Molecular Characterization of Mitochondrial Genome From *Trichostrongylus* Species (Nematoda: Trichostrongylidae) in Northern Iran

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Research

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Abstract

Background: The members of *Trichostrongylus* spp. are gastrointestinal nematodes of ruminants with worldwide distribution. The nematodes are considered as major health challenges especially in endemic regions of Iran. Several species of the parasite are reported from humans and animals in the country. Frequently, molecular analyses for identification of different species focus on nuclear regions and there is lack of information regarding the mitochondrial genes of *Trichostrongylus* spp*.* Therefore, the aim of the present study was to identify *Trichostrongylus* species by molecular analysis of mitochondrial gene in Guilan province, northern Iran.

Methods: The nematodes were collected from the abomasum contents of sheep, goats and cattle. Morphological survey was performed for initial screening. Total DNA was extracted, and the partial region of cytochrome c oxidase subunit I (*Cox1*) gene was amplified and sequenced. Genetic diversity was calculated and phylogenetic analysis of the nucleotide sequence data was conducted.

Results: Three species of *Trichostrongylus* including *T. colubriformis*, *T. vitrines* and *T. axei* were identified by morphological characterizations. 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 *Trichostrongylus* species obtained in this study was 14.4- 15.4%.

Conclusions: The *Cox1* sequences of members of the *Trichostrongylus* spp*.* are highly variable and this could be used as a valuable measure for achieving a proper assessment of biodiversity. Sequence data generation from additional species of *Trichostrongylus* will be needed to reconstruct the phylogenetic relationships of this genus of nematodes.

Background

Trichostrongylus nematodes are highly prevalent and considered as gastrointestinal parasite pathogens among ruminants with a worldwide distribution [1, 2]. *Trichostrongylus* spp. usually infect human by ingesting the infective-stage larvae [1]. Clinical symptoms of humans are mild, though in some patients gastrointestinal signs and eosinophilia may occur [3, 4]. These nematodes are major health challenges, causing reduced animal production or even death of the infected animal in severe infection, as well as economic burden with the cost of treatment, considered as a problem especially in developing countries [5, 6]. Several species of the parasite have been reported from herbivores with approximately 12 species identified in humans [2, 7]. Also, the frequency of *Trichostrongylus* from human and animal hosts has been repeatedly reported in Iran [8, 9, 10, 11]. Ruminant infection was reported from Isfahan, Khuzestan, Mazandaran, Kermanshah, Hormozgan and West Azerbaijan provinces, with human infections found in Khuzestan, Isfahan, Tehran, Hormozgan, Kermanshah, Mazandaran, Guilan, Sistan & Baluchestan, and West Azerbaijan provinces [2, 12, 13, 14, 15].

Identification of the parasite species could be useful in preventing and controlling the disease. According to the morphological futures 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* [8, 9, 14]. In recent years, some studies clarified the human infections with *T. vitrinus, T. axei, T. colubriformis* and *T. longispicularis* species in endemic areas of northern Iran with *T. colubriformis* considered as a predominant species [2, 12, 16, 17]. Infection with various species of Trichostrongylus including *T. colubriformis* [10, 11, 14, 18, 19], *T. vitrinus* [10, 11, 14, 18, 19], *T. axei* [14], *T. capricola* [10, 14], *T. probolurus* [10, 11, 14, 18, 19], *T. longispicularis* [10], *T. orientalis* [14], *T. lerouxi* [20], *T. skrjabini* [14] and *T. hamatus* [18] were reported in different herbivores such as sheep [10, 11, 14], goat [10, 14], cattle [10, 14], buffalo [10, 14] and camel [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 [14]. Also, *T. colubriformis, T. vitrines*, and *T. axei* were common species among different herbivores in most parts of the country [14].

There is a tremendous diversity of the nematodes in the country [10, 21] however the molecular approaches, currently available and easily applicable, could accurately identify these species. Molecular studies based on internal transcribed spacer (ITS) and 28S regions of ribosomal DNA were applied for genetic variation and phylogenetic analysis of Trichostrongylina [16, 22, 23, 24]. Frequently, many studies focus on ITS2 for analysis of genetic variation, species detection, and phylogenetic relationships [2, 16, 21]. Mitochondrial (mt) genomes could 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 [25, 26, 27]. There are few studies that have investigated the mitochondrial gene of Trichostrongylidae family, in which the mtDNA of the species of *Marshallagia marshalli*, *Haemonchus placei, Haemonchus contortus, T. vitrinus*, *T. axei*, *Ostertagia trifurcate*, and *Teladorsagia circumcincta* were evaluated for phylogenetic relationship and species identification [25, 28, 29, 30, 31, 32, 33]. Taxonomy studies of the nematodes based on sequences of coding mitochondrial genes are more accurate than non-coding ribosomal genes. Meanwhile, the mitochondrial genomes are considered as suitable markers for population evolution studies [31, 32], while the studies targeting the mtDNA for identification of Trichostrongylidae are very limited worldwide with even no single study on mitochondrial gene of the nematodes reported from Iran, so, the present study focused on molecular phylogenetic analysis based on cytochrome c oxidase subunit I (*Cox1*) mitochondrial gene of *Trichostrongylus* species in northern Iran.

Methods

Sample collection and morphological identification

This study was conducted in Guilan province, located in the littoral of Caspian Sea, northern Iran. The nematodes were collected from abomasum of sheep, goats, and cattle in the abattoir of the town of Talesh, the center of animal husbandry in Guilan province (Fig 1). The Trichostrongylidae family members were isolated by washing the abomasum content followed by passing through the 20, 40, and 100 mesh screens. The helminths 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 [34].

DNA extraction and PCR amplification

Male parasites were isolated for DNA extraction. Total genomic DNA was extracted from one male worm of each species of trichostrongyloid nematodes from every kind of livestock using a commercial DNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instructions. The partial region of *cox1* gene with approximately 700bp was amplified using the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') sequences as *cox1* gene forward and reverse primers (Folmer, Black et al. 1994). 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 ºC 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 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 sequence of *Trichostrongylus* species deposited in the GenBank database were given the 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 with ClustalW incorporated in the BioEdit software. Phylogenetic tree was constructed by the MEGA7 software (Molecular and Evolution Genetic Analysis v7). The maximum likelihood method based on the Tamura 3-parameter model and maximum–likelihood algorithm was applied. Bootstrap value was done based on 1000 replications in the topology of the tree and *Dictyocaulus capreolus* considered as out group (Fig 2). Pairwise distance comparisons were clarified for seven sequences of *Trichostrongylus* species isolated in the present study as well as other species with BioEdit software.

Results

All of the study male worms were identified based on the morphological characteristics of male copulatory spicules and gubernaculum (Fig 1). *Trichostrongylus axei* was isolated from cattle, sheep and goat but *T. colubriformis* and *T. vitrines* only detected from sheep and goats. The isolates were successfully amplified for *Cox1* gene with specific band. Sequence results confirmed three species of *T. colubriformis*, *T. vitrines*, 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 distinct cluster of the tree (Fig 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 sheep and goats were quite similar. The intra-species distance rate within the specimens of *T. axei*, *T. colubriformis*, and *T. vitrinus* obtained in this study and those available in the GenBank amounted to 0.95-3.1% (1.9%), 0.19-4.08% (2.4%) and 0-2.32% (1.5%), respectively.

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, compared with *T. vitrinus*, was 15.4%.

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 the isolates of *T. vitrinus* was 14.9%.

Discussion

Three species of *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 were *T. colubriformis*, *T. vitrinus*, and *T. axei* [14]. Iran is one of the most important foci for *Trichostrongylus* infection among human and animal hosts [12, 16, 35, 36]. Proper conditions such as humidity and climate in 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, 16, 35].

In the present study the authors used the sequence analysis protocol for detecting mitochondrial *cox1* gene, whereas several other studies reported from Iran employed ITS-rDNA gene specific for the phylogenetic analysis of *Trichostrongylus* species [2, 12, 16]. The nuclear ribosomal gene is widely applied to studies of deep and shallow phylogenetic relationship in the phylum Nematoda [2, 16, 37, 38]. Recent studies elustrated that the mitochondrial genes to be the proper options for phylogenomics approach and specifically for the *CoxI* gene that has mainly been used in population genetic surveys for various nematode parasites of the vertebrates [38, 39, 40].

Several studies have illustrated that the sequence differences between the members of the *Trichostrongylus* genus is not noticeable based on the ITS2 gene [2, 12, 16] . For example, Ashrafi et al. (2020) 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 [21]. The phylogenetic tree constructed in our study represented that three species of *T. colubriformis*, *T. vitrines,* and *T. axei* were separated in distinct cluster along with the same species obtained from other studies in different countries (Fig 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.

Little information on mitochondrial genes of Trichostrongyloidea superfamily is available. Palevich et al. (2020) in New Zealand investigated the complete mitochondrial genomes of *H. contortus* and *T. circumcincta* by phylogenetic analysis [32]. Another study in Uzbekistan was performed based on ribosomal (ITS2) and mitochondrial (*Cox1*) of *Marshallagia* sp. the result of the study indicate that ITS2 sequences had little variation, therefor the gene did not allow diagnosing species, while *Cox1*is more verified [31]. *Ostertagia trifurcata* and *Marshallagia marshalli* in China were evaluated by phylogenetic analysis of the complete mitochondrial genes. The studies introduced complete mt genome sequence of the nematods as a novel genetic markers for population genetic and molecular epidemiology [28, 33]. Two studies reported from Brazil and Australia evaluated the complete mitochondrial genes of *H. placei, T. circumcincta, T. vitrines*, 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 [25, 30]. *Cox1* and *nad4* genes of *T. axei* were analyzed for population genetic structure of the nematode in USA [29]. However, additional sequence studies, especially the analysis of both nuclear and mitochondrial genes, are needed to provide a comprehensive understanding of genetic variation of *Trichostrongylus* spp. in endemic areas and other parts of Iran.

In conclusion, our study presented three species of *T. colubriformis*, *T. vitrines* and *T. axei* among sheep, cattle and goats in Guilan province, northern Iran. This study investigated the sequence of mitochondrial *Cox1* gene region and concluded that the genetic diversity of the *Cox1* gene is notable and the gene is suitable for analysis of gene diversity of intra-species distance among helminthes. The scarcity of molecular *Cox1* data within *Trichostrongylus* spp. in various geographical regions and hosts will be needed 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.

Declarations

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Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Competing interests

The authors declared no conflict of interests.

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Authors' contributions

Project conceptualization and management: MS: Data collection. Formal analysis. Writing, review and editing. EH: Study design. Data curation. Writing: original draft

Availability of data and material

The datasets used and/or analysed during the current study are available on reasonable request. Representative nucleotide sequences generated in this study were submitted to the GenBank database under the accession numbers MW051250- MW051256.

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Figures

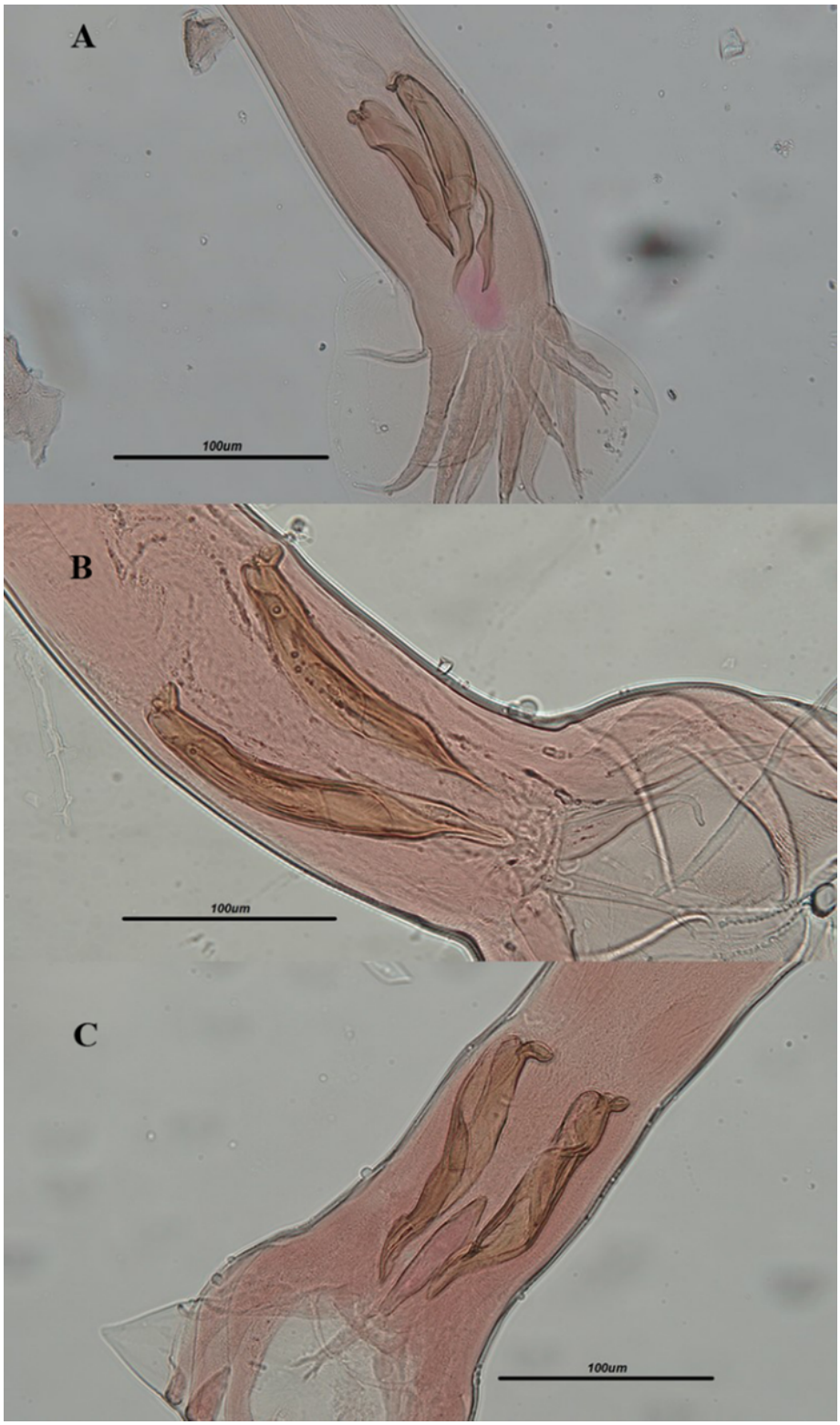

Figure 1

Copulatory bursa and spicules of T. axei (A), T. vitrines (B) and T. colubriformis (C)

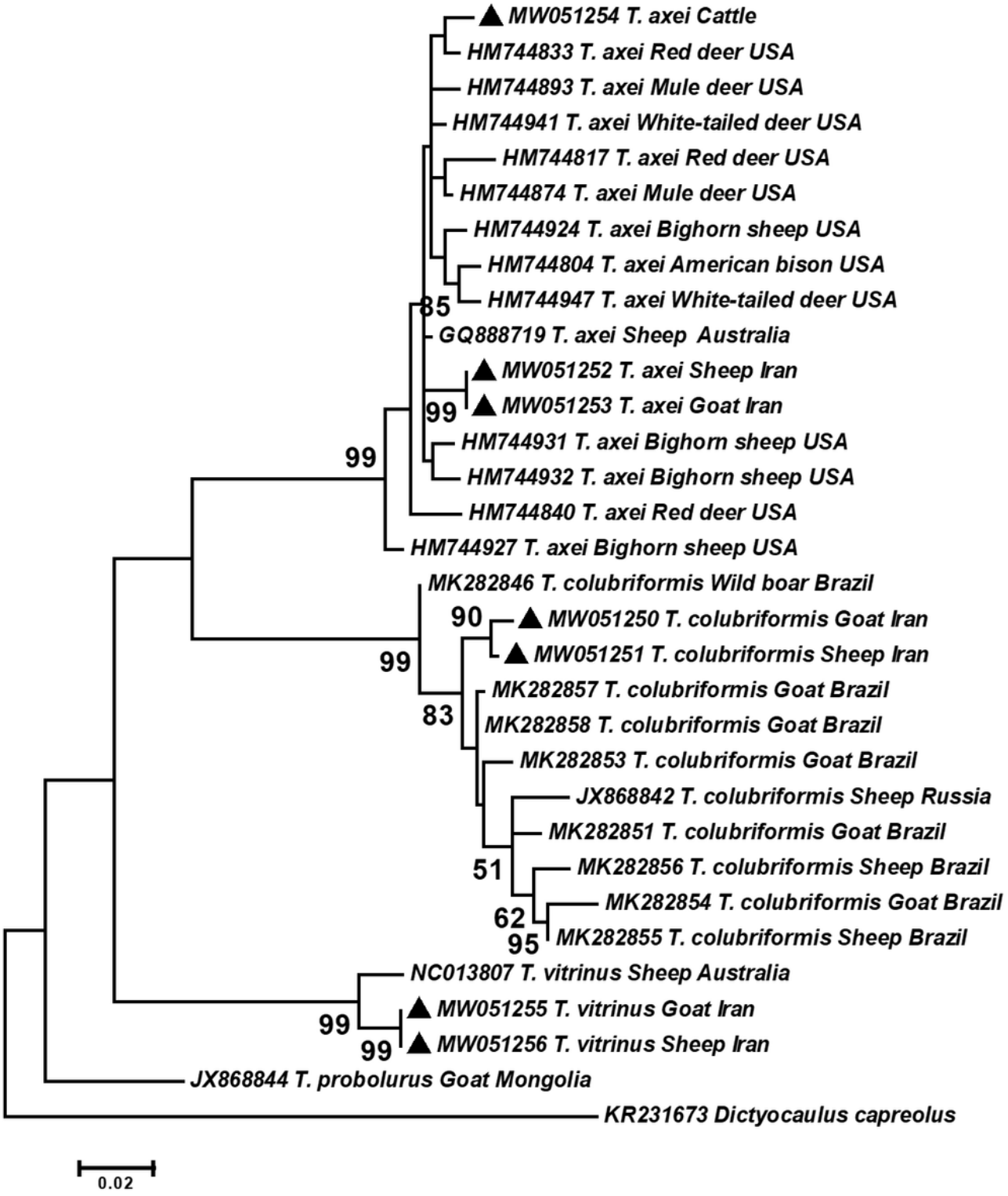

Figure 2

Phylogenetic tree of isolates of Trichostrongylus spp. obtained in this study (▲) and other isolates of Trichostrongylus 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.

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