Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.124

SHORT COMMUNICATION

Genetic Mapping of *Marshallagia* and *Ostertagia* Nematodes Isolated from Free-Range Sheep in Northern Xizang

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ARTICLE HISTORY (26-188)

Received:	February 26, 2026
Revised:	May 13, 2026
Accepted:	May 15, 2026
Published online:	May 18, 2026

Key words:
Gastrointestinal nematodes
Gene sequencing
Marshallagia
Ostertagia
Phylogenetic tree

ABSTRACT

Livestock in the northern areas of Xizang are increasingly affected by nematodes belonging to multiple families, resulting in an expanded geographic distribution of parasitic infections. We analyzed genetic classification status and evolutionary relationships of the ITS1 gene in free-range Tibetan sheep nematodes in the jurisdiction of Xizang. The gastrointestinal tract nematodes of free-range sheep were collected and the ITS1 gene fragment was amplified by PCR. The amplified products were subjected to agarose gel electrophoresis and sequenced. The results showed that 1-1, 1-2, 1-3, 3-1, 3-2, 3-3, 3-4 and 3-6 belong to the same branch as *Marshallagia* nematodes, whereas 1-2, 1-3, 1-4, 3-1 and 3-5 had the TTT sequence motif, indicating a distinct phylogenetic position distant from *Marshallagia* and *Ostertagia* nematodes while samples 3-7 clustered with *Trichuris* nematodes. These findings provide a reference basis for clarifying the typing of nematodes and the genetic evolution of pathogens in northern Xizang.

To Cite This Article: Zhao X, Du S, Zhang Q, Deji Q, Lv Q and Tang W, 2026. Genetic mapping of *Marshallagia* and *Ostertagia* nematodes isolated from free-range sheep in northern Xizang. Pak Vet J, 46(5): 1372-1375. http://dx.doi.org/10.29261/pakvetj/2026.124

INTRODUCTION

Ruminants are facing significant challenge of parasitic infections, particularly those caused by nematodes (families *Trichostrongylidae* and *Trichuridae)*, resulting in severe health impairment, reduced productivity and substantial economic losses (Hayward and Beal, 2024; Asad *et al*., 2025). These nematodes are distributed worldwide and exhibit a high infection rate in Xizang, China (Ai et al., 2023; Mo *et al*., 2026). They are important pathogens that cause parasitic diseases in yaks and sheep, and pose a risk of zoonotic transmission (Presswell and Bennett, 2022). Infection can lead to adverse symptoms such as weight loss, diarrhea, and in severe cases, death even, resulting in significant economic losses to the livestock industry (Ai *et al*., 2023; Strydom *et al*., 2023). Currently, there is insufficient consideration of how some ecological environmental elements in the Xizang region affect nematode classification (Liu *et al*., 2022). The distribution and types of nematodes, for example, may be impacted by elements including soil type, vegetation cover and plateau climate, which could have an impact on classification outcomes. Molecular biology techniques have been used in a few studies to classify and identify nematodes in Tibetan sheep and cattle, although this research is still quite limited (Bhat *et al*., 2022; Ai *et al*., 2023; Yan *et al*., 2023). This study focuses on gastrointestinal (GI) nematodes in yaks and sheep in the northern region of Xizang. Through morphological identification and amplification of ITS sequences using specific primers, sequence information was obtained, and a phylogenetic tree was constructed using the neighbor-joining method to analyze the phylogenetic relationships of nematode populations in this region.

MATERIALS AND METHODS

Collection and processing of specimens: The northern Xizang's Geji County, herdsmen killed the yaks and the sheep, and the visual observation of the internal organs was done on the site between November and December 2023. A total of 11 nematode samples (n=11) were collected. These nematode samples were collected from the stomach, liver and spleen of the yaks and the liver and stomach of the sheep from the Xizang region. Each nematode sample represented a nematode isolate, which was placed in a 75% alcohol reagent bottle and stored in a refrigerator at 4°C with the sample number attached. All the experiment operations were under the instructions and approval of the Laboratory Animals Research Centre of Xizang Academy of Agricultural and Animal Husbandry Sciences (XAAA2025020309).

PCR amplification and sequencing of the ribosomal ITS sequence: All nematode samples (n=11) were processed for DNA extraction through kit while amplification was carried out through primers of ITS ribosomal sequences (Gasser and Chilton, 1995; Gasser and Hoste, 1995), focusing NC5 for ITS-F: (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 for ITS-R: (5′-TTAGTTTCTTTCCTCCGCT-3′), which were synthesized at Suzhou Genewiz Biotechnology Co., Ltd. *In silico* thermodynamic analysis (OligoCalc/ Primer3Plus) indicated melting temperatures of 62.4°C (48% GC) and 58.1°C (41% GC) for the 25 nt and 22 nt primers, respectively, with $\Delta G > -2.0$ kcal/mol excluding significant hairpin structures or primer-dimer formations. The 4.3°C Tm differential is within acceptable ranges for PCR compatibility. The DNA was amplified by using genomics DNA as a template using premix Taq™ Mix (12.5μL), upstream & downstream primers each 1μL (10 pmol/L), template DNA 1μL, and ddH2O 9.5μL. PCR amplification conditions were as follows: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, 35 cycles and final extension at 72°C for 7 min. A 5μL of PCR product used for electrophoresis at 1.0% agarose gel.

Ribosomal gene sequence analysis: Sequence assembly was performed using DNASTAR software and sequence alignment was conducted through the NCBI/BLAST web page. After determining the genotype, alignment was performed using the reference sequence of the ITS gene of nematodes from the GenBank database. The Neighbor Joining (NJ) method in MEGA11.0 software was utilized, with *Taenia asiatica* (KR422379) as the out group to construct a phylogenetic tree (Gao *et al.*, 2024). The Kimura 2-parameter model was selected, and bootstrap testing was conducted (repeated 1000 times).

Statistical analysis: All nucleotide sequences were edited and aligned using BioEdit and MEGA11.0. Sequence similarity (%) was determined using BLAST against the NCBI. Genetic distances were calculated using the Kimura 2-parameter model. Phylogenetic analysis was performed using the Maximum Likelihood method with 1,000 bootstrap replicates to assess tree reliability. The descriptive statistics was applied for percentages and univariate data using SPSS version 22 of statistical computer software programme.

RESULTS AND DISCUSSION

The features of female worm tail were relatively straight, with the vulva located in the mid-body while that of male showed a special morphological structure, such as a copulatory organ (Fig. 1). The ITS1 gene of the scattered Tibetan sheep nematode in Ali, Xizang, and the ITS sequence size of all 11 nematodes was focused at 864 bp (Fig. 2). A phylogenetic tree was constructed based on ITS sequences and samples 1-4 collected from sheep in Geji County, Xizang, were grouped with the *Mongolian Marshallagia* nematode (JQ925871) on the same branch.

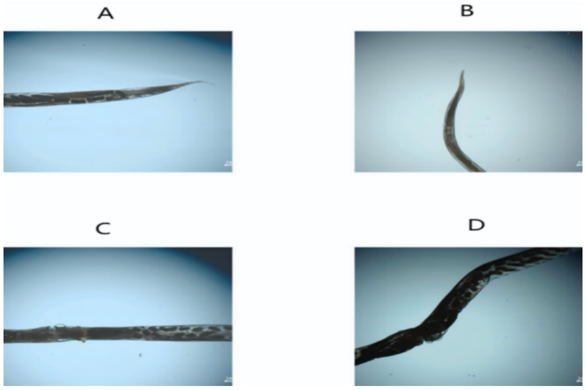

Fig. 1: Morphological observation of nematodes. A: Female tail B: Anterior dorsal surface C: Vulva of *Mongolian Marshallagia* nematode D: Vulva of Western *Ostertagia* nematode.

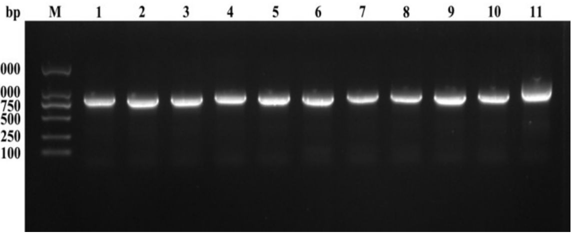

Fig. 2: The agarose gel electrophoresis pattern of amplified ITS genes from isolated nematodes. M: Marker, 1: 1-1, 2: 1-2, 3: 1-3, 4: 1-4, 5: 3-1, 6: 3-2, 7: 3-3, 8: 3-4, 9: 3-5, 10: 3-6, 11: 3-7.

Such clustering patterns may indicate possible genetic conservation and ecological adaptation among *Marshallagia* populations across high-altitude pastoral ecosystems of Central and East Asia (Yan *et al.*, 2023; Kuchboev *et al.*, 2020). Samples 1-1, 1-2, 1-3, 3-1, 3-2, 3-3, 3-4, and 3-6 were grouped with the *Marshallagia* nematodes on the same branch. Samples 1-2, 1-3, 1-4, 3-1, and 3-5 all had TTT nucleotide motifs, indicating a relatively distant relationship with *Marshallagia* and *Ostertagia* nematodes. Samples 3-7 were closely related to *Trichuris* nematodes (Fig. 3). This distinct nucleotide motifs may suggest evolutionary divergence of nematodes associated with environmental selection pressure, host adaptation and/or geographic isolation. Similar type of adaptive response of *Marshallagia* spp. to ecological variations were recently reported by Aleuy *et al.* (2023).

Nematodes of the genus *Trichuris* exhibit morphological differences that were frequently linked to host adaptation, as described in a previous research (Stroehlein *et al.*, 2017). At the molecular level, the ITS region sequence of *Trichuris* sp. NMG 2014 differs by 5-8% from 3-6%. Phylogenetic analysis showed that it was closely related to *Marshallagia (M.) mongolica and Ostertagia trifurcata* in Mongolia and the West. The data in the above experiments were all independent branches of *Fasciola (F.) hepatica.*

Marshallagia nematodes are found in Xinjiang, Uzbekistan and some areas from Spitsbergen Island to the New World (Kuchboev *et al.*, 2020; Bhat *et al.*, 2023). They were also distributed in small ruminants in North Africa, Kashmir, Algeria, the Caucasus, and other regions (Hoberg *et al.*, 2008; Zouyed *et al.*, 2018). The discovery in the Ali region of Xizang shows that the transmission of *Marshallagia* nematode has been widespread. This broad geographical spreading of *Marshallagia* spp. has provided sound evidence about strong ecological plasticity and ability to persist under diverse ecological conditions. Such adaptability may facilitate long-term spread of infective larvae in grazing lands used by nomadic pastoral communities.

In contrast to Fu *et al.*, (2023) study, small ruminants were found to be the primary hosts of the exceedingly common and harmful *Trichostrongylus* as GI worm. A significant incidence and abundance of the zoonotic parasite *Trichostrongylus (T.) colubriformis* (Bhat *et al.*, 2023) was also noted. On the other hand, *T. colubriformis* remains one of the most pathogenic and economically important GI nematodes in sheep due to its effects on intestinal nutrient absorption and production losses (Tafere *et al.*, 2022). While GI parasitic infections in livestock from the Xilingol League were shown to have distinct host- and region-associated patterns. Sheep had greater infection rates than cattle and goats, with *Eimeria* spp. being the most common parasites, followed by helminths and intestinal protozoa (Zhang *et al.*, 2024).

Field investigations and experimental studies can be conducted to explore the interactions between nematodes and hosts, as well as the impact of environmental factors on nematode distribution and evolution (Williamson and Gleason, 2003; Midha *et al.,* 2017). As Fakhrahmad *et al.* (2021) studied the presence of beta tubulin SNPs linked to Benzimidazole (BZ) resistance in *M. marshalli*, a common trichostrongylid member showed the contradiction with the current study. Another study was in contradiction to the present results that in Riyadh, Saudi Arabia, a population of *Fasciola* was separated from sheep. Sheep were found to be infected with both *F. hepatica* and *F. gigantica*, with *F. hepatica* predominating (80%) (Alajmi *et al.*, 2019). This study was limited by a small sample size (n=11) from a single county during winter months and reliance on single-gene (ITS1) analysis, which may not fully resolve cryptic species or represent regional diversity across northern Xizang.

Conclusions: The study provides a valuable resource for the identification of the different kinds of nematodes and the genetic evolution of them in northern Xizang. By doing a more thorough study, we should be able to offer better scientific and practical approaches to the prevention and management of nematode diseases in Tibetan sheep, which would guarantee the industry's continued growth. This study is limited due to its geographic focus on northern Xizang, which may not fully indicate diversity of nematodes in other regions. Also, the sample size and reliance of molecular data limit comprehensiveness of evolutionary analysis.

Data availability statement: All raw sequence data from animals were deposited in the NCBI Sequence Read Archive database under accession number: PZ051164-PZ051174.

Ethics statement: All the experiment operations were under the instructions and approval of the Laboratory Animals Research Centre of Xizang Academy of Agricultural and Animal Husbandry Sciences (XAAA2025020309).

Authors contribution: WT: research idea and methodology. XZ, SD, QZ, QD and QL: reagents, materials, and analysis tools. WT: writing – original draft and preparation. WT: writing – review and editing. WT: visualization and supervision. All authors have known and approved the final manuscript.

Funding: This study was supported by the Base and Talent Project of the Science and Technology Program of Xizang, China (XZ202401JD0012).

Acknowledgement: None.

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