the GenBank database, into a distinct cluster of the tree (Figure 2). The genetic divergence within the species of *T. axei, T. colubriformis*, and *T. vitrinus* obtained in this study were 0-2.5%, 0.77%, and 0%, respectively. Two species of *Trichostrongylus* including *T. axei* and *T. vitrinus* isolated from the sheep and goats were quite similar. The intra-species distance rate within the specimens of *T. axei*, *T. colubriformis*, and *T. vitrinus* found in the present study and those available in the GenBank database amounted to 0.95-3.1% (1.9%), 0.19-4.08% (2.4%), and 0-2.32% (1.5%), respectively.

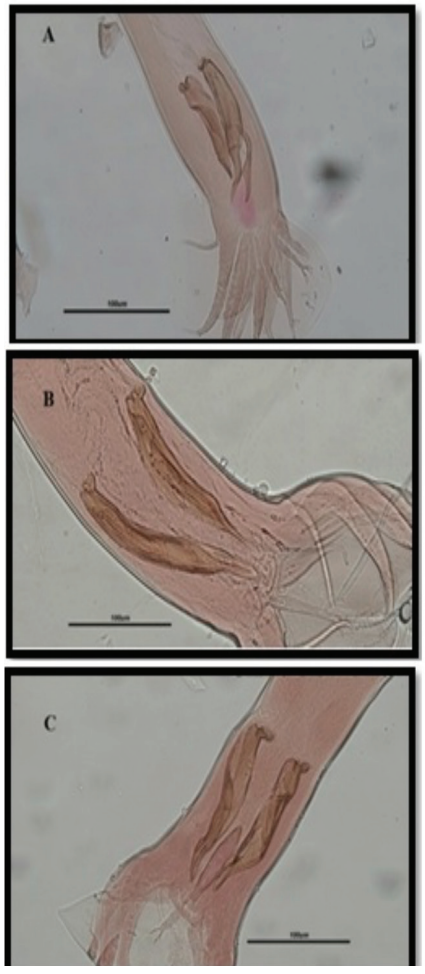

Figure 1. Posterior ends of males of *T. axei* (A), *T. vitrines* (B) and *T. colubriformis* (C)

In this study, the mean inter-species differences between our *T. axei* specimens, compared with *T. colubriformis* and *T. vitrinus* isolates, were 14.4% and 14.6%, respectively. Also, the mean genetic difference between the *T. colubriformis* specimens was 15.4% when compared with *T. vitrinus*.

Based on our sequences and those deposited in the GenBank, the mean inter-species distance rates between the isolates of *T. axei* and those of *T. colubriformis* and *T. vitrinus* were 13.5% and 14.5%, respectively. Also, the mean genetic diversity between the isolates of *T. colubriformis* and those of *T. vitrinus* was 14.9%. The isolates were categorized into 5 haplotypes. The haplotype diversity for 7 isolates was calculated as Hd =0.9048. The distribution of *Trichostrongylus* isolates in each haplotypes is shown in Table 1.

DISCUSSION

The three species of the *Trichostrongylus* including *T. colubriformis*, *T. vitrinus*, and *T. axei* identified in the present study, along with the data already reported from Iran confirm that the predominant species in herbivorous animals (15). *T. axei* was isolated from the cattle, sheep, and goats while *T. colubriformis* and *T. vitrinus* were only detected among the sheep and goats. *T. axei* (2.86%) was the most predominant nematodes of cattle in Ethiopia (41).

Iran is one of the most important foci for *Trichostrongylus* infection among human and animal hosts (12,16,42,43). Proper conditions such as humidity and climate in the northern parts of the country including Mazandaran and Guilan provinces lead to permanent establishment of the life cycle process of soil transmitted helminthes in the regions (2,8,42).

In the present study the authors used the sequence analysis protocol for detecting the mitochondrial *Cox1* gene, whereas several other studies, reported from Iran, employed *ITS-rDNA* gene specific for the phylogenetic analysis of *Trichostrongylus* species (2,8,13). The nuclear ribosomal gene is widely applied to the studies of deep and shallow phylogenetic relationship in the phylum Nematoda (2,8,44,45). Recent studies illustrated that the mitochondrial genes to be the proper options for phylogenetic approach and specifically for the *Cox1* gene that has mainly been used in population genetic surveys for various nematode parasites of the vertebrates (45-47).

Several studies have demonstrated that the sequence differences between the members of the *Trichostrongylus* are not noticeable when the detection protocol is based on the *ITS2* gene (2,8,13). Ashrafi et al. (13) reported a mean inter-species distance rate of 2.6% within different species of *Trichostrongylus* while in

the current study the mean inter-species variation within our specimens and those available in the GenBank was 13.5-14.9%. Due to the high-level divergence in the *Cox1* gene, it could be considered as a valuable genetic tool for phylogenetic and taxonomic studies on the members of the *Trichostrongylus* genus. The phylogenetic tree constructed in our study represented that the three species of *T. colubriformis*, *T. vitrinus*, and *T. axei* were separated in distinct cluster along with the same species obtained from other studies in different countries (Figure 2). The results of genetic diversity within the species showed that the intra-species distance rate among the present isolates was so close, indicating high proximity of the sequences in the region.

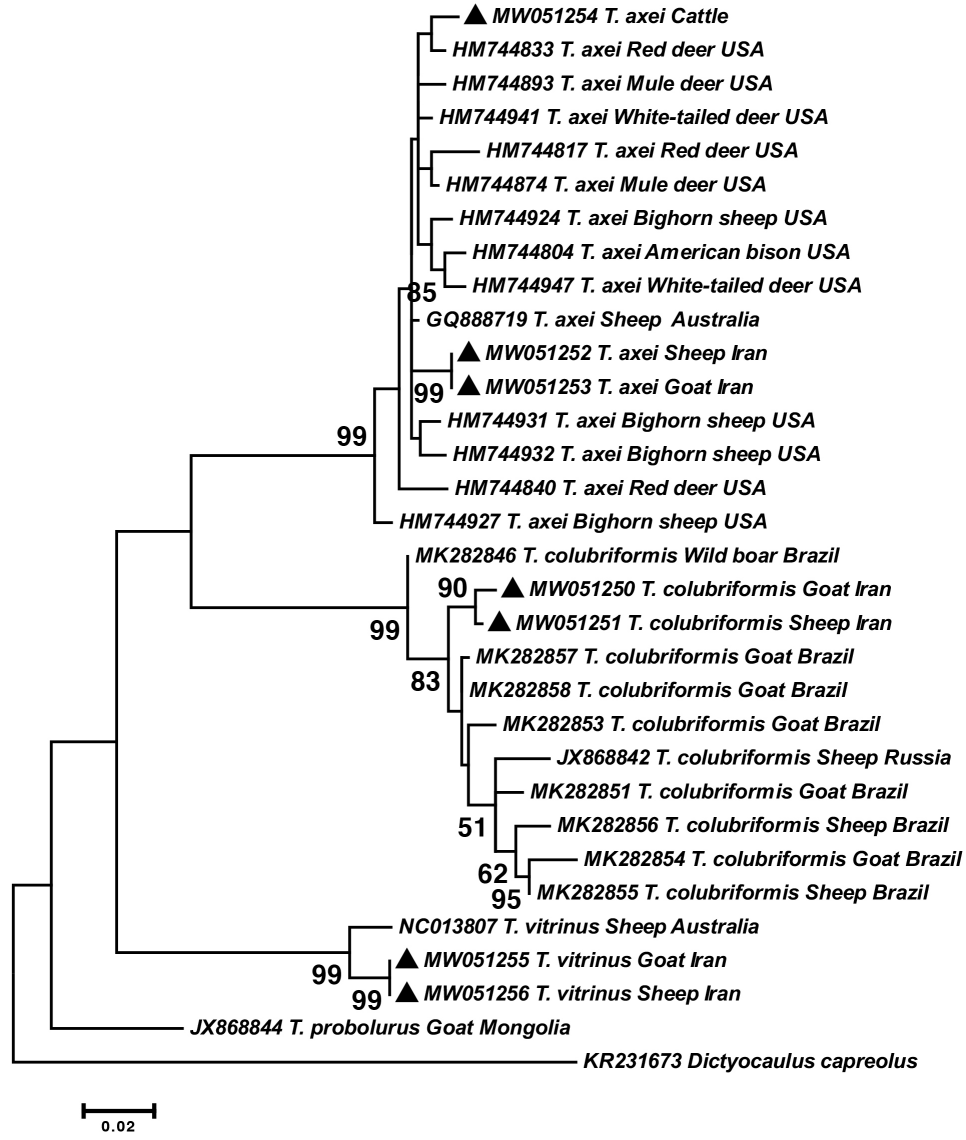

Figure 2. Phylogenetic tree of isolates of *Trichostrongylus* spp. obtained in this study (▲) and other isolates of *Trichostrongylus* spp. retrieved from GenBank based on *Cox1* gene. The tree was designed by using the maximum-likelihood test and the Tamura 3-parameter model as implemented in the MEGA7 software. *Dictyocaulus capreolus* was used as an out group

Table 1. Haplotypes groupings for *Trichostrongylus* isolates of partial cytochrome c oxidase gene subunit 1 *(Cox1)* and the accession numbers of isolates in each group

Haplotype	Number of isolates	Species	Accessions
Hap-1	1	*T. colubriformis*	MW051250
Hap-2	1	*T. colubriformis*	MW051251
Hap-3	2	*T. axei*	MW051252, MW051253
Hap-4	1	*T. axei*	MW051254
Hap-5	2	*T. vitrinus*	MW051255, MW051256

Little information on mitochondrial genes of Trichostrongyloidea superfamily is available. Palevich et al. (33) in New Zealand investigated the complete mitochondrial genomes of *H. contortus* and *T. circumcincta* by phylogenetic analysis. Another study, reported from Uzbekistan, was based on ribosomal (ITS2) and mitochondrial (*Cox1*) of *Marshallagia* sp. and concluded that the ITS2 sequences has little variation and is not a suitable gene for diagnosing different species, while *Cox1* gene shows more diversities (32). *Ostertagia trifurcata* and *Marshallagia marshalli* were evaluated by phylogenetic analysis of the complete mitochondrial genes in China and the findings introduced complete mt genome sequence of the nematodes as a novel genetic marker for population genetic and molecular epidemiology (29,34). Two other studies, reported from Brazil and Australia, evaluated the complete mitochondrial genes of *H. placei, T. circumcincta, T. vitrinus*, and *T. axei* and suggested that the phylogenomics approach of mtDNA could be applied as a new genetic marker in phylogenetic analysis and geographic relationships among different isolates in population genetic studies (26,31). Moreover, the *Cox1* and *nad4* genes of *T. axei* were also analyzed for population genetic structure of the nematode in USA (30). Our study could be the basis for further sequence studies with greater sample sizes, especially the analysis of both nuclear and mitochondrial genes, are needed to provide a comprehensive understanding of the genetic variations of *Trichostrongylus* spp. in endemic areas and other parts of Iran.

CONCLUSION

In the present study three species of *T. colubriformis*, *T. vitrinus,* and *T. axei* were observed among the specimens of Guilan province, northern Iran. This study concluded the genetic diversity of the *Cox1* gene is notable and the gene is suitable for analyzing the gene diversity of intra-species distance among helminthes. The scarcity of molecular data on *Cox1* gene within *Trichostrongylus* spp. in various geographical regions and hosts makes it necessary to produce sufficient data on diversities of this gene which eventually leads to reconstruct the total phylogenetic relationships of this group of nematode. Thus, the findings of the present study suggest that the analysis of complete mitochondrial genome to be the focus of further experiments in the future research.

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*** Ethics**

Ethics Committee Approval: This study was approved by the Medical Ethics Committee of Guilan University of Medical Sciences (Approval code: IR.GUMS.REC.1397.176).

Informed Consent: Not applicable.

Peer-review: Internally and externally peer-reviewed.

*** Authorship Contributions**

Concept: M.S., Design: M.S., E.H., Data Collection or Processing: H.H., Analysis or Interpretation: M.S., M.A.M., Literature Search: E.H., Writing: E.H., Critical Review: M.S., E.H.

Conflict of Interest: No conflict of interest was declared by the authors

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