Molecular identification and phylogenesis analysis of gastrointestinal nematode in different populations of Kazakh sheep

Xiaofei yan
Xinjiang Agricultural University
Sangang He
Xinjiang Academy of Animal Science
Yiyong Liu
Animal Husbandry Terminus of Ili Kazakh Autonomous Prefecture
Bing Han
Xinjiang Academy of Animal Science
Ning Zhang
Xinjiang Academy of Animal Science
Haifeng Deng
Zhaosu Horse Farm, Ili Kazakh Autonomous Prefecture
Yuqi Wang
Xinjiang Agricultural University
Mingjun Liu (xjlmj2006@126.com)
Xinjiang Academy of Animal Science

Research Article

Keywords: Kazakh sheep, Gastrointestinal nematodes, Molecular epidemiology, Phylogenesis, The second internal transcription spacer of ribosomal DNA

Posted Date: September 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1978124/v1

Abstract

Three dominant species of gastrointestinal nematodes, *Haemonchus contortus*, *Trichostrongylus* spp., and *Teladorsagia circumcincta* from the various populations of Kazakh sheep were subjected to molecular identification and phylogenetic analysis. The internal transcribed spacer 2 (ITS-2) sequences of ribosomal DNA (rDNA) was used as the target sequence and specific primers. The results showed that all the three species had their ITS-2 specific bands amplified. The infection rates based on fecal DNA were 35.59% of *H. contortus*, 25.55% of *Trichostrongylus* spp., and 11.24% of *T. circumcincta* respectively, and the infection rates based on larva DNA were 19.75% of *H. contortus*, 23.54% of *Trichostrongylus* spp., and 10.03% of *T. circumcincta* respectively. The results were consistent with the PCR results of fecal samples and larval DNA from *Trichostrongylus* spp. and *T. circumcincta*, except *H. contortus*. The phylogenetic tree showed that the ITS-2 sequences of the three species were on the same branch as the ITS-2 sequences of the same species in NCBI, and on different branches from those of the ITS-2 sequences of different families, genera and species. The same 93 fecal samples were analyzed by molecular identification and saturated saline solution floatation method respectively. The molecular identification of hatched larva from fecal samples exhibited an infection rate of 100.00% with respect to the three dominant species, higher than the infection rate of 96.77% by the saturated saline solution floatation method. Therefore, the present molecular identification is more reliable in specificity and accuracy of these three species.

Introduction

Chronic wasting disease (CWD) caused by gastrointestinal nematode (GIN) infection is one of the important factors affecting sheep health and production performance. Weight loss, death, and direct costs of deworming drugs caused by GIN infection have caused tremendous losses to farming enterprises and herdsmen. It was reported that the annual cost of *H. contortus* treatment was $26 million in Kenya, $46 million in South Africa, and $103 million in India (Peter et al. 2005). Various epidemiological surveys have shown that the infection rates and infection intensities of *H. contortus*, *Trichostrongylus spp*., and *T. circumcinctar* in GIN were generally higher than the other species worldwide (Baihaqi et al. 2019; Domke et al. 2013; Zajac et al. 2020). Our previous study also demonstrated that these three species were the dominant species of GIN in the study area (Zhaosu County, Yili) (Yan et al. 2021). GIN usually presents as a mixed infection, and the infectious species distribution and numbers vary with the season, weather conditions (especially precipitation and temperature), ruminant species and individual differences (Waller 2006). There may be significant differences in fecundity and pathogenicity among different species, and they are also factors contributing to differences in infectivity. Accurate diagnosis of GIN infection is the core of epidemiological survey and disease control. The conventionally adopted method is the saturated saline flotation microscopy (Bowman 2009), which is labor-intensive and time-consuming, and cannot accurately identify or differentiate single eggs or larva of different species of GIN. In particular, culturing larva is laborious and takes one week (Dobson et al. 1992). In addition, the morphological identification of third stage (L3) larva as a genus or species requires experience but may still be unreliable. Therefore, there is an urgent need to establish molecular identification techniques.

The internal transcribed spacer (ITS) of ribosomal DNA (rDNA) usually contains many insertion-deletion mutations (INDELs), and the interspecific differences of INDELs can be used as markers for taxonomic identification (Blouin 2002). As of now, genetic markers in the first and second ITS regions (ITS-1 and ITS-2, respectively) and external transcribed spacer regions (ETS) of rDNA have been used to successfully develop PCR assays for identification and analysis of GIN eggs and larva (Gasser et al. 1993, 1997; Newton et al. 1998; Heise et al. 1999; Samson-Himmelstjerna et al. 2002). For example, Zarlenga et al. (1998, 2001) used rDNA ITS and ETS sequences to develop a sensitive PCR method for specific identification of GIN eggs in cattle for the first time. Subsequently, Schnieder et al. (1999) reported genus-specific PCR for the identification of eggs or larva of *Ostertagia*, *Cooperia*, *Nematodirus*, *Haemonchusand*, and *Trichostrongylus* in ruminants. Zhao (2013) used the ITS-1 and ITS-2 sequences of rDNA to develop polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and specific PCR technology, and established molecular identification method for three types of nematodes. Avramenko et al. (2015) applied deep sequencing of ITS-2 to investigate nematode community structure and further estimated the relative abundance of GINs in different species of ruminants. In addition, understanding the genetic variation within and between GIN species is helpful to learn the transmission of GIN and develop control strategies. Kampfer et al. (1998) proposed a molecular phylogeny of Nematoda based on ITS-2. However, the available high-rank-level molecular phylogenetic study was restricted to *Strongylida* based on the ITS-2 sequences (Chilton et al. 1997). Studies in Italy (Cerutti et al. 2010) and Brazil (Brasil et al. 2012) reported that *H. contortus* in domestic and wild animals showed high genetic variation and relatively low host specificity. *H. contortus* population genetics surveys have been conducted worldwide, involving Australia, Brazil, Europe, Malaysia, and the United States (Blouin et al. 1995; Troell et al.2006; Hunt et al. 2008). In this study, based on the previous epidemiological survey results in Zhaosu County, Xinjiang (Yan et al. 2021), combined with the feeding practices on pasture, we took fecal samples and hatched single larva as test materials and used the ITS-2 sequences of rDNA as genetic markers. We then conducted molecular identification and phylogenetic analysis of *H. contortus*, *Trichostrongylus spp*., and *T. circumcinctar* in Kazakh sheep and the F1- and F2-generations of Kazakh × Texel sheep crosses. Our study laid a foundation for the establishment of GIN-specific molecular diagnostic methods.

Materials And Methods

Sample collection

All animals utilized in this research were prospectively approved and granted a formal waiver of ethics approval by the Animal Welfare Committee of Shihezi University (Xinjiang, China). In the four seasons, April (spring), July (summer), September (autumn), and December (winter) 2020, in Zhaosu County and the Nilka County, Yili, the rectal fecal samples were collected from the female adult Kazakh sheep (4–5 years old), the female adult F1-generation (3–4 years old) of Texel × Kazakh crosses, and the female adult F2-generation (2 years old) of Texel × F1 crosses. The net sample count was 916 with the details shown in Table 1. Each fecal sample weighed 20 g ~ 30 g, was put in a clean zip bag, with variety, gender, age, chip number (unique for each sheep), and clinical symptoms recorded. The samples were transported to the laboratory and stored in the refrigerator at 4 °C. Deworming was carried out in 1–2 days after sampling in Spring and Autumn, respectively. In Spring, intramuscular injection of ivermectin (0.04 ml/kg) and closantel sodium (0.1–0.2 ml/kg) was administered in April, and in Autumn, intramuscular injection of ivermectin (0.04 ml/kg) and oral albendazole (0.1–0.15 ml/kg) were administered in September. Both the sampling intervals and deworming intervals were at least 3 months.

Table 1
Number of fecal samples (number of samples)

Population	Spring	Summer	Autumn	Winter	Overall
Kazakh	137	100	126	102	465
F1	122	50	80	79	331
F2	44	5	41	30	120
Overall	303	155	247	211	916

Reagents and instruments

The fecal DNA kit (spin-column type) and 2 × Taq PCR Master Mix were purchased from Tiangen Biotech (Beijing) Co., Ltd. The 10 × Taq PCR buffer with KCl was purchased from Thermo Fisher Scientific, and etc. Optical microscope (Motic, model: SK200); Gradient thermal cycler (Eppendorf, Germany, model: Mastercycler pro); microcentrifuge (Eppendorf, German, model: Eppendorf 5424); Nucleic acid and protein quantification instrument (Eppendorf, Germany, model: BioSpectrometer basic); multifunctional gel imaging system (ProteinSimple, the United States, model: AlphaImager HP); UV meter (Beijing Liuyi Biotechnology Co., LTD., model: WD-9403 F), and etc.

Primer synthesis

The specific primers for rDNA ITS-2 sequences of the three species of nematodes reported by Bott NJ et al. (2009) were synthesized by Sangon Biotech (Shanghai) Co. Ltd. (Table 2).

Table 2
rDNA ITS-2 specific primers of three parasites species

Species	Primer sequence	Amplification length (bp)
H. contortus	Forward: CAAATGGCATTTGTCTTTTAG	265
	Reverse: TTAGTTTCTTTTCCTCCGCT	
T. circumcinctar	Forward: TATGCAACATGACGTACGACGG	218
	Reverse: TTAGTTTCTTTTCCTCCGCT	
Trichostrongylus spp.	Forward: TATGCAACATGACGTACGACGG	267–268

Experiment method

Conventional fecal analysis

This entailed the use of the egg/oocyst floating method (Yan et al. 2021). Saturated NaCl was used as the floating fluid (specific gravity 1.2 kg/m3) to check the infecting species in the feces samples. The cover slip was then subjected to microscopic examination. The digital microscope and microscopic image acquisition and analysis system were used to observe the morphology, structure, color, and size of the samples. Subsequently, the images of eggs were captured and saved. The species identification was conducted in accordance with reference literature (Bott et al. 2009; Kong 2016).

Fecal DNA extraction

Fecal DNA extraction was performed following the fecal DNA extraction kit instruction. An amount of 2 μl of the extracted DNA was used to measure the OD and determine the concentration with a BioSpectrometer.

Incubation and isolation of L3 larva

Partial fecal samples were randomly selected from each population in each season. The single and the mixed fecal samples were placed in an electrothermal constant temperature incubator and cultured at 25°C for 7 days. After hatching, L3 larvae were isolated using Baermann's Technique (Zajac et al. 2012), and they were picked under the microscope.

Genomic DNA extraction of single L3 larva

The single larva was rinsed and added with 8 μl ddH_2O, 1 μl 10 × Taq Buffer with KCl, and 15 mM $MgCl_2$, followed by flash frozen in liquid nitrogen for 1 min, and then heated at 85°C for 2 min. Then the sample was added with 1 μl proteinase K (20 mg/μL), incubated at 56°C for 15 min, 95°C for 10 min, and stored at -20°C.

PCR identification

Using the DNA of fecal samples and the DNA of single L3 larvae as templates, ITS-2 sequences of the three species *H. contortus*, *Trichostrongylus spp.*, and *T. circumcinctar*, were amplified. The PCR reaction system had a total volume of 20.0 μL, including Mix 10.0 μL, fecal/larva DNA 2.0 μL, ddH_2O 6.0 μL, forward primer 1.0 μL, and reverse primer 1.0 μL. The PCR reaction program was as follows, 94°C for 5 min, a total of 35 cycles of (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s), and then 72°C for 7 min. Samples without genomic DNA were included as negative controls for each PCR. The PCR products were subjected to electrophoresis on 2% agarose gel at 100 V for 30 min, photographed and documented by a gel imaging system.

Data analysis

The samples with positive PCR results were sent to Sangon Biotech (Shanghai) Co. Ltd. for sequencing. The obtained sequences were subjected to BLAST homology analysis with the sequences of these three species published in Genbank to verify their species. DNAMAN8 was used to compare and analyze the sequencing results, which were further subjected to homology analysis with the three species sequences (MN845169.1, KP663663.1, and JQ989274.1) published in Genbank

Construction of the phylogenetic tree

The phylogenetic tree was constructed using MEGA 6.0. A total of 25 sequences of each of the three species and the ITS-2 sequences of the three species in Genbank were included. The sequences of *Nematodirus oiratianus* (AJ239112.1), *Marshallagia marshallli* (AJ400715.1), *Cooperia oncophora* (AJ544389.1), and *Oesophagostomum dentatum* (AJ889571.1), and the ITS-1 sequences of the three species were taken as outgroups. The phylogenetic tree was constructed with MEGA 6.0 using neighbor joining method (NJ). Then Bootstrap with 500 replications was conducted for confidence interval test, using 50% as the critical value of confidence, and those less than 50% would not be displayed. The other settings were as default. SPASS 19.0 was used to analyze the difference in calculated infection rates between conventional fecal assay and molecular identification method.

Results And Analysis

PCR amplification of ITS-2 sequences of the dominant species of GIN in sheep

The ITS-2 sequences of *H. contortus*, *Trichostrongylus spp.*, and *T. circumcincta* were amplified respectively using the fecal genomic DNA of Kazakh sheep and the F1- and F2-generations of sheep crosses and the DNA from the lysate of hatched larva as templates. The PCR products were subjected to agarose gel electrophoresis, showing specific single bands at 200–300 bp, with a size of 265 bp for *H. contortus*, 267 ~ 268 bp for *Trichostrongylus spp.*, and 218 bp for *T. circumcincta*, respectively (Fig. 1). To test the specificity of the three pairs of primers, the DNA template of a single larva was used to check whether there was crossover among the three pairs of primers, and the results revealed no non-specific amplification for any pair of primers (Fig. 2).

ITS-2 sequences analysis of the dominant species of GIN in sheep

The obtained sequences of the three species were analyzed and compared online by BLAST. It was found that that these sequences were highly (all > 95%) homologous to the ITS-2 sequences of *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* in GenBank. DNAMAN8 was used to conduct intraspecific comparison between the obtained six sequences of each species and the ITS-2 sequences of the three species in GenBank, and the results showed small intraspecific differences. In detail, they shared 99.31% homology with the ITS-2 sequence of H. contortus in GenBank (accession number MN845169.1), as shown in Fig. 3-N; shared 99.12% homology with the ITS-2 sequence of *Trichostrongylus colubriformis* in GenBank (accession number KP663663.1), as shown in Fig. 3-M; and shared 98.76% homology with the ITS-2 sequence of *T. circumcincta* in GenBank (accession number JQ989274.1), as shown in Fig. 3-O.

The *H. contortus* study sequence had four mutation sites, the G-C transversion at position 27, the G-A transition and G-T transversion at position 48, the T-A transversion at position 57, and the G-C transversion at position 103 (as shown in Table 3). The *Trichostrongylus* spp. study sequence had 19 mutation sites, including 8 transitions, 13 transversions, and 5 deletions. Among them, M-F-H (the *Trichostrongylus* spp. ITS-2 sequence of the fecal DNA from Kazakh sheep) had the most mutation sites (as shown in Table 4). The *T. circumcincta* study sequence had 20 mutation

sites, including 4 transitions, 26 transversions, and 2 deletions. Among them, O-Y-F2-41 (the *T. circumcincta* ITS-2 sequence of the larva DNA from the F2-generation sheep crosses) has 8 mutation sites (as shown in Table 5).

Table 3
H.contortus ITS-2 gene sequence nucleotide variation sites

□□	□□			
	27	**48**	**57**	**103**
MN845169.1	G	G	T	G
N-F-F1	C[b]	.	.	C[b]
N-F-F2	.	.	.	.
N-Y-F1	.	.	.	.
N-Y-F2	.	A[a]	.	.
N-Y-H	.	.	A[b]	.
N-Y-F2-16	.	.	.	.
N-Y-F2-17	.	.	.	.
N-F-H-50	.	.	.	.
N-F-H-46	.	.	.	.
N-Y-F2-20	.	T[b]	.	.
N-Y-F2-10	.	A[a]	.	.
N-Y-F2-11	.	.	.	.
N-Y-F2-15	.	.	.	.
N-Y-F2-8	.	.	A[b]	.
N-Y-F2-5	.	.	A[b]	.
N-Y-F2-13	.	.	A[b]	.
N-F-H-49	.	.	.	.
N-Y-H-25	.	.	.	.

Note: N: *H.contortus*; F: ITS-2 sequence obtained from fecal DNA; Y: ITS-2 sequence obtained from larval DNA; h: Kazakh sheep; F1: hybrid F1 sheep; F2: hybrid F2 sheep ; shoulder a: conversion, b: transversion, c: deletion, d: insertion.

Table 4
Trichostrongylus spp. ITS-2 gene sequence nucleotide variation sites

□□	□□																		
	1	**2**	**3**	**9**	**22**	**23**	**35**	**37**	**59**	**60**	**61**	**70**	**115**	**123**	**126**	**144**	**145**	**146**	**147**
KP663663.1	G	T	A	A	T	A	T	T	A	G	T	G	A	T	A	A	T	T	A
M-F-F1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-F-F2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-F-H	T[b]	G[b]	T[b]	.	C[a]	.	C[a]	C[a]	.	.	A[b]	.	.	.	.	.	.	.	.
M-Y-F1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-Y-F2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-Y-H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-F-F1-4	.	.	A[c]	.	.	.	.	.	.	.	.	.	.	G[b]	C[b]	.	.	.	.
M-Y-F2-15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C[b]
M-Y-F2-16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T[b]	C[a]	C[a]	.
M-Y-H-24	.	.	A[c]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-F-F2-12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-F-F1-6	.	.	.	.	.	.	.	.	.	A[a]	.	.	.	G[b]	.	.	.	.	C[b]
M-Y-H-21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C[a]	.
M-F-F1-7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-F-F2-14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-Y-F1-9	.	.	.	.	.	.	.	.	C[b]	.	.	A[a]	.	.	.	.	.	.	.
M-Y-H-22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-Y-H-19	.	.		A[c]	G[b]	A[c]	.	.	.	.	.	.	T[b]	.	.	.	A[c]	.	.

Note: M: *Trichostrongylus* spp.; other labels are the same as in Table 3

Table 5
T. circumcincta ITS-2 gene sequence nucleotide variation sites

□□	□□																			
	0	**2**	**15**	**18**	**19**	**20**	**22**	**23**	**24**	**31**	**36**	**78**	**96**	**103**	**110**	**115**	**116**	**118**	**119**	**134**
MN845174.1	T	A	T	A	G	A	A	G	A	T	T	A	A	G	A	G	C	G	A	A
O-F-F1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-F-F2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-F-H	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.
O-Y-F1	T[c]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-Y-F2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-Y-H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-F-F2-40	.	.	.	.	.	.	.	.	.	.	.	.	.	C[b]	.	C[b]	.	.	.	.
O-Y-H-47	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-Y-F1-33	.	.	.	.	C[b]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-Y-H-48	.	C[b]	.	.	.	.	.	A[b]	T[b]	.	.	.	.	.	.	C[b]	.	.	.	.
O-F-F1-28	.	C[b]	.	C[b]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-F-F2-38	.	C[b]	.	C[b]	.	.	C[b]	.	.	.	C[a]	C[b]	C[b]	.	.	.	.	.	.	.
O-F-F1-30	.	.	.	C[b]	C[b]	.	.	.	.	.	.	.	.	.	.	.	G[b]	.	.	.
O-Y-F2-41	.	.	.	.	C[b]	C[b]	C[b]	.	.	C[a]	.	.	.	.	T[b]	C[b]	.	A[a]	.	A[d]
O-Y-F1-32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-F-H-43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-Y-H-49	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-F-F2-35	.	.	T[c]	C[b]	C[b]	C[b]	.	.	.	.	.	.	.	.	.	C[b]	.	.	G[a]	.

Note: O: *T. circumcincta*; other labels are the same as in Table 3

Cluster analysis of ITS-2 sequences of the three dominant species of GIN

MEGA 6.0 was used to conduct multiple sequence alignment and construct a phylogenetic tree involving the fecal DNA and larva DNA ITS-2 sequences of different populations of the three nematode species, ITS-2 and ITS-1 sequences of the three nematode species from NCBI, and the ITS-2 sequences of other nematode species. In this study, the 25 obtained *H. contortus* ITS-2 sequences were closely related to the *H. contortus* ITS-2 sequences AY647245.1 and X78803.1 from NCBI, sharing the same branch. They were remotely related to the *H. contortus* ITS-1 sequences JX289536.1 and MG675030.1 from NCBI, presenting on different branches. They were also remotely related to the ITS-2 sequence of other genus of *Trichostrongylidae*, such as *Cooperia oncophora* (AJ544389.1), *Marshallagia marshalli* (AJ400715.1), and *Nematodirus oiratianus* (AJ239112.1), and *Oesophagostomum dentatum* (AJ889571.1) of *Strongylida*, locating in different branches (Fig. 4). The 25 obtained *Trichostrongylus spp.* ITS-2 sequences in this study were closely related to the ITS-2 sequences of *Trichostrongylus spp.* (MH047849.1) and *Trichostrongylus colubriformis* (KP663663.1) from NCBI, locating in the same branch, closer to *T. colubriformis*. They were remotely related to the ITS-1 sequences of *T. colubriformis* (Y15876.1) and *Trichostrongylus spp.* (MG707739.1), on different branches. And they were also remotely related to the ITS-2 sequences of other genus of *Trichostrongylidae*, such as *H. contortus* (AY647245.1), *N. oiratianus* (AJ239112.1), *M. marshalli* (AJ400715.1), *C. oncophora* (AJ544389.1), and *O. dentatum* (AJ889571.1), on different branches (Fig. 5). The 25 obtained *T. circumcincta* ITS-2 sequences obtained in this study were closely related to the ITS-2 sequences of *Teladorsagia circumcincta* (JQ989274.1, JF747153.1) and *Ostertagia trifurcata* (MN845174.1) from NCBI, locating in the same branch. And *Ostertagia* and *Teladorsagia* were closely related, belonging to the same genus. They were remotely related to the ITS-1 sequences of *Ostertagia ostertagi* (AF044933.1) and *Ostertagia circumcincta* (AF044934.1), locating in different branches. They were also remotely related to the ITS-2 sequences of other genus of *Trichostrongylidae*, such as *H.contortus* (AY647245.1), *O. dentatum* (AJ889571.1), and *N. oiratianus* (AJ239112.1), in different branches. The results indicated that the adopted three pairs of ITS-2 primers could accurately separate *H.contortus, Trichostrongylus spp*, and *T. circumcincta* (Fig. 6).

Infection of the three dominant species of GIN

Detection of infection of the three dominant species in fecal DNA

A total of 916 fecal samples from the four seasons were subjected to molecular identification (Table 1). A number of 477 samples showed infection with at least one dominant species, with an overall infection rate of 52.07%. Among them, 326 samples showed infection with *H. contortus*, with an infection rate of 35.59%, 234 samples had infection with *Trichostrongylus spp*., showing an infection rate of 25.55%, and 103 samples were infected with *T. circumcincta*, having an infection rate of 11.24%. A number of 242 samples collected in the spring showed infection (infection rate 80%), 69 summer samples showed infection (infection rate 70%), 108 autumn samples showed infection (infection rate 43.72%), and 58 winter samples showed infection (infection rate 37.42%). A number of 253 samples from Kazakh sheep showed infection, with an infection rate of 55.05%; 159 samples from the F1-generation sheep crosses showed infection, with an infection rate of 48.04%; and 65 samples from the F2-generation sheep crosses showed infection, with an infection rate of 54.17%.

Detection of infection of the three dominant species in larva DNA

Molecular identification of the 977 larvae hatched from mixed fecal samples in the four seasons was carried out (Table 6). A total of 521 nematodes were identified in the three dominant species, including 193 *H. contortus* (infection rate 19.75%), 230 *Trichostrongylus spp.* (infection rate 23.54%) and 98 *T. circumcincta* (infection rate 10.03%). A total of 95 nematodes in the three dominant species were identified from the sample collected in the spring (infection rate 70.59%), 138 were identified from the summer samples (infection rate 46.46%), 45 from the autumn samples (infection rate 18.44%), and 243 from the winter samples (infection rate 81.00%). A number of 165 samples from Kazakh sheep showed infection, with an infection rate of 48.25%; 185 samples from the F1-generation sheep crosses showed infection, with an infection rate of 53.62%; and 171 samples from the F2-generation sheep crosses showed infection, with an infection rate of 58.97%.

Table 6
Number of hatched larvae samples in mixed fecal samples

Spring	Summer	Autumn	Winter	Overall	Kazakh	F1	F2	Overall
136	297	244	300	977	342	345	290	977

Comparison of saturated saline solution floatation method and molecular identification

A number of 93 fecal samples were used to compare the two detection methods. The infection rate given by the saturated saline flotation method was 96.77% regarding the three dominant species (90/93). Furthermore, the 93 fecal samples were cultured to collect individual larva, which was subsequently subjected to molecular identification. Molecular identification demonstrated an infection rate of 100.00% (93/93). The saturated saline flotation method revealed 37 fecal samples infected with all three dominant species, while molecular identification only revealed three such samples. The differences in the average infection rates of *H. contortus* and *Trichostrongylus spp.* revealed by the two methods were extremely significant ($P < 0.001$), but the difference in the average infection rates of *T. circumcincta* revealed by the two methods was not significant ($P > 0.001$) (Table 7).

Table 7
he average infection rate of the three dominant species was detected by the two methods

Methods	*H.contortus*(%)	*Trichostrongylus* spp.(%)	*T. circumcincta*(%)
Floatation method	59.62 ± 30.79^{A}	6.34 ± 9.47^{A}	11.62 ± 23.00^{A}
Molecular identification	15.72 ± 20.73^{B}	25.05 ± 24.07^{B}	12.44 ± 16.93^{A}
Note: Data in the same column, without the same uppercase superscripts (A, B) indicate a highly significant difference ($P < 0.01$)			

Discussion

In order to effectively prevent and treat sheep GIN infection, we need to deeply understand its epidemiological pattern. Infection rate is an essential indicator to determine the risk factors of population health, and a same infection intensity of different species may result different pathogenic potential. Therefore, accurate identification of different species and understanding of the parasitic epidemiology are the basis for developing sustainable parasite control measures. The conventional method of sheep GIN diagnosis is mostly by fecal examination. The nematode species are identified based on the biological characteristics of eggs and infectious larva found in fecal. For example, *Trichuris* and *Nematodirus* are easily identified according to their shapes and sizes. However, most species of *Strongyloides* are similar in size and shape, and usually cannot be accurately identified at the genus level, such as *Haemonchus*, *Trichostrongylus*, *Teladorsagia*, *Cooperia*, *Bunostomum*, etc.

Therefore, fecal culture and morphometric analysis of the third stage larva (L3) must be conducted to identify the species, which requires considerable time and effort to identify the morphology of larva at various stages.

DNA molecular diagnostic technology has excellent specificity and sensitivity, and it is often used for the specific identification of GINs in livestock (Santos et al. 2020; Gasser 1999, 2006; Zarlenga et al. 2001). The most popular molecular markers are cytochrome c oxidase subunit I (cox1) of mitochondrial DNA (mtDNA), NADH dehydrogenase subunit 4 (Nad4) and the first and second internal transcribed spacers (ITS1 and ITS2, respectively) of ribosomal DNA (rDNA). Most studies consistently demonstrated that the ITS regions of rDNA could serve as reliable genetic markers for the specific identification of *Strongyloides* in domestic animals (Gasser 1999, 2006; Zarlenga et al. 2001; Chilton et al. 2004; Wimmer et al. 2004). The results of other studies have shown that the intraspecies sequence variation of ITS-1 and ITS-2 (usually < 1.5%) was much smaller than interspecies variation (Huby-Chilton et al. 2006). In this study, the sequence homological analysis of the three nematode species revealed intraspecific differences between 0.88% and 1.24%. By amplification and sequence analysis of the ITS-2 regions of parasite eggs or larva DNA, Santos et al. (2020) identified 12 species of seven genera, including *Chabertia*, *Cooperia*, *Haemonchus*, *Oesophagostomum*, *Ostertagia*, *Teladorsagia*, and *Trichostrongylus*. Elmahalawy (2018) used Droplet Digital™ PCR with designed ITS-2 primer/probe combinations of *Haemonchus*, *Trichostrongylus*, and *Teladorsagia*to and achieved effective results, distinguishing the most important sheep GINs in Sweden. In the present study, three pairs of ITS-2 primers targeting *H.contortus, Trichostrongylus spp*, and *T. circumcincta* were selected to amplify the fecal DNA and single larva DNA of naturally infected Kazakh sheep. As a result, single clear bands were obtained. In addition, no PCR amplification was detected using single larva DNA templates that belonged to different nematode species from the primers. It also provided evidence for the specificity of PCR. Through phylogenetic tree analysis, sequences of the same genus were clustered in one branch, while sequences of different genera were present in different branches, showing clear interspecific grouping. The ITS-2 sequences from different samples that located in the same branch of the same species cannot be effectively distinguished, therefore such intraspecies conserved genes with large interspecies differences could be used as ideal molecular markers for the taxonomic identification and evolutionary genetic research of various GIN species. In this study, *Bunostomum trigoncephalum*, *Oesophagostomum*, *Nematodirus*, and *Marshallagia* were all attempted to amplify respectively, but not all species could be detected. Presumably, it is because of the difference in infection levels of nematode species from different samples.

In this study, the same 93 fecal samples were used to carry out morphological identification of eggs and DNA molecular identification of hatched larva, respectively, and given different results. The infection rate tested by the saturated saline solution flotation method was 96.77%, whereas that by molecular identification was 100.00%, providing evidence for the high sensitivity of PCR. In addition, 37 samples were infected with all three dominant species as shown by saline solution flotation method, inconsistent with the results from molecular identification, which only showed three 3 triple infected fecal samples. Because of the similar size and shape of the eggs of *Trichoencephalae*, different species of nematodes may not be accurately distinguished when examining the eggs following the saturated saline solution flotation method. Different genera of nematodes may be mixed up too. For example, the egg size of *H. contortus* is (70–81) μm × (39–55) μm, and the egg size of *Oesophagostomum. spp* is (70–90) μm × (34–45) μm, and both of them are oval-shaped (Kong 2016; Zajac et al., 2012). Hence, the saturated saline solution flotation method may mistake the eggs of other species of nematodes as one of the three dominant species, thereby increasing the triple infection rate. In contrast, the primers for molecular identification were species-specific. The sequences of the obtained PCR products only identified nematodes of these three species and will not be mixed up with other species of nematodes. Therefore, molecular identification has a higher sensitivity that made the triple infection rate lower than the result of the saline solution flotation method. Other scholars have also conducted related research. For instance, to overcome the limitations of conventional methods such as fecal egg count (FEC) and/or larva culture (LC) in terms of sensitivity and specificity, Roeber et al. (2012)developed and evaluated a semi-automated, high-throughput multiplexed-tandem PCR (MT-PCR) platform that can be used for species- or genus-specific diagnosis of GIN infection within 24 hours, while the LC method requires 7–10 days. Hence the primary advantage of molecular identification is that it could run at least 96 samples in one day and eliminate any potential risk of “cross contamination”. Though the conventional saline solution flotation methods can diagnose an overt infection of *Strongyloides* within 1–2 days, only after the larva culture and microscopic identification by an experienced specialist, genus or species-specific diagnosis can be achieved. However, this culture method requires at least one week, and the fecal composition and culture conditions may cause significant changes in larva development, leading to bias in identification results (Roeber et al. 2011).

Conclusion

In this study, the ITS-2 sequences of rDNA were used to perform molecular identification on fecal samples and hatched larvae of Kazakh sheep and the F1- and F2-generations of Kazakh × Texel sheep crosses.That infections with three dominant species, *H. contortus*, *Trichostrongylus spp*, and *T. circumcincta* were successfully identified. The molecular identification method showed more accurate and convenient than the conventional saline solution flotation method regarding specific identification of nematode species by differential analysis of the two methods based on the same fecal samples. Therefore, the present study laid a foundation for the establishment molecular identification method and the grasp molecular epidemiology of GIN.

Declarations

Acknowledgments

We would like to thank all organizations which funded this work and all the teachers who cooperated in technical assistance.

Funding Information

This study was funded by the Scientific Research Project of University of Xinjiang Autonomous Region (No. XJED2020Y049), and the National innovation and entrepreneurship Training Program for college students of China (No.2020KJ0018), and the KeyLab funding of Xinjiang Ugrus Autonomous region: identification of genes with resistance to sheep gastrointestinal nemotode infection by genome-wide association study, The Tianshan Innvation Team (No. 2018D14004), and the Regional Collaborative Innovation Project of Xinjiang Uygur Autonomous Regioin (Shanghai Cooperation Organization Science and Technology Partnershi and International Cooperation Program).Cooperative Research and Application of Genomic Selection Technology for sheep Meat Traits (No. 2020E01004).

Conflict interest

The authors declare that they have no competing interests.

Availability of data and material

The date that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Conceived and designed the experiments: Xiaofei Yan, Mingjun Liu, Sangang He.

Performed the experiments: Xiaofei Yan, Yiyong Liu, Haifeng Deng, Bing Han.

Analyzed the data: Xiaofei Yan, Ning Zhang, Yuqi Wang.

Contributed reagents/materials/analysis tools: Mingjun Liu, Sangang He, Yiyong Liu, Haifeng Deng.

Wrote the paper: Xiaofei Yan, Mingjun Liu.

Ethics approval

All animal procedures were approved by the Animal Welfare Committee of Shihezi University (Xinjiang, China). Experiments were conducted in accordance with animal ethics guidelines and approved protocols.

Consent to participate and Consent for publication

All authors read and approved the final manuscript, and consent for publication.

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Figures

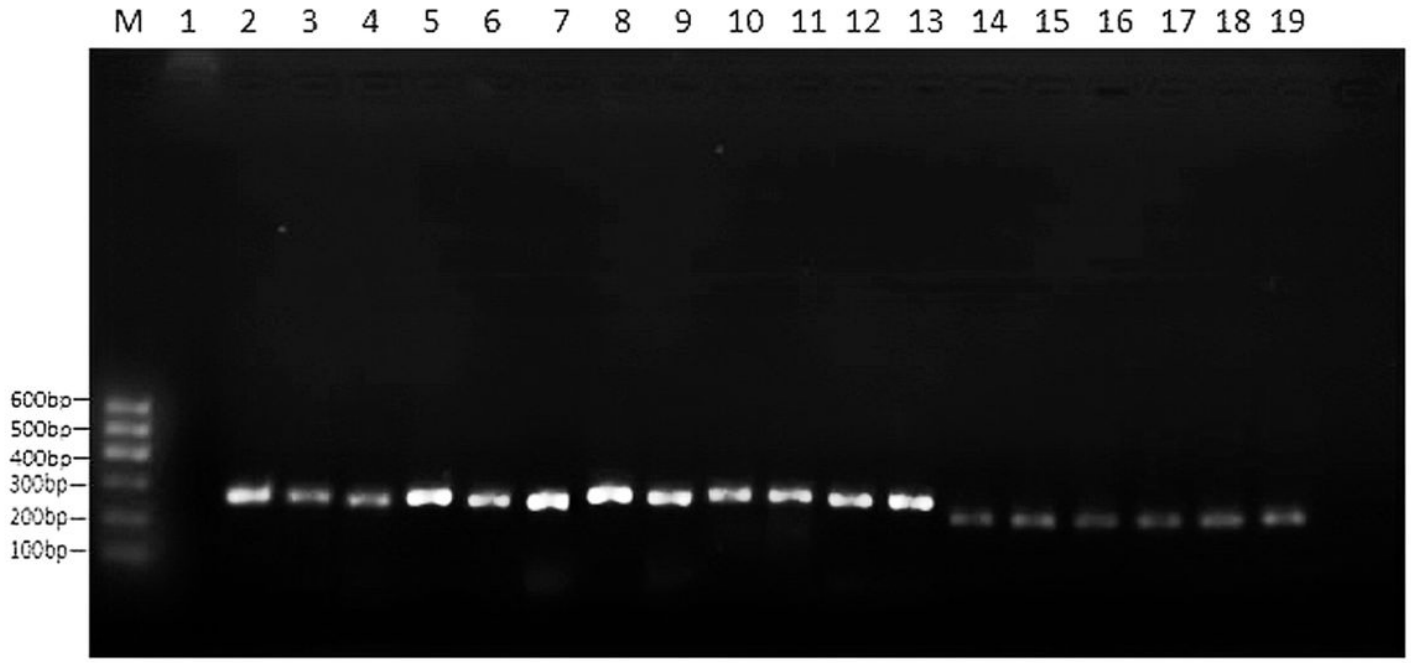

Figure 1

PCR amplification products of *H.contortus, Trichostrongylus spp., T. circumcincta* ITS-2 sequences

Note: M: 100 bp DNA Mark; 1: negative; 2-4: *H.contortus* larval DNA PCR product; 5-7: *H.contortus* fecal DNA PCR product; 8-10: *Trichostrongylus* spp. larval DNA PCR product; 11-13 : *Trichostrongylus* spp. fecal DNA PCR product; 14-16: *T. circumcincta* larvae DNA PCR product; 17-19: *T. circumcincta* fecal DNA PCR product

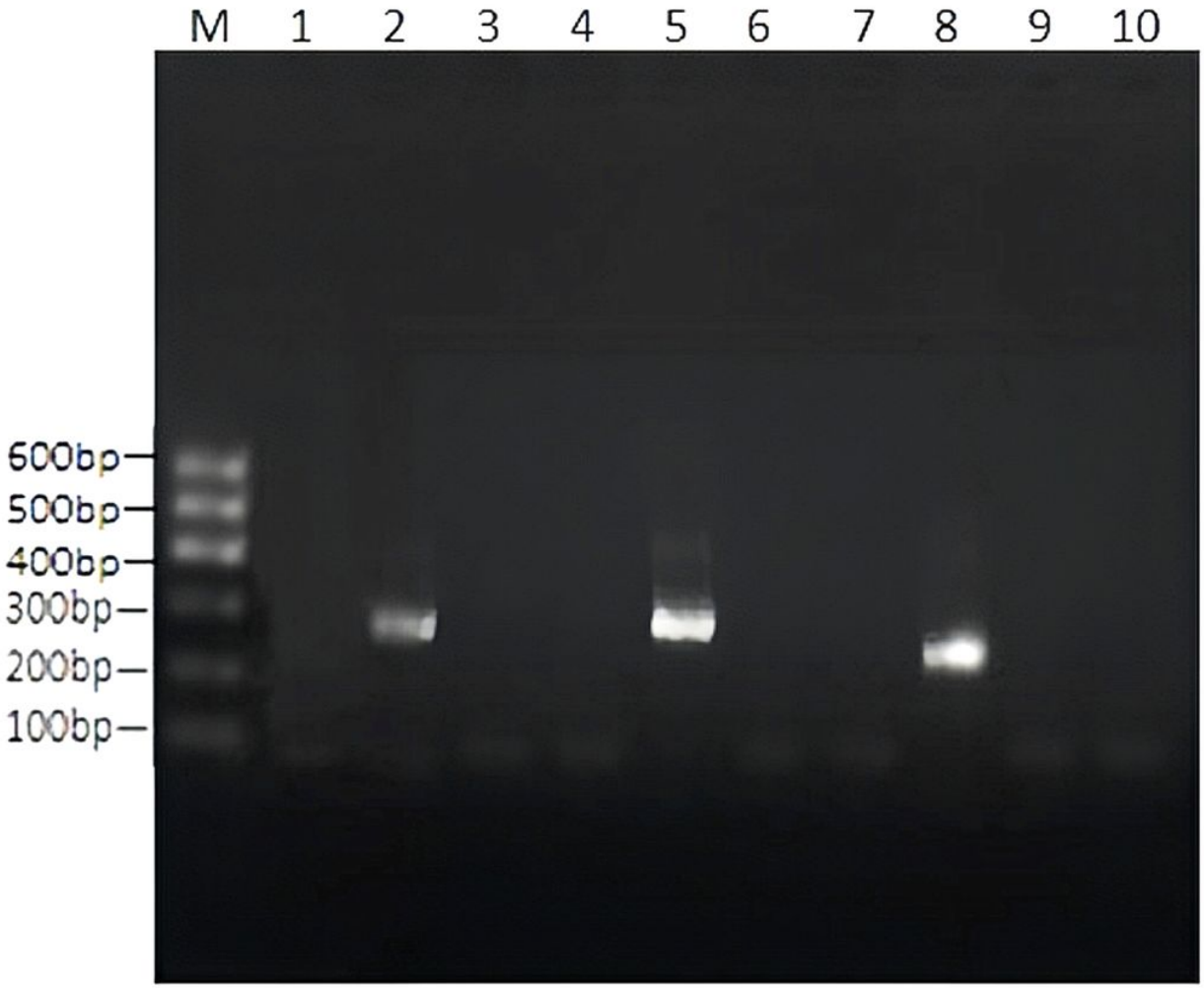

Figure 2

***H.contortus*, *Trichostrongylus spp.*, *T. circumcincta* ITS-2 sequence primer specificity detection**

Note: M: 100 bp DNA Mark; 1: negative; 2: *H.contortus* primer positive sample; 3: *Trichostrongylus* spp. positive sample; 4: *T. circumcincta* positive sample; 5: *Trichostrongylus* spp. primer positive sample; 6: *H.contortus* positive sample; 7: *T. circumcincta* positive sample; 8: *T. circumcincta* primer positive sample; 9: *H.contortus* positive sample; 10: *Trichostrongylus* spp. positive sample.

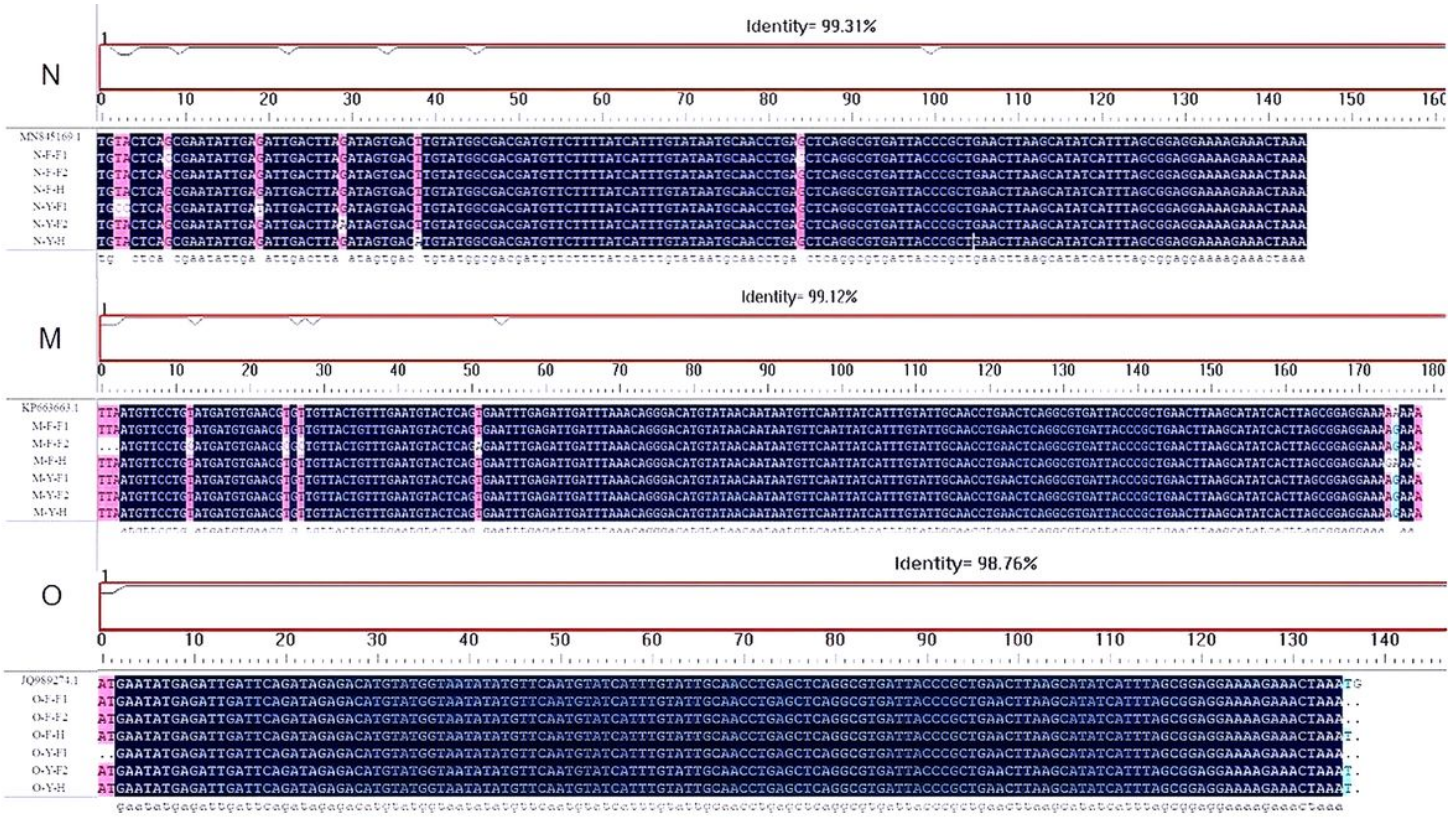

Figure 3

Homology analysis of ITS-2 sequences

Note: *H.contortus* ITS-2 sequence homology analysis; M: *Trichostrongylus* spp. ITS-2 sequence homology analysis; O: *T. circumcincta* ITS-2 sequence homology analysis

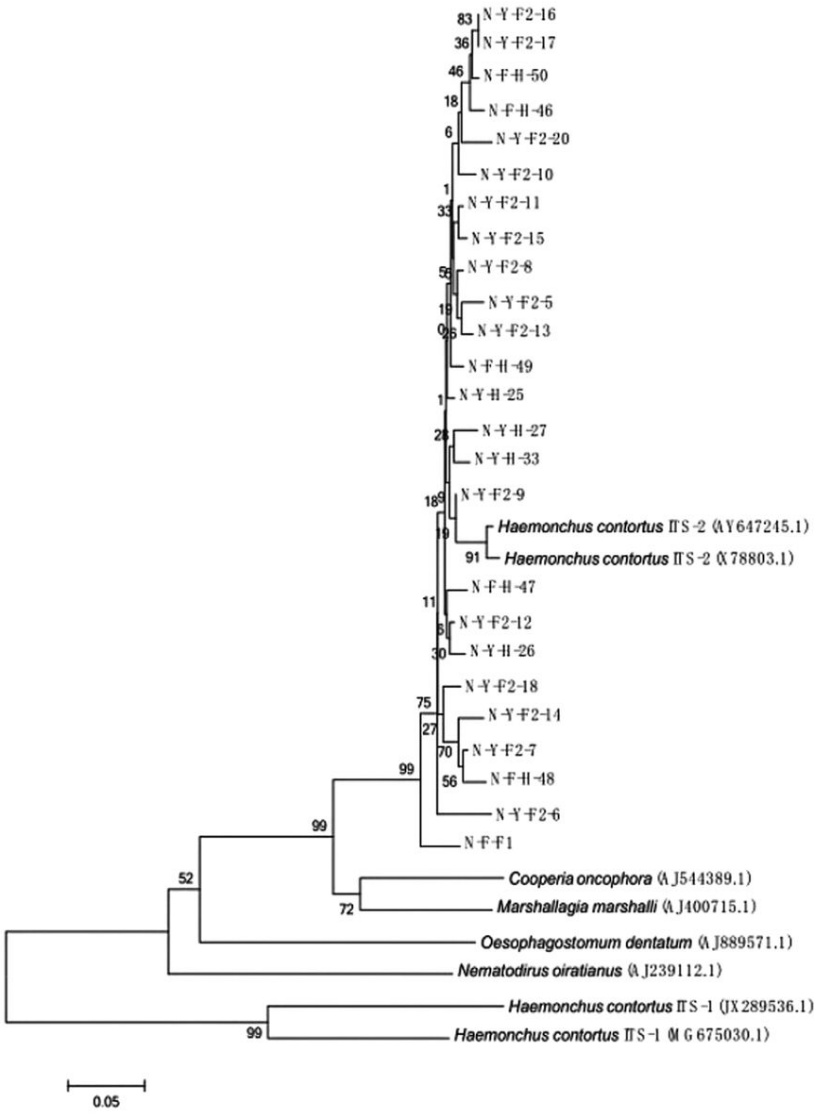

Figure 4

Neighbour joining tree of the ITS-2 sequences from *H.contortus* isolated in this study and selected data base entries

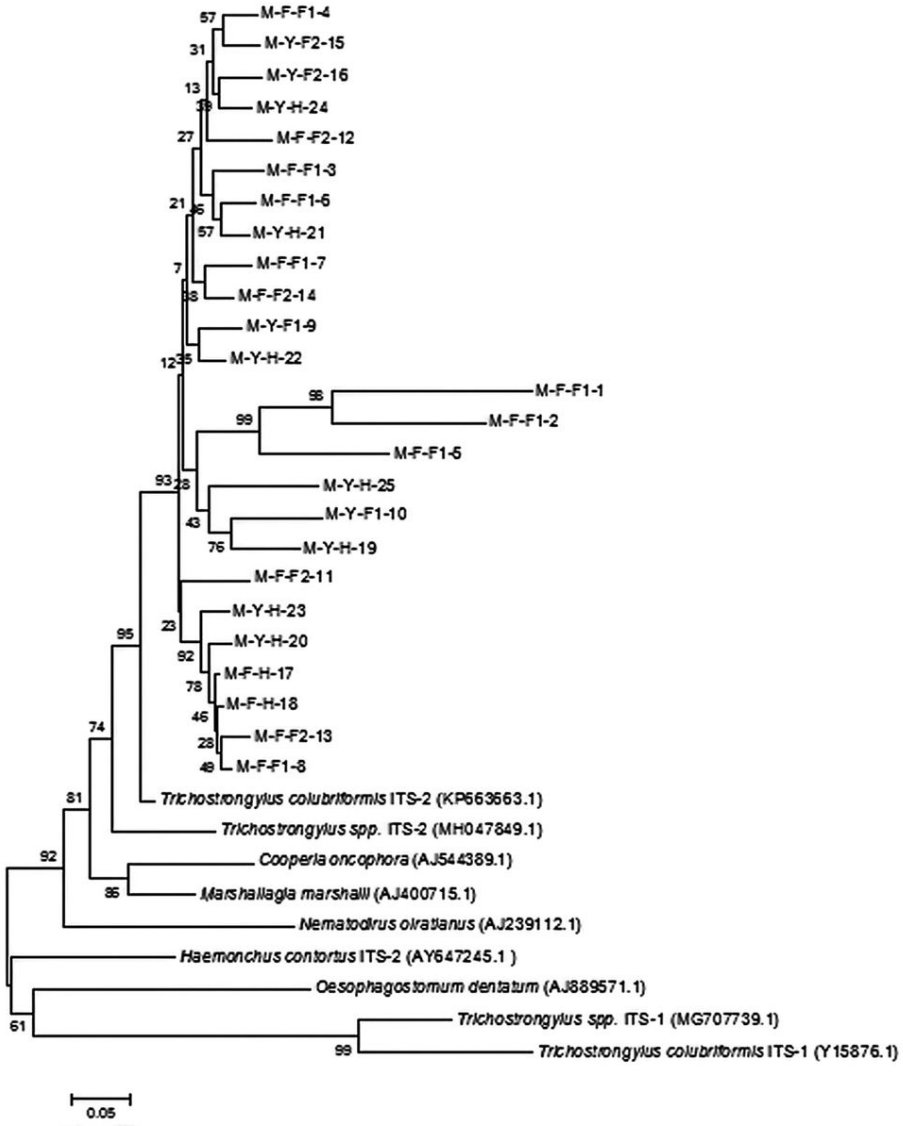

Figure 5

Neighbour joining tree of the ITS-2 sequences from *Trichostrongylus* spp. isolated in this study and selected data base entries

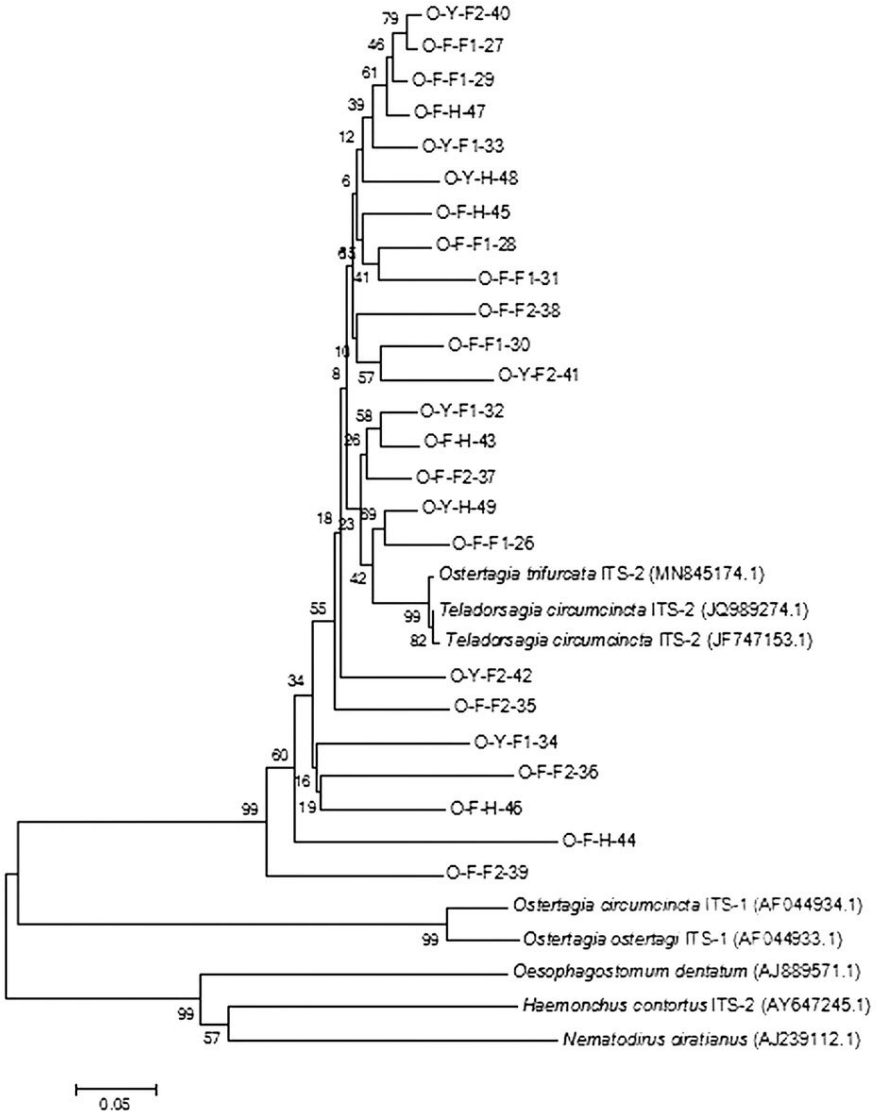

Figure 6

Neighbour joining tree of the ITS-2 sequences from *T. circumcincta* isolated in this study and selected data base entries