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

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Abstract

The objective of the present study was to identify *Trichostrongylus* species by molecular analysis and also phylogenetic relationships of *Trichostrongylus* species by mitochondrial *Cox1* gene in Guilan province, northern Iran. 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. 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%. 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.

Introduction

Trichostrongylus nematodes are highly prevalent and considered as gastrointestinal parasitic pathogens among ruminants with worldwide distribution (Roberts and Janovy, 2009, Sharifdini et al., 2017b). Clinical symptoms of humans are mild although in some patients gastrointestinal signs and eosinophilia may occur (Ghanbarzadeh et al., 2018, Wall et al., 2011). These nematodes are major health challenges, causing reduced animal products or even death among the infected animals in severe cases (da Rocha et al., 2020, McLeod, 1995). Several species of the parasite have been reported from herbivores with approximately 12 species identified in humans (Phosuk et al., 2013, Sharifdini et al., 2017b). Also, the frequency of *Trichostrongylus* spp. in human and animal hosts has been repeatedly reported in Iran (Ghadirian, 1977, Ghadirian et al., 1974, Ghasemikhah et al., 2011, Shahbazi et al., 2012). 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 (Ashrafi et al., 2020, Ashrafi et al., 2015, Ghadirian and Arfaa, 1975, Sharifdini et al., 2020, Sharifdini et al., 2017b).

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* (Ghadirian, 1977, Ghadirian and Arfaa, 1975, Ghadirian et al., 1974). 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 the predominant species (Sharifdini et al., 2017b, Sharifdini et al., 2017a, Gholami et al., 2015, Ashrafi et al., 2020). Infection with various species of Trichostrongylus including *T. colubriformis* (Anvari-Tafti et al., 2013, Borji et al., 2010, Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011, Shahbazi

et al., 2012), *T. vitrinus* (Anvari-Tafti et al., 2013, Borji et al., 2010, Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011, Shahbazi et al., 2012), *T. axei* (Ghadirian and Arfaa, 1975), *T. capricola* (Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011), *T. probolurus* (Anvari-Tafti et al., 2013, Borji et al., 2010, Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011, Shahbazi et al., 2012), *T. longispicularis* (Ghasemikhah et al., 2011), *T. orientalis* (Ghadirian and Arfaa, 1975), *T. lerouxi* (Biocca et al., 1974), *T. skrjabini* (Ghadirian and Arfaa, 1975), and *T. hamatus* (Anvari-Tafti et al., 2013) were reported in different herbivores such as sheep (Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011, Shahbazi et al., 2012), goats (Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011), cattle (Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011), buffalos (Ghadirian and Arfaa, 1975, Ghasemikhah et al., 2011), and camels (Anvari-Tafti et al., 2013, Borji et al., 2010) 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 (Ghadirian and Arfaa, 1975).

There is a tremendous diversity of the nematodes in the country (Ghasemikhah et al., 2011, Ghasemikhah et al., 2012) 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 Trichostrongylina (de Bellocq et al., 2001, Hoberg et al., 1999, Sharifdini et al., 2017a, von Samson-Himmelstjerna et al., 2002, Pandi et al., 2021). Although, numerous number of studies have focused on ITS2 for analysis of the Trichostrongylidae family in genetic variation, species detection, and phylogenetic relationships (Ghasemikhah et al., 2012, Sharifdini et al., 2017a, Sharifdini et al., 2017b), 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 (dos Santos et al., 2017, Hu et al., 2004, Saccone et al., 1999). 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 (Ahmad et al., 2019, Archie and Ezenwa, 2011, dos Santos et al., 2017, Jex et al., 2010, Kuchboev et al., 2020, Palevich et al., 2020, Sun et al., 2018). 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 (Kuchboev et al., 2020, Palevich et al., 2020), 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 cytochrome c oxidase subunit I (*Cox1*) from mitochondrial gene of *Trichostrongylus* species in northern Iran.

Material And 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 (Hosseinnezhad et al., 2021) (Figure. 1).

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 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 (Barghandan et al., 2019).

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 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 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 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 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 while *Dictyocaulus capreolus* considered as out group (Figure. 2). Pairwise distance comparisons clarified the presence of the seven sequences of the three species of *Trichostrongylus*, isolated in the present study as well as other species using BioEdit software.

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.

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%.

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 (Ghadirian and Arfaa, 1975). Iran is one of the most important foci for *Trichostrongylus* infection among human and animal hosts (Alemi and Arfaa, 1978, Ashrafi et al., 2020, Massoud et al., 1980, Sharifdini et al., 2017a). 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 (Alemi and Arfaa, 1978, Sharifdini et al., 2017a, Sharifdini et al., 2017b).

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 (Ashrafi et al., 2020, Sharifdini et al., 2017a, Sharifdini et al., 2017b). The nuclear ribosomal gene is widely applied to the studies of deep and shallow phylogenetic relationship in the phylum Nematoda (Holterman et al., 2006, Kiewnick et al., 2014, Sharifdini et al., 2017a, Sharifdini et al., 2017b). Recent studies illustrated that the mitochondrial genes to

be the proper options for phylogenomic approach and specifically for the *CoxI* gene that has mainly been used in population genetic surveys for various nematode parasites of the vertebrates (Kiewnick et al., 2014, Nadler and Hudspeth, 2000, Otranto et al., 2005).

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 (Ashrafi et al., 2020, Sharifdini et al., 2017a, Sharifdini et al., 2017b). 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 (Ghasemikhah et al., 2012). 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.

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 (Palevich et al., 2020). 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 (Kuchboev et al., 2020). *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 (Ahmad et al., 2019, Sun et al., 2018). 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 (dos Santos et al., 2017, Jex et al., 2010). Moreover, the *Cox1* and *nad4* genes of *T. axei* were also analyzed for population genetic structure of the nematode in USA (Archie and Ezenwa, 2011). However, additional sequence studies, 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.

Declarations

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References

1. AHMAD, A. A., YANG, X., ZHANG, T., WANG, C., ZHOU, C., YAN, X., HASSAN, M., IKRAM, M. & HU, M. 2019. Characterization of the complete mitochondrial genome of Ostertagia trifurcata of small ruminants and its phylogenetic associations for the Trichostrongyloidea superfamily. *Genes,* 10**,** 107.
2. ALEMI, A. & ARFAA, F. 1978. Prevalence of intestinal helminthiasis in the rural area of gilan province (caspian littoral). *Iranian Journal of Public Health***,** 25-34.
3. ANVARI-TAFTI, M., SAZMAND, A., HEKMATIMOGHADDAM, S. & MOOBEDI, I. 2013. Gastrointestinal helminths of camels (Camelus dromedarius) in center of Iran. *Trop Biomed,* 30**,** 56-61.
4. ARCHIE, E. A. & EZENWA, V. O. 2011. Population genetic structure and history of a generalist parasite infecting multiple sympatric host species. *International journal for parasitology,* 41**,** 89-98.
5. ASHRAFI, K., SHARIFDINI, M., HEIDARI, Z., RAHMATI, B. & KIA, E. B. 2020. Zoonotic transmission of Teladorsagia circumcincta and Trichostrongylus species in Guilan province, northern Iran: molecular and morphological characterizations. *BMC Infectious Diseases,* 20**,** 1-9.
6. ASHRAFI, K., TAHBAZ, A., SHARIFDINI, M. & MAS-COMA, S. 2015. Familial Trichostrongylus infection misdiagnosed as acute fascioliasis. *Emerging infectious diseases,* 21**,** 1869.
7. BARGHANDAN, T., HAJIALILO, E., SHARIFDINI, M. & JAVADI, A. 2019. Prevalence and phylogenetic analysis of gastrointestinal helminths (Nematoda: Trichostrongylidae) in ruminant livestock of northwest Iran. *Ankara Üniversitesi Veteriner Fakültesi Dergisi,* 67**,** 65-72.
8. BIOCCA, E., CHABAUD, A. & GHADIRIAN, E. 1974. Trichostrongylus lerouxi n. sp., parasite of Bos taurus. *Parassitologia,* 16**,** 199.
9. BORJI, H., RAZMI, G. R., MOVASAGHI, A., NAGHIBI, A. A. & MALEKI, M. 2010. SHORT PAPER: A STUDY ON GASTROINTESTINAL HELMINTHS OF CAMELS IN MASHHAD ABATTOIR, IRAN.

10. DA ROCHA, L. O., DA SILVA LEMOS, G. C., VIEIRA, I. J. C., BRAZ-FILHO, R., DE PAIVA FREITAS, S., GLÓRIA, L. S. & DE PAULA SANTOS, C. 2020. Chemical characterization and in vitro biological activity of Cymbopogon citratus extracts against Haemonchus spp. and Trichostrongylus spp. nematodes from sheep. *Parasitology,* 147**,** 1559-1568.
11. DE BELLOCQ, J. G., FERTE, H., DEPAQUIT, J., JUSTINE, J.-L., TILLIER, A. & DURETTE-DESSET, M.-C. 2001. Phylogeny of the Trichostrongylina (Nematoda) inferred from 28S rDNA sequences. *Molecular Phylogenetics and Evolution,* 19**,** 430-442.
12. DOS SANTOS, L. L., PROSDOCIMI, F., LIMA, N. C. B., DA COSTA, I. R., CARDOSO, D. C., DRUMMOND, M. G., BRASIL, B. D. S. A. F., BASTIANETTO, E. & DE OLIVEIRA, D. A. A. 2017. Comparative genomics and phylogenomics of Trichostrongyloidea mitochondria reveal insights for molecular diagnosis and evolutionary biology of nematode worms. *Gene Reports,* 9**,** 65-73.
13. GHADIRIAN, E. 1977. Human infection with Trichostrongylus lerouxi (Biocca, Chabaud, and Ghadirian, 1974) in Iran. *The American journal of tropical medicine and hygiene,* 26**,** 1212-1213.
14. GHADIRIAN, E. & ARFAA, F. 1975. Present status of trichostrongyliasis in Iran. *The American journal of tropical medicine and hygiene,* 24**,** 935-941.
15. GHADIRIAN, E., ARFAA, F. & SADIGHIAN, A. 1974. Human infection with Trichostrongylus capricola in Iran. *The American journal of tropical medicine and hygiene,* 23**,** 1002-1003.
16. GHANBARZADEH, L., SARAEI, M., KIA, E., AMINI, F. & SHARIFDINI, M. 2018. Clinical and haematological characteristics of human trichostrongyliasis. *J Helminthol,* 93**,** 149-53.
17. GHASEMIKHAH, R., MIRHENDI, H., KIA, E., MOWLAVI, G., SARMADIAN, H., MESHGI, B., GOLESTAN, B. & MOBEDI, I. 2011. Morphological and morphometrical description of Trichostrongylus species isolated from domestic ruminants in Khuzestan province, southwest Iran. *Iranian journal of parasitology,* 6**,** 82.
18. GHASEMIKHAH, R., SHARBATKHORI, M., MOBEDI, I., KIA, E., HARANDI, M. F. & MIRHENDI, H. 2012. Sequence analysis of the second internal transcribed spacer (ITS2) region of rDNA for species identification of Trichostrongylus nematodes isolated from domestic livestock in Iran. *Iranian journal of parasitology,* 7**,** 40.
19. GHOLAMI, S., BABAMAHMOODI, F., ABEDIAN, R., SHARIF, M., SHAHBAZI, A., PAGHEH, A. & FAKHAR, M. 2015. Trichostrongylus colubriformis: possible most common cause of human infection in Mazandaran province, North of Iran. *Iranian journal of parasitology,* 10**,** 110.
20. HOBERG, E. P., MONSEN, K. J., KUTZ, S. & BLOUIN, M. S. 1999. Structure, biodiversity, and historical biogeography of nematode faunas in holarctic ruminants: morphological and molecular diagnoses fo
21. r Teladorsagia boreoarcticus n. sp.(Nematoda: Ostertagiinae), a dimorphic cryptic species in muskoxen (Ovibos moschatus). *The Journal of parasitology***,** 910-934.
22. HOLTERMAN, M., VAN DER WURFF, A., VAN DEN ELSEN, S., VAN MEGEN, H., BONGERS, T., HOLOVACHOV, O., BAKKER, J. & HELDER, J. 2006. Phylum-wide analysis of SSU rDNA reveals deep

phylogenetic relationships among nematodes and accelerated evolution toward crown clades. *Molecular biology and evolution,* 23**,** 1792-1800.

23. HOSSEINNEZHAD, H., SHARIFDINI, M., ASHRAFI, K., ATRKAR ROUSHAN, Z., MIRJALALI, H. & RAHMATI, B. 2021. Trichostrongyloid nematodes in ruminants of northern Iran: prevalence and molecular analysis. *BMC Veterinary Research,* 17**,** 1-12.
24. HU, M., CHILTON, N. B. & GASSER, R. B. 2004. The mitochondrial genomics of parasitic nematodes of socio-economic importance: recent progress, and implications for population genetics and systematics. *Advances in parasitology,* 56**,** 134-213.
25. JEX, A. R., HALL, R. S., LITTLEWOOD, D. T. J. & GASSER, R. B. 2010. An integrated pipeline for next-generation sequencing and annotation of mitochondrial genomes. *Nucleic Acids Research,* 38**,** 522-533.
26. KIEWNICK, S., HOLTERMAN, M., VAN DEN ELSEN, S., VAN MEGEN, H., FREY, J. E. & HELDER, J. 2014. Comparison of two short DNA barcoding loci (COI and COII) and two longer ribosomal DNA genes (SSU & LSU rRNA) for specimen identification among quarantine root-knot nematodes (Meloidogyne spp.) and their close relatives. *European Journal of Plant Pathology,* 140**,** 97-110.
27. KUCHBOEV, A., SOBIROVA, K., KARIMOVA, R., AMIROV, O., VON SAMSON-HIMMELSTJERNA, G. & KRÜCKEN, J. 2020. Molecular analysis of polymorphic species of the genus Marshallagia (Nematoda: Ostertagiinae). *Parasites & vectors,* 13**,** 1-12.
28. MASSOUD, J., ARFAA, F., JALALI, H. & KEYVAN, S. 1980. Prevalence of intestinal helminths in Khuzestan, Southwest Iran, 1977. *The American Journal of Tropical Medicine and Hygiene,* 29**,** 389-392.
29. MCLEOD, R. 1995. Costs of major parasites to the Australian livestock industries. *International journal for parasitology,* 25**,** 1363-1367.
30. NADLER, S. A. & HUDSPETH, D. S. 2000. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. *Journal of Parasitology,* 86**,** 380-393.
31. OTRANTO, D., TESTINI, G., DE LUCA, F., HU, M., SHAMSI, S. & GASSER, R. 2005. Analysis of genetic variability within Thelazia callipaeda (Nematoda: Thelazioidea) from Europe and Asia by sequencing and mutation scanning of the mitochondrial cytochrome c oxidase subunit 1 gene. *Molecular and cellular probes,* 19**,** 306-313.
32. PALEVICH, N., MACLEAN, P. H., CHOI, Y.-J. & MITREVA, M. 2020. Characterization of the complete mitochondrial genomes of two sibling species of parasitic roundworms, Haemonchus contortus and Teladorsagia circumcincta.
33. PANDI, M., SHARIFDINI, M., ASHRAFI, K., ATRKAR ROUSHAN, Z., RAHMATI, B. & HAJIPOUR, N. 2021. Comparison of Molecular and Parasitological Methods for Diagnosis of Human Trichostrongylosis. *Frontiers in Cellular and Infection Microbiology***,** 959.
34. PHOSUK, I., INTAPAN, P. M., SANPOOL, O., JANWAN, P., THANCHOMNANG, T., SAWANYAWISUTH, K., MORAKOTE, N. & MALEEWONG, W. 2013. Molecular evidence of Trichostrongylus colubriformis and

Trichostrongylus axei infections in humans from Thailand and Lao PDR. *The American journal of tropical medicine and hygiene,* 89**,** 376-379.

35. ROBERTS, L. & JANOVY, J. 2009. Foundations of parasitology. 8th edn. 701 pp. New York, McGraw-Hill.
36. SACCONE, C., DE GIORGI, C., GISSI, C., PESOLE, G. & REYES, A. 1999. Evolutionary genomics in Metazoa: the mitochondrial DNA as a model system. *Gene,* 238**,** 195-209.
37. SHAHBAZI, A., FALLAH, E., KOSHKI, M. K., NEMATOLLAHI, A., GHAZANCHAEI, A. & ASFARAM, S. 2012. Morphological characterization of the Trichostrongylus species isolated from sheep in Tabriz, Iran. *Research Opinions in Animal and Veterinary Sciences,* 2**,** 309-312.
38. SHARIFDINI, M., DERAKHSHANI, S., ALIZADEH, S. A., GHANBARZADEH, L., MIRJALALI, H., MOBEDI, I. & SARAEI, M. 2017a. Molecular identification and phylogenetic analysis of human Trichostrongylus species from an endemic area of Iran. *Acta tropica,* 176**,** 293-299.
39. SHARIFDINI, M., GHANBARZADEH, L., BARIKANI, A. & SARAEI, M. 2020. Prevalence of intestinal parasites among rural inhabitants of Fouman, Guilan Province, Northern Iran with emphasis on Strongyloides stercoralis. *Iranian Journal of Parasitology,* 15**,** 91.
40. SHARIFDINI, M., HEIDARI, Z., HESARI, Z., VATANDOOST, S. & KIA, E. B. 2017b. Molecular phylogenetics of Trichostrongylus species (Nematoda: Trichostrongylidae) from humans of Mazandaran province, Iran. *The Korean Journal of Parasitology,* 55**,** 279.
41. SUN, M.-M., HAN, L., ZHANG, F.-K., ZHOU, D.-H., WANG, S.-Q., MA, J., ZHU, X.-Q. & LIU, G.-H. 2018. Characterization of the complete mitochondrial genome of Marshallagia marshalli and phylogenetic implications for the superfamily Trichostrongyloidea. *Parasitology research,* 117**,** 307-313.
42. VON SAMSON-HIMMELSTJERNA, G., HARDER, A. & SCHNIEDER, T. 2002. Quantitative analysis of ITS2 sequences in trichostrongyle parasites. *International Journal for Parasitology,* 32**,** 1529-1535.
43. WALL, E. C., BHATNAGAR, N., WATSON, J. & DOHERTY, T. 2011. An unusual case of hypereosinophilia and abdominal pain: an outbreak of Trichostrongylus imported from New Zealand. *Journal of travel medicine,* 18**,** 59-60.

Figures

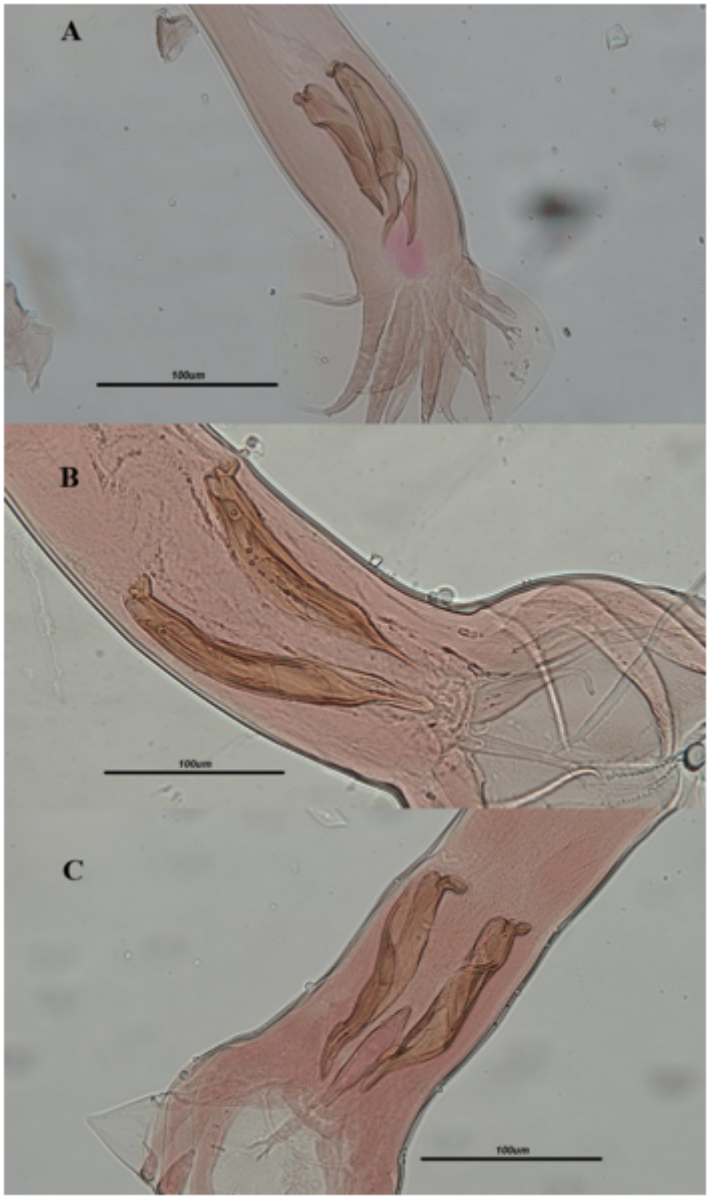

Figure 1

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

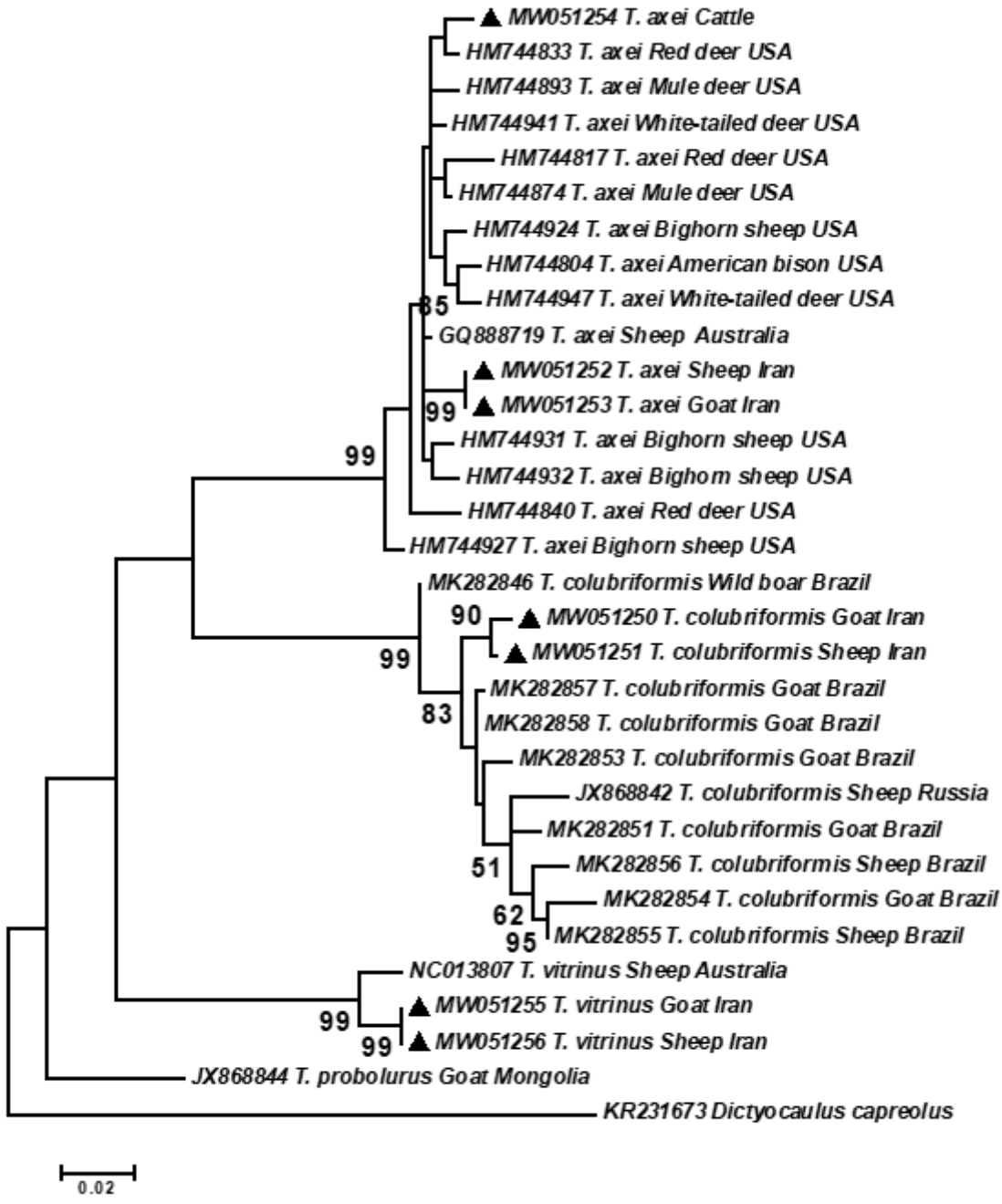

Figure 2

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.