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RESEARCH ARTICLE

ITS2-Based Molecular Identification and Phylogenetic Analysis of Major Gastrointestinal Strongyles in Sheep from Slovenia

Laura Šimenc Kramar and Janez Posedi*

Institute of Microbiology and Parasitology, Parasitology Unit, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia
*Corresponding author: Janez.Posedi@vf.uni-lj.si

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ABSTRACT

Gastrointestinal strongyle infections are common in sheep and require accurate species identification for effective diagnosis and control. Morphological identification is limited, and molecular markers such as the internal transcribed spacer 2 (ITS2) region are widely used for species differentiation. However, variability of ITS2 sequences at different biological levels may influence interpretation of molecular data. The aim of this study was to analyze ITS2 sequence variability at three levels—within individual parasites (intra-individual variability), within species (intraspecific variability), and between species (interspecific variability)—using cloned sequences obtained from morphologically identified adult strongyles in sheep in Slovenia. Adult nematodes were collected from four naturally infected sheep, and five species were identified: *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, *Cooperia curticei* and *Chabertia ovina*. A total of 45 ITS2 sequences were obtained from 15 parasite specimens (three clones per specimen) and analyzed using sequence alignment and phylogenetic methods. Intra-individual variability was generally low, with identical sequences observed in some species and minor differences in others. Intraspecific variability ranged from zero to 3.8%, depending on the species, while clear interspecific differences enabled reliable species discrimination. Phylogenetic analysis confirmed clustering of Slovenian isolates with corresponding reference sequences. These findings indicate that ITS2 is a suitable marker for distinguishing major gastrointestinal strongyle species, while highlighting the presence of intra-individual and intraspecific variation that should be considered when interpreting molecular data. The results represent preliminary ITS2 sequence data for strongyles in sheep in Slovenia and provide a basis for future molecular studies.

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INTRODUCTION

Gastrointestinal helminth infections are widespread in grazing livestock and represent a major constraint to animal health, productivity, and welfare. In sheep, infections are typically caused by mixed populations of strongyle nematodes, including species of the genera *Teladorsagia*, *Trichostrongylus*, *Cooperia*, and *Chabertia*. Accurate identification of these parasites is essential for effective diagnosis, treatment, and control strategies.

In small ruminants, such as sheep, most of the common and pathogenic helminth species belong to the strongyle group (Sargison *et al.*, 2007). Sheep are most often affected by multiple strongyle nematodes, including species of *Teladorsagia, Trichostrongylus, Haemonchus, Nematodirus, Chabertia, Cooperia,* and *Oesophagostomum* (Zajac *et al.*, 2006). In Slovenia, extensive research on gastrointestinal helminths in sheep has not been conducted since the 1980s. It was reported that *Ostertagia circumcincta* (52.47%) and *Haemonchus contortus* (38.61%) were among the most dominant helminths in sheep, along with notable occurrences of *Trichostrongylus axei, T. vitrinus, T. capricola, Nematodirus filicollis, Ostertagia trifurcata, Strongyloides papillosus and Trichostrongylus colubriformis* (Kopitar, 1984). A decade later, significant shifts were observed in helminth prevalence. The findings showed that *O. circumcincta* increased to 82%, *T. axei* to 57%, while *Nematodirus spathiger* and *N. filicollis* were

found in 55% and 42% of sheep, respectively. Other frequently detected species included *T. vitrinus* (22%), *H. contortus* (18%), and *Chabertia ovina* (25%) (Brglez, 1996). These results suggest dynamic changes in helminth populations over time, potentially influenced by environmental and management factors. Since then, no data on small ruminant helminths from Slovenia have been published. Although helminth control in grazing cattle in Slovenia has been evaluated within the national animal welfare programme (Podpečan *et al.*, 2023), data on gastrointestinal strongyles in sheep remain scarce. This presents a big gap in research also of Jezersko-Solčava sheep bread which is one of two most represented sheep breeds in Slovenia (Šterbenc *et al.*, 2025).

Conventional diagnostic methods, such as faecal egg counts and coproculture, are limited by the morphological similarity of strongyle eggs and larvae, which often prevents reliable species-level identification. Morphological identification of adult parasites requires specialised expertise and is not applicable in routine diagnostics. These limitations highlight the need for molecular approaches that enable precise and reproducible identification of strongyle species. Among molecular markers, the ITS2 region of ribosomal DNA is widely used for species identification and phylogenetic analysis of nematodes and has been successfully applied in large-scale surveys of gastrointestinal nematode communities in grazing livestock across different geographical regions (Redman *et al.*, 2019; De Seram *et al.*, 2022; Airs *et al.*, 2023). The ITS2 region provides sufficient interspecific variation for species discrimination while maintaining relative conservation within species. However, due to its multicopy nature, intra-individual sequence variability may occur, which can influence the interpretation of molecular identification results. Despite the increasing use of ITS2-based approaches, data on ITS2 sequence variability in gastrointestinal strongyles from sheep in Slovenia are lacking. In addition, the extent of variability at different levels—within individual parasites, within species, and between species remains insufficiently characterised for these parasites.

In this study, 45 ITS2 rDNA sequences from adult parasites of the species *Trichostrongylus axei, Trichostrongylus colubriformis, Teladorsagia circumcincta, Cooperia curticei,* and *Chabertia ovina* from sheep were determined using biotechnological methods and deposited in GenBank NCBI. Therefore, the aim of this study was to analyse ITS2 sequence variability at three levels within individual parasites (intra-individual variability), within species (intraspecific variability), and between species (interspecific variability) using cloned sequences obtained from morphologically identified adult strongyles in sheep in Slovenia. Specifically, 45 ITS2 rDNA sequences from adult parasites were determined and deposited in GenBank NCBI; inter- and intraspecific differences were identified, and a phylogenetic comparison with other sequences from GenBank was conducted.

MATERIALS AND METHODS

Sampling: The research samples were collected from a herd of 18 rams of the indigenous Slovenian Jezersko-Solčava sheep breed, bred at the Institute of Microbiology and Parasitology. Four one-year-old sheep that were naturally infected with gastrointestinal strongyles and excreted their eggs were removed from the herd at the start of the experiment. The sheep were not treated with antiparasitic drugs. All procedures involving animals were conducted in accordance with institutional and national guidelines for animal welfare. Samples were collected during post-mortem from animals slaughtered as part of routine commercial slaughter.

Acquisition of adult parasites: The four previously mentioned sheep were sacrificed for the experimental diagnostic procedures using a standard slaughter method. After death, the abomasum, small intestine, caecum, and large intestine were removed. The contents of these four sections of the digestive tract were individually transferred into 0.9% NaCl solution heated to 38–39°C. A 2% agarose gel (BioLife, US) was prepared, cooled to 50°C, and mixed with the intestinal content solution in a 1:1 ratio. This mixture was poured onto cloths to a thickness of 3–4mm and left to harden. The cloths with the agarose gel mixture were suspended in containers filled with 0.9% NaCl solution heated to 30°C and incubated for 2 to 3 hours. During incubation, live adult parasites migrated from the agarose gel mixture into the NaCl solution and settled at the bottom of the containers. Recovered adult parasites were clean following the isolation procedure and were transferred into 70% ethanol for preservation. The specimens were stored at 4°C until further morphological identification.

Determination of adult parasites: Adult parasites were identified based on morphological features described in the literature using a stereo microscope (Barth and Visser, 1991).

Extraction of genomic DNA, PCR and cloning of PCR products: Genomic DNA was extracted from adult parasites according to the manufacturer's instructions using the NucleoSpin Tissue (Macherey-Nagel, Germany) kit. A total of 100µL of genomic DNA was eluted in a buffer and stored at -20°C until further use. For amplification of the ITS1-5.8S-ITS2 region and the initial 28S segment of the ribosomal DNA, the primers NC5 F (5′-GTAGGTGAACCTGCGGAA GGATCATT-3′) and NC2 R (5′-TTAGTTTCTTTTCCT CCGCT-3′) were used (Gasser *et al.*, 1993). The reaction mixture contained 10µL of 10× PCR buffer (Invitrogen, US), 0.5µL Platinum® Taq DNA polymerase (Invitrogen, US), 3µL 50mM MgCl2 (final concentration 1.5mM), 200µM of each dNTP, 0.5µM of each primer (NC5 F and NC2 R), and 5µL of extracted DNA in a total reaction volume of 100µL (sterile deionised water used to adjust to final volume; 5µL of sterile deionised water replaced template DNA in the negative control). The thermal cycling conditions were 94°C for 2 min; 94°C for 30 sec; 25 cycles of 56 °C for 30 sec and 72°C for 30 sec; followed by 72°C for 7 min and 4°C indefinitely. PCR products were separated by gel electrophoresis, excised, and purified. For cloning of PCR products, the vectors pCR®2.1-TOPO and pCR®II-TOPO, cell components DH5α-T1R, and the TOPO TA Cloning® kit (Invitrogen, US) were used according to the manufacturer's instructions. Both vectors belong to the TOPO TA Cloning® system and are functionally equivalent for cloning PCR products with 3′-A overhangs. Their use at

different stages reflected reagent availability during the experimental work. Plasmids were extracted using the Wizard™ Minipreps (Promega, US) and NucleoSpin® Plasmid (Macherey-Nagel, Germany) kits. To test whether the correct DNA fragments were present in the plasmids, restriction digestion with the *EcoRI* enzyme was performed, followed by electrophoresis. For plasmid concentration control, a BioPhotometer (Eppendorf, Germany) was used at wavelengths of 230, 260, 280, and 320nm.

Sequencing: Sanger sequencing was performed twice (forward and reverse) on three clones from three individual parasites of each investigated parasite species, using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, US) on a Perkin-Elmer GeneAmp PCR System 2400 sequencer. The primers used for sequencing are listed in Table 1. Two sequencing primers (NC13 F and NC13 R) were modified in this study. Initial testing revealed sequencing difficulties, including base-calling errors and unclear signal peaks at the 5′ and 3′ ends of the ITS2 region, likely due to mismatches or secondary structures at the original primer binding sites. To address this, the modified primers were designed to improve annealing specificity and sequencing clarity. These primer sequences are reported here and may facilitate future studies targeting similar genomic regions in strongyle nematodes (Table 1). To minimize potential sequencing artefacts or cloning-induced errors, all sequences were obtained by bidirectional sequencing, and only consistent base calls across reads were considered.

Table 1: List of primers used for sequencing.

Name	Sequence	Reference
NC5 F	5¢-GTAGGTGAACCTGCGGAAGGAT CATT-3¢	Gasser *et al.*, 1993
NC2 R	5¢- TTAGTTTCTTTTCCTCCGCT-3¢	Gasser *et al.*, 1993
NC1 F	5¢- ACGTCTGGTTCAGGGTTGTT-3¢	Gasser *et al.*, 1993
NC13 F	5¢- ATCGATGAAAAACGCAGC-3¢	Modified (this study)
NC13 R	5¢- GCTGCGTTTTTCATCGAT-3¢	Modified (this study)
M13 F	5¢- GTAAAACGACGGCCAG-3¢	Invitrogen
M13 R	5¢- CAGGAAACAGCTATGAC-3¢	Invitrogen

Definition of variability levels: Intra-individual variability was defined as sequence differences among the three clones obtained from a single parasite specimen. Intraspecific variability was defined as sequence differences among clones from different individuals of the same species. Interspecific variability refers to sequence differences between species.

Sequence analysis and phylogeny: Sequencing products were analysed using the ABI PRISM 310 Genetic Analyser (PE Biosystems, US). Sequences were checked and processed with the Chromas v1.45 programme (Technelysium Pty Ltd, Australia). For the preparation of alignments and consensus sequences, the ClustalW v1.4 method from the BioEdit v5.0.9 programme package (Tom Hall, Department of Microbiology, NCSU) was used. The same programme package was also used to determine the percentage of G+C content and intraspecies matching. All obtained sequences were submitted to and published in GenBank (NCBI, USA) under the accession numbers listed in Table 2. Comparison sequences were selected from GenBank (NCBI, USA), aligned, and trimmed using the Geneious Prime Version 2025.0.3 Software Suite (Biomatters, New Zealand) with default parameters. The MEGA11 version 11.0.13 programme was used for phylogenetic analysis, and the trees were constructed using the maximum likelihood method with the Tamura 3-parameter model and bootstrapping with 1000 replicates.

RESULTS

Determination of adult parasites: Five different strongyle species were determined based on morphological features of adult parasites. These species were *Trichostrongylus axei, Trichostrongylus colubriformis, Teladorsagia circumcincta, Cooperia curticei* and *Chabertia ovina* (Fig. 1) (Barth and Visser, 1991).

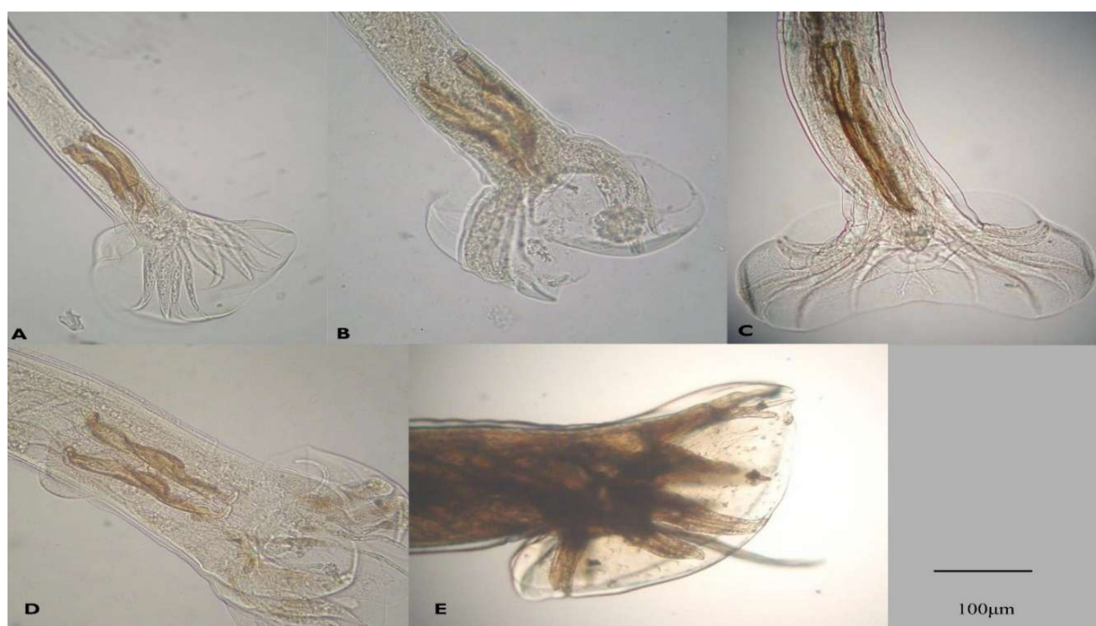

Fig. 1: Bursa copulatrix of various strongyle species. (A) *T. axei.* (B) *T. colubriformis*, (C) *T. circumcincta,* (D) *C. curticei,* (E) *C. ovina.*

Sequencing results of the ITS-5,8S-ITS2 region, start of 28S region of ribosomal DNA and cloning: By amplifying genomic DNA extracted from adult parasites, PCR products approximately 850bp in length were obtained. Fresh PCR products were used for cloning. Table 2 presents the clones obtained from five different strongyle species.

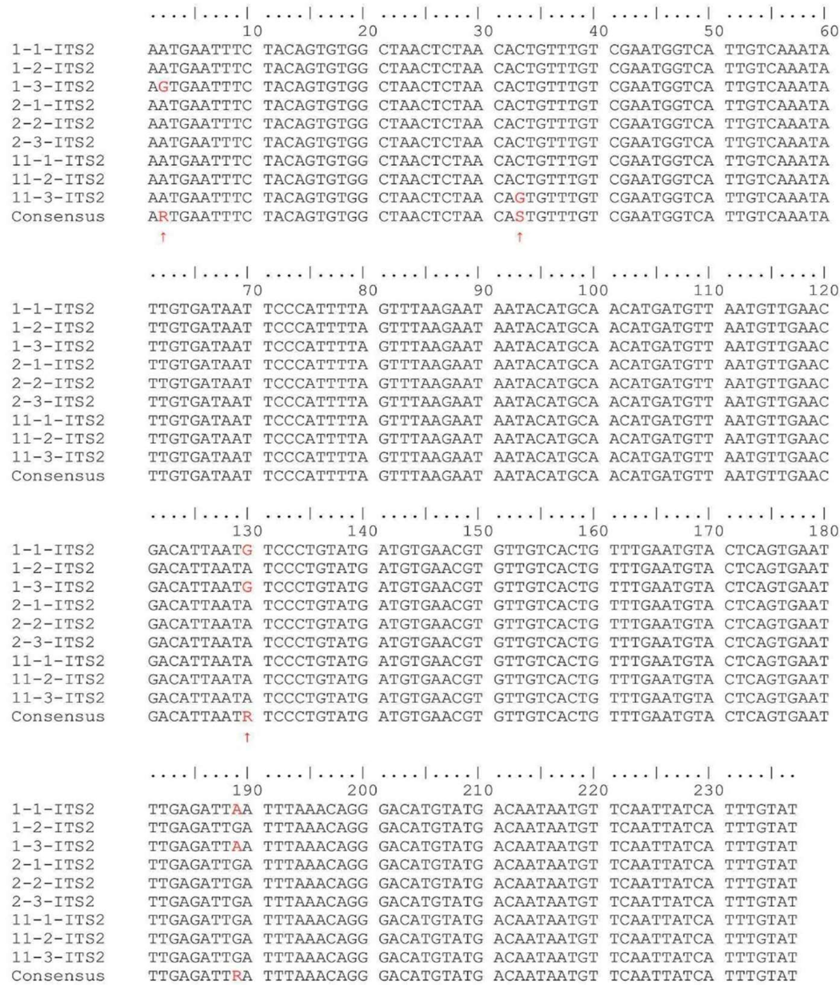

Fig. 2: The ITS2 sequences alignment from nine obtained *T. axei* clones from three individual adult parasites. The mutation sites are marked with red arrow under the consensus sequence.

Table 2: List of clones from which ITS2 sequences were obtained.

Consecutive no.	Parasite no.	Parasite species	Clone mark	Accession no.	Consecutive no.	Parasite no.	Parasite species	Clone mark	Accession no.
1	11	*T. axei*	1-1	PV346002	25	26	*T. colubriformis*	19-2	PV356714
2	11	*T. axei*	1-2	PV346003	26	26	*T. colubriformis*	19-3	PV356715
3	11	*T. axei*	1-3	PV346004	27	26	*T. colubriformis*	19-4	PV356716
4	14	*T. axei*	2-1	PV346005	28	16	*C. curticei*	12-1	PV345993
5	14	*T. axei*	2-2	PV346006	29	16	*C. curticei*	12-2	PV345994
6	14	*T. axei*	2-3	PV346007	30	16	*C. curticei*	12-3	PV345995
7	15	*T. axei*	11-1	PV346008	31	18	*C. curticei*	13-1	PV345996
8	15	*T. axei*	11-2	PV346009	32	18	*C. curticei*	13-2	PV345997
9	15	*T. axei*	11-3	PV346010	33	18	*C. curticei*	13-3	PV345998
10	2	*T. circumcincta*	3-1	PV364283	34	19	*C. curticei*	16-2	PV345999
11	2	*T. circumcincta*	3-2	PV364284	35	19	*C. curticei*	16-3	PV346000
12	2	*T. circumcincta*	3-3	PV364285	36	19	*C. curticei*	16-4	PV346001
13	6	*T. circumcincta*	4-1	PV364286	37	30	*C. ovina*	9-2	PV346011
14	6	*T. circumcincta*	4-2	PV364287	38	30	*C. ovina*	9-3	PV346012
15	6	*T. circumcincta*	4-3	PV364288	39	30	*C. ovina*	20-1	PV346013
16	7	*T. circumcincta*	5-1	PV364289	40	31	*C. ovina*	10-2	PV346014
17	7	*T. circumcincta*	5-2	PV364290	41	31	*C. ovina*	10-3	PV346015
18	7	*T. circumcincta*	5-4	PV364291	42	31	*C. ovina*	21-1	PV346016
19	23	*T. colubriformis*	6-4	PV356708	43	32	*C. ovina*	15-2	PV346017
20	23	*T. colubriformis*	17-2	PV356709	44	32	*C. ovina*	15-3	PV346018
21	23	*T. colubriformis*	17-3	PV356710	45	32	*C. ovina*	15-4	PV346019
22	24	*T. colubriformis*	7-3	PV356711					
23	24	*T. colubriformis*	18-2	PV356712					
24	24	*T. colubriformis*	18-3	PV356713					

***Trichostrongylus axei*:** The ITS2 ribosomal DNA sequences of all nine *T. axei* clones obtained were 237bp in length. The G+C content ranged from 31.22 to 31.65% (Fig. 2). The percentage of intraspecies identity between the ITS2 sequences of *T. axei* ranged from 98.3 to 100.0%. Some closely related sequences from GenBank include those from cattle in Cameroon (MN709032), sheep in New Zealand (KC998724, Bisset *et al.*, 2014), and a human in Thailand (KC337066, Phosuk *et al.*, 2013), all of which share 100% nucleotide identity in the ITS2 region with sequences from Slovenia (**Fig. 3**). A polymorphic site in *T. axei* sequence was identified at position 130. Furthermore, polymorphisms were discovered at positions 2, 33, and 189. At position 2, either nucleotide A or G was present; at position 33, either C or G; and at position 189, either G or A. Polymorphic sites at positions 130 and 189 were observed in two sequenced clones, while sites 2 and 33 were found in one clone each. Intra-individual variability in the ITS2 region was observed across several strongyle species, ranging from 0 to 2.6%. Intraindividual variability for the ITS2 region in *T. axei* in our study ranged from 0 to 1.3%, while intraspecies variability was 1.7%.

Fig. 3: The phylogenetic comparison of 22 sequences of ITS2 from ***T. axei*** from GenBank of which nine sequences from Slovenia were determined in this study (marked with bold text). The sequences are named by organism name, GenBank accession number, host and country. The maximum likelihood tree was computed based on the T-92+G model with a bootstrap support of 1000 repetitions.

Trichostrongylus colubriformis: All clones obtained by cloning the rDNA of the *T. colubriformis* species shared 100% identity in the ITS2 region. The sequence length was 238bp, and the G+C content was 31.9%. The most closely related ITS2 sequences from GenBank are from a human host in Laos (KC337064, Phosuk *et al.*, 2013), a human in Thailand (KC337065, Phosuk *et al.*, 2013), and a sheep in New Zealand (KC998728, Bisset *et al.*, 2014), all of which share 100% nucleotide identity in the ITS2 region with the Slovenian sequences (Fig. 4).

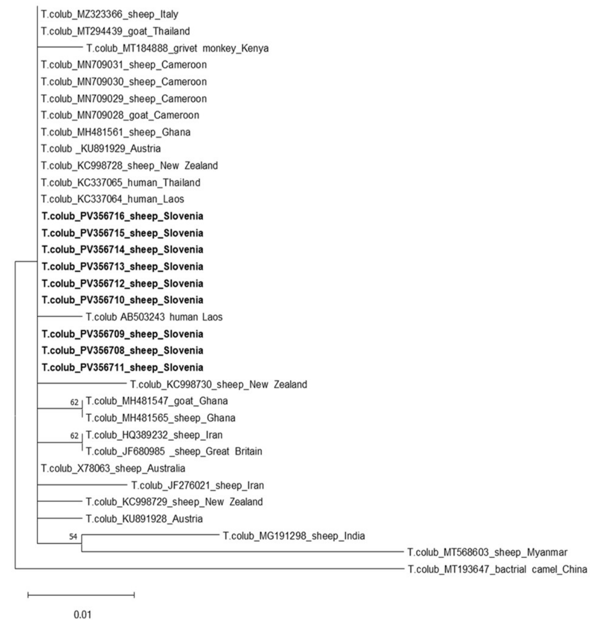

Fig. 4: The phylogenetic comparison of 34 sequences of ITS2 from *T. colubriformis* from GenBank of which nine sequences from Slovenia were determined in this study (marked with bold text). The sequences are named by organism name, GenBank accession number, host and country. The maximum likelihood tree was computed based on the T-92+G model with a bootstrap support of 1000 repetitions.

***Teladorsagia circumcincta*:** In clones obtained from *T. circumcincta*, the ITS2 sequences were 246bp long, except for clone 3-2, which had a 248bp long ITS2 sequence. The G+C content ranged from 33.47% to 34.55% (Fig. 5). In our study, nine polymorphic sites were identified in the ITS2 region. Intraindividual variability reached up to 2.1%, and intraspecies variability up to 2.9%. The percentage of intraspecies identity between the ITS2 sequences of *T. circumcincta* varied from 97.1% to 100.0%. Some of the most closely related sequences from GenBank to the Slovenian sequences include a sequence from a goat host from China (100% nucleotide identity, MT193650), from a Soay sheep host from Scotland (99.59% nucleotide identity, AY439025) (Wimmer *et al.*, 2003), and from a sheep host from Cyprus (99.59% nucleotide identity, DQ354330) (Liénard *et al.*, 2006) (Fig. 6).

Cooperia curticei: The length of the ITS2 fragment was 242bp (Fig. 7). The G+C content ranged from 30.17 to 32.23%. The percentage of intraspecies identity between the ITS2 sequences of *C. curticei* varied from 96.2 to 100.0%. The intraspecies variability in *Cooperia curticei* in our study was found reaching up to 3.8%. Thirteen polymorphic sites were identified in this species. Some closely related sequences from GenBank to the Slovenian sequences include sequence KU891909 from Austria (97.11% nucleotide identity), a sequence from a sheep host in New Zealand (99.17% nucleotide identity, KC998735) (Bisset *et al.*, 2014), and a sequence from a sheep host in Ghana (98.35%, MH481604) (Fig. 8).

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               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       10         20         30         40         50         60
3-1-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--T ATAACACTGT TTGTCGAATG GCATTTATCA
3-2-ITS2       AATGAAACTA CTACAGTGTG GCTAACACAT ATAACACTGT TTGTCGAATG GCATTTATCA
3-3-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--T ATAACACTGT TTGTCGAATG GCATTTATCA
4-1-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--T ATAACACTGT TTGTCGAATG GCATTTATCA
4-2-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--T ATAACACTGT TTGTCGAATG GCATTTATCA
4-3-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--A ATAACACTGT TTGTCGAATG GCATTTATCA
5-1-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--T ATAGCACTGT TTGTCGAATG GCATTTATCA
5-2-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--T ATAACACTGT TTGTCGAATG GCATTTATCA
5-4-ITS2       AATGAAACTA CTACAGTGTG GCTAACA--T ATAACACTGT TTGTCGAATG GCATTTATCA
CONSENSUS      AATGAAACTA CTACAGTGTG GCTAACACAW ATARCACTGT TTGTCGAATG GCATTTATCA
                                             ↑↑↑    ↑

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       70         80         90        100        110        120
3-1-ITS2       CTTTATCGTG GTAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG ACGTACGACG
3-2-ITS2       CTTTATTGTG GTAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAATATG ACGTACGACG
3-3-ITS2       CTTTATCGTG GTAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG ACGTACGACG
4-1-ITS2       CTTTATCGTG ATAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG ACGTACGACG
4-2-ITS2       CTTTATCGTG ATAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG ACGTACGACG
4-3-ITS2       CTTTATTGTG GTAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG GCGTACGACG
5-1-ITS2       CTTTATTGTG ATAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG ACGTACGACG
5-2-ITS2       CTTTATTGTG ATAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG ACGTACGACG
5-4-ITS2       CTTTATTGTG ATAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAACATG ACGTACGACG
CONSENSUS      CTTTATYGTG RTAATTCCCA TTTCAGTTCA AGAATAACAT ATGCAAYATG RCGTACGACG
                     ↑    ↑                                     ↑    ↑

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      130        140        150        160        170        180
3-1-ITS2       GTATTACCGT CGTAACGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
3-2-ITS2       GTATTACCGT CGTAACGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
3-3-ITS2       GTATTACCGT CGTAACGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
4-1-ITS2       GTATTACCGT CGTAACGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
4-2-ITS2       GTATTACCGT CGTAACGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
4-3-ITS2       GTATTACCGT CGTAACGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
5-1-ITS2       GTATTACCGT CGTAGCGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
5-2-ITS2       GTATTACCGT CGTAGCGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
5-4-ITS2       GTATTACCGT CGTAGCGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
CONSENSUS      GTATTACCGT CGTARCGTTC CTGAATGATA TGAACGCGTA TTGCTACTAT TTGAATGTAC
                              ↑

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      190        200        210        220        230        240
3-1-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
3-2-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
3-3-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
4-1-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
4-2-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
4-3-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATACATGT TCAATGTATC
5-1-ITS2       TCAATGAACA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
5-2-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
5-4-ITS2       TCAATGAATA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATATATGT TCAATGTATC
CONSENSUS      TCAATGAAYA TGAGATTGAT TCAGATAGAG ACATGTATGG TAATAYATGT TCAATGTATC
                       ↑                                       ↑

               ....|....|
                      250
3-1-ITS2       ATTTGTAT
3-2-ITS2       ATTTGTAT
3-3-ITS2       ATTTGTAT
4-1-ITS2       ATTTGTAT
4-2-ITS2       ATTTGTAT
4-3-ITS2       ATTTGTAT
5-1-ITS2       ATTTGTAT
5-2-ITS2       ATTTGTAT
5-4-ITS2       ATTTGTAT
CONSENSUS      ATTTGTAT
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Fig. 5: The ITS2 sequences alignment from all nine obtained *T. circumcincta* clones from three individual adult parasites. The mutation sites are marked with red arrow under the consensus sequence.

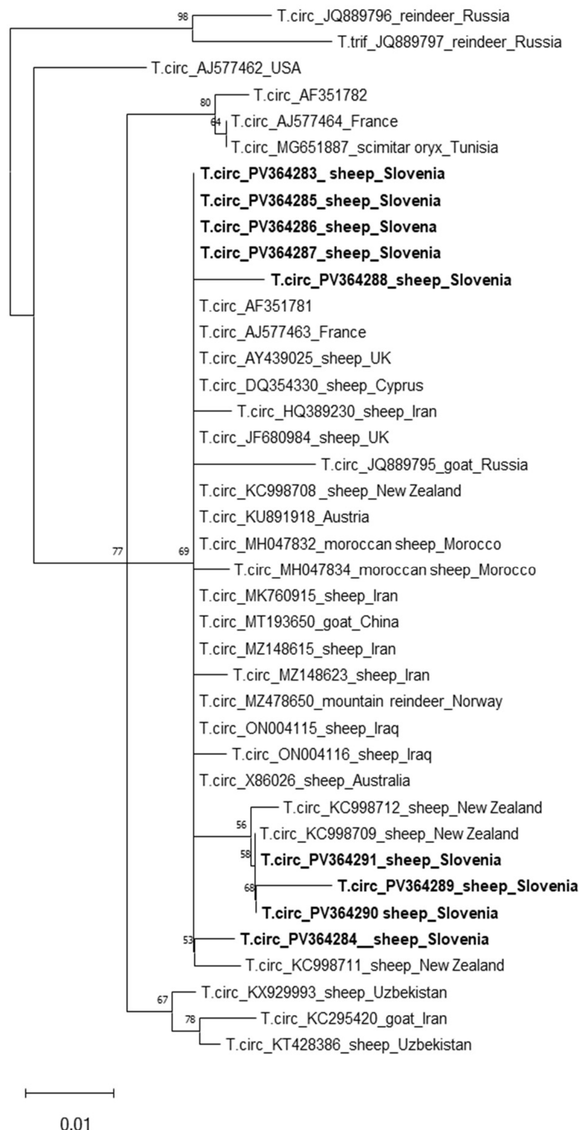

Fig. 6: The phylogenetic comparison of 40 sequences of ITS2 from ***T. circumcincta*** from GenBank of which nine sequences from Slovenia were determined in this study (marked with bold text). The sequences are named with organism name, GenBank accession number, host and country. The maximum likelihood tree was computed based on the T-92+G model with a bootstrap support of 1000 repetitions.

***Chabertia ovina*:** The length of the ITS2 sequences was 233bp for five clones and 235bp for four clones. The G+C content ranged from 44.26% to 45.06% (Fig. 9). The percentage of intraspecies identity between the ITS2 sequences of *C. ovina* varied from 97.4 to 100.0%. In our study, the 233bp sequences exhibited a deletion of two nucleotides at positions 142 and 143. Additional polymorphic sites were found at positions 56, 60, 62, and 80. Intraindividual and intraspecies variability in the ITS2 nucleotide sequences in our study was up to 2.6%. Some closely related sequences from GenBank to the Slovenian sequences include sequence KC998759 from a sheep host in New Zealand (up to 97.87%) (Bisset *et al.*, 2014), from a goat host in China (up to 98.72%, KF913471) (Liu *et al.*, 2014), and from a goat host in Tunisia (up to 98.28%, KY930445) (Said *et al.*, 2018) (Fig. 10).

DISCUSSION

Advancements in molecular biology are crucial for the diagnosis of animal and human diseases. Many new molecular methods for diagnosing animal diseases exist, but currently, many are too costly for routine use in veterinary medicine. To establish molecular diagnostics in veterinary parasitology, specific genetic markers are required to distinguish between morphologically similar specimens. In this study, ITS2 ribosomal DNA genetic markers were identified in gastrointestinal strongyles of sheep. Intra-individual variability in the ITS2 region was observed across several strongyle species, ranging from 0% to 2.6%. This variation is consistent with the multicopy nature of ribosomal DNA, where multiple ITS2 variants may exist within a single genome. Although some polymorphisms were detected among clones from the same individual, these did not hinder species-level identification. The high sequence similarity among intraindividual clones, particularly in *T. colubriformis* and *T. axei*, indicates that ITS2 remains a reliable molecular marker, even in the presence of minor sequence heterogeneity. Future applications of this marker should consider such variability when designing diagnostic or phylogenetic tools. Intraindividual variability for the ITS2 region in *T. axei* in our study ranged from 0 to 1.3%, while intraspecies variability was 1.7%. In our study, all clones obtained from *T. colubriformis* had identical nucleotide sequences (100% identity) in the ITS2 region. Based on our research, we conclude that intraspecies variability for the ITS2 region in *T. colubriformis* was very low. Intraindividual variability in *Teladorsagia circumcincta* reached up to 2.1%, and intraspecies variability up to 2.9%. Based on our findings, both intraindividual and intraspecific variability in *T. circumcincta* are higher than in *Trichostrongylus* species. The polymorphisms demonstrate the presence of minor ITS2 variants within individual parasites, consistent with the multicopy and slightly heterogeneous nature of ribosomal DNA arrays. The intraindividual variation is commonly reported in nematodes and has been shown not to affect the robustness of molecular identification. In our study, clones from the same specimen showed only minor sequence variation and consistently grouped with their species in phylogenetic trees. Therefore, the ITS2 region remains a reliable molecular marker for species-level resolution, even in the presence of some intraindividual heterogeneity. The highest intraspecies variability in our study was found in *Cooperia curticei*, reaching up to 3.8%. In *Chabertia ovina* intraindividual and intraspecies variability in the ITS2 nucleotide sequences in our study was up to 2.6%. Based on our research, it can be concluded that intraspecific variability in the ITS2 region is higher than in *Trichostrongylus* species and lower than in *T. circumcincta* and *C. curticei*.

Slovenia's strongyle populations, which infect locally adapted breeds, may harbour unique ITS2 haplotypes or cryptic species not yet represented in global repositories. For example, the first complete ITS2 sequences of *Strongylus vulgaris* and *S. edentatus* were only recently published, highlighting persistent gaps even for well-studied species (Halvarsson *et al.*, 2023). Slovenian samples could similarly help resolve ambiguities in

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                   10         20         30         40         50         60
12-1-ITS2 AACGATATAC TACAGTGTGG CTAACGTTTT AACGCTGTCT GTCTAGTGGC ATTTGTCTAC
12-2-ITS2 AACGATATAC TACAGTGTGG CTAAACTTTT AACACTGTCT GTCTAATGGC ATTTGTCTAC
12-3-ITS2 AACAATATAC TACAGTGTGG CTAACGTTTT AACACTGTCT GTCTAATGGC ATTTGTCTAC
13-1-ITS2 AACGATATAC TACAGTGTGG CTAACGTTCA AACACTGTCT GTCTAATGGC ATTTGTCTAC
13-2-ITS2 AACGATATAC TACAGTGTGG CTAAACTTTT AACACTGTCT GTCTAATGGC ATTTGTCTAC
13-3-ITS2 AACAATATAC TACAGTGTGG CTAACGTTTT AACACTGTCT GTCTAATGGC ATTTGTCTAC
16-2-ITS2 AACGATATAC TACAGTGTGG CTAACGTTCA AACACTGTCT GTCTAATGGC ATTTGTCTAC
16-3-ITS2 AACGATATAC TACAGTGTGG CTAACGTTTT AACACTGTCT GTCTAATGGC ATTTGTCTAC
16-4-ITS2 AACGATATAC TACAGTGTGG CTAACGTTTA AACACTGTCT GTCTAATGGC ATTTGTCTAC
CONSENSUS AACRATATAC TACAGTGTGG CTAAMSTTYW AACRCTGTCT GTCTAATGGC ATTTGTCTAC

                   70         80         90        100        110        120
12-1-ITS2 ATTGGTTCTG ACAAATCCCA TTATAGTTCA AGAATAACAT ATGCAACATA ATGTTTGTAT
12-2-ITS2 ATTGGTTCTG ACAAATCTCA TTATAGTTCA AGAATAACAT GTGCAACATA ATGTTCGTAT
12-3-ITS2 ATTGGTTCTG ACAAATCCCA TTATAGTTCA AGAATAACAT ATGCAACATA ATGTTCGTAT
13-1-ITS2 ATTGGTTCTG ACAAATCCCA TTATAGTTCA AGAATAACAT ATGCAACATA ATGTTCGTAT
13-2-ITS2 ATTGGTTCTG ACAAATCTCA TTATAGTTCA AGAATAACAT GTGCAACATA ATGTTCGTAT
13-3-ITS2 ATTGGTTCTG ACAAATCCCA TTATAGTTCA AGAATAACAT ATGCAACATA ATGTTCGTAT
16-2-ITS2 ATTGGTTCTG ACAAATCCCA TTATAGTTCA AGAATAACAT ATGCAACATA ATGTTCGTAT
16-3-ITS2 ATTGGTTCTG ACAAATCCCA TTATAGTTCA AGAATAACAT ATGCAACATA ATGTTCGTAT
16-4-ITS2 ATTGGTTCTG ACAAATCCCA TTATAGTTCA AGAATAACAT ATGCAACATA ATGTTCGTAT
CONSENSUS ATTGGTTCTG ACAAATCYCA TTATAGTTCA AGAATAACAT RTGCAACATA ATGTTYGTAT

                  130        140        150        160        170        180
12-1-ITS2 AGTAATATGA ACATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAAT GTACTCAATG
12-2-ITS2 AGTAATATGA ACATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAAT GTACTCAATG
12-3-ITS2 AGTAATACGA ACATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAGT GTACTCAATG
13-1-ITS2 AGTAATATGA ACATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAGT GTACTCAATG
13-2-ITS2 AGTAATATGA ACATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAAT GTACTCAATG
13-3-ITS2 AGTAATACGA ACATTTCTGA ATGGTATGAA TCTATTGTCA CTGTTTAAGT GTACTCAATG
16-2-ITS2 AGTAATATGA ACATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAGT GTACTCAATG
16-3-ITS2 AGTAATATGA ATATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAGT GTACTCAATG
16-4-ITS2 AGTAATATGA ACATTTCTGA ATGGTATGAA TCTATTATCA CTGTTTAAGT GTACTCAATG
CONSENSUS AGTAATAYGA AYATTTCTGA ATGGTATGAA TCTATTRTCA CTGTTTAART GTACTCAATG

                  190        200        210        220        230        240
12-1-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT TGGAGATAGT GGTATTCGAT TATCATTTGT AT
12-2-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT TGGAGATAGT AGTATTCAAT TATCATTTGT AT
12-3-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT TGGAGATAGT AGTATTCGAT TATCATTTGT AT
13-1-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT CGGAGATAGT AGTATTCGAT TATCATTTGT AT
13-2-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT TGGAGATAGT AGTATTCAAT TATCATTTGT AT
13-3-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT TGGAGATAGT AGTATTCGAT TATCATTTGT AT
16-2-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT CGGAGATAGT AGTATTCGAT TATCATTTGT AT
16-3-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT TGGAGATAGT AGTATTCGAT TATCATTTGT AT
16-4-ITS2 AATATGAGAT TGATTTAAAC AGGGACATGT CGGAGATAGT AGTATTCGAT TATCATTTGT AT
CONSENSUS AATATGAGAT TGATTTAAAC AGGGACATGT YGGAGATAGT AGTATTCRAT TATCATTTGT AT
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Fig. 7: The ITS2 sequences alignment from all nine obtained *C. curticei* clones from three individual adult parasites. The mutation sites are marked with red arrow under the consensus sequence.

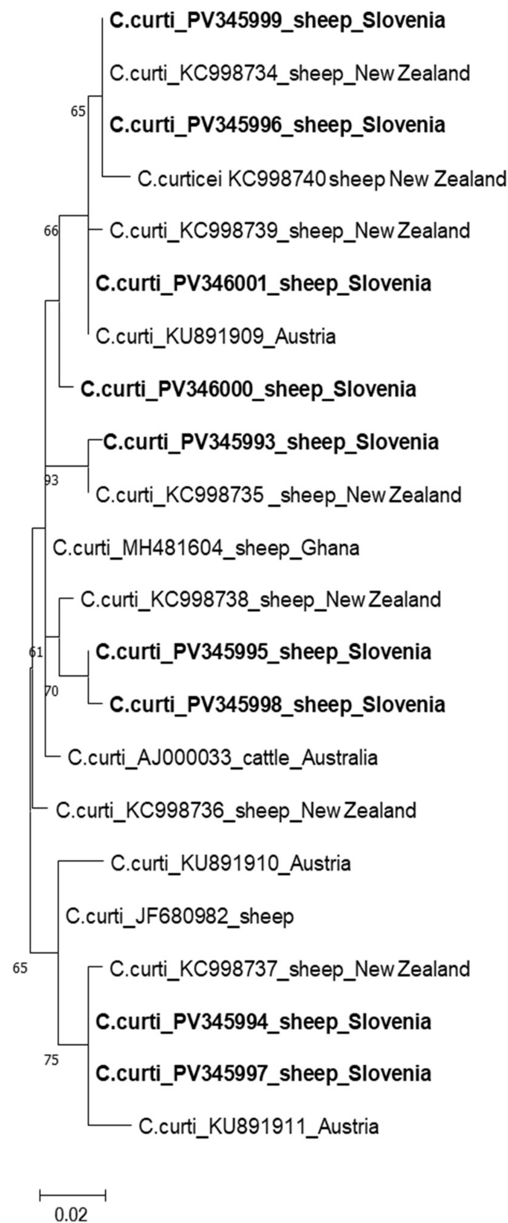

Fig. 8: The phylogenetic comparison of 22 sequences of ITS2 from ***C. curticei*** from GenBank of which nine sequences from Slovenia were determined in this study (marked with bold text). The sequences are named with organism name, GenBank accession number, host and country. The maximum likelihood tree was computed based on the T-92+G model with a bootstrap support of 1000 repetitions.

existing phylogenetic trees, particularly for clades that remain poorly defined due to incomplete or regionally biased data. Slovenia's smallholder farming systems, which prioritise sustainable practices, provide a unique context to study the early emergence of anthelmintic resistance. The management of local sheep breeds often involves rotational grazing and limited drug use. ITS2-based monitoring of Slovenian strongyles could detect novel resistance-associated mutations or gene expression profiles before they become widespread, as demonstrated in recent nemabiome studies on sheep farms in Europe (Krücken *et al.*, 2024). Additionally, zoonotic parasites such as *Haemonchus contortus* and *Trichostrongylus spp.*, which are prevalent in Slovenian sheep, pose cross-species transmission risks. Molecular characterization of their ITS2 regions aids in identifying genetic markers linked to host switching, informing public health strategies to mitigate spillover events.

This study has several limitations that should be acknowledged. First, the host sample comprised only four sheep from a single herd; however, the primary experimental unit was the individual parasite, with three clones sequenced from three individual parasites of each species (45 sequences total). This design was appropriate for characterising ITS2 variability at intra-individual and intraspecific levels and is consistent with comparable published studies (Gasser *et al.*, 1993; Bisset *et al.*, 2014). The results should nonetheless be regarded as preliminary, and future studies with larger and more geographically diverse datasets are warranted. Second, only adult parasites collected post-mortem were analysed. Third, the study relied on a single genetic marker; use of additional markers in future work would allow more comprehensive phylogenetic resolution. Future studies should also consider increasing the number of clones per individual to better capture intra-individual ITS2 variability, particularly for species with higher polymorphism rates such as *Cooperia curticei*. Furthermore, the application of next-generation sequencing approaches such as ITS2-based nemabiome metabarcoding, which has been employed to characterise mixed strongyle infections in various host species including horses (Hamad *et al.*, 2024), would complement cloning-based methods by enabling simultaneous species-level quantification from faecal samples without the need for adult parasite recovery. *Ostertagia* and *Trichostrongylus*, which frequently co-occur (Desalegn *et al.*, 2023), may require further investigation to better understand their genetic diversity. Improved diagnostic accuracy supports targeted treatment regimens, reducing drug use and delaying resistance. Globally, shared ITS2 data enables real-time tracking of invasive species or drug-resistant strains migrating through livestock trade, as demonstrated by the transboundary spread of *S. vulgaris* haplotypes across Europe and Asia (Halvarsson *et al.*, 2023). The economic importance of the local sheep breed in Slovenia, valued for meat, milk and wool, makes parasite control a priority for rural livelihoods. ITS2-based research tailored to local strongyle populations provides farmers with precise tools for monitoring infections. For instance, semi-quantitative PCR assays using Slovenian-derived ITS2 primers could quantify parasite loads in faecal samples more accurately than faecal egg counts, which do not differentiate species. This enables farmers to administer anthelmintics only when necessary, aligning with EU mandates for sustainable agriculture. Furthermore, identifying Slovenian-specific ITS2 markers could inform breeding programmes to enhance innate resistance in local sheep breeds, reducing reliance on chemicals. Therefore, the results should be interpreted with caution and considered as preliminary, particularly with respect to broader epidemiological conclusions.

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              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     10         20         30         40         50         60
9-2-ITS2      TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACACAAAA
9-3-ITS2      TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
20-1-ITS2     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
10-2-ITS2     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
10-3-ITS2     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
21-1-ITS2     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
15-2-ITS2     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
15-3-ITS2     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
15-4-ITS2     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACATAAAT
CONSENSUS     TATATAGTTA CTACAGTGTG GCTTGTGACA CTGTTTGTCG TACGACGCTT GCACAYAAAW
                                                                          ↑   ↑

              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     70         80         90        100        110        120
9-2-ITS2      ATTTCACATG TGTGATCCTC GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
9-3-ITS2      ACTTCACATG TGTGATCCTC GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
20-1-ITS2     ACTTCACATG TGTGATCCTT GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
10-2-ITS2     ACTTCACATG TGTGATCCTT GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
10-3-ITS2     ACTTCACATG TGTGATCCTT GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
21-1-ITS2     ACTTCACATG TGTGATCCTC GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
15-2-ITS2     ACTTCACATG TGTGATCCTC GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
15-3-ITS2     ACTTCACATG TGTGATCCTC GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
15-4-ITS2     ACTTCACATG TGTGATCCTC GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
CONSENSUS     AYTTCACATG TGTGATCCTY GTACTAGATA AGAAATATAT TGCAACAGGT ACCTTGGAGC
               ↑                  ↑

              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    130        140        150        160        170        180
9-2-ITS2      AATCCAAGGT GCACGGATGT GTGATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
9-3-ITS2      AATCCAGGGT GCACGGATGT G--ATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
20-1-ITS2     AATCCAAGGT GCACGGATGT GTGATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
10-2-ITS2     AATCCAAGGT GCACGGATGT GTGATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
10-3-ITS2     AATCCAAGGT GCACGGATGT GTGATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
21-1-ITS2     AATCCAAGGT GCACGGATGT G--ATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
15-2-ITS2     AATCCAAGGT GCACGGATGT G--ATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
15-3-ITS2     AATCCAAGGT GCACGGATGT G--ATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
15-4-ITS2     AATCCAAGGT GCACGGATGT G--ATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
CONSENSUS     AATCCARGGT GCACGGATGT GTGATGACCT CGTTGTCACC GTGAAAGCGT TCAGCGACTA
                    ↑                 ↑↑

              ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    190        200        210        220        230        240
9-2-ITS2      AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
9-3-ITS2      AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
20-1-ITS2     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
10-2-ITS2     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
10-3-ITS2     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
21-1-ITS2     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
15-2-ITS2     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
15-3-ITS2     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
15-4-ITS2     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
CONSENSUS     AGAATGCTTT GGCGGGGCTT GTTTGACAAC GGTGTACGGT TCATGTCATT TGCAA
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Fig. 9: The ITS2 sequences alignment from all nine obtained *C. ovina* clones from three individual adult parasites. The mutation sites are marked with red arrow under the consensus sequence.

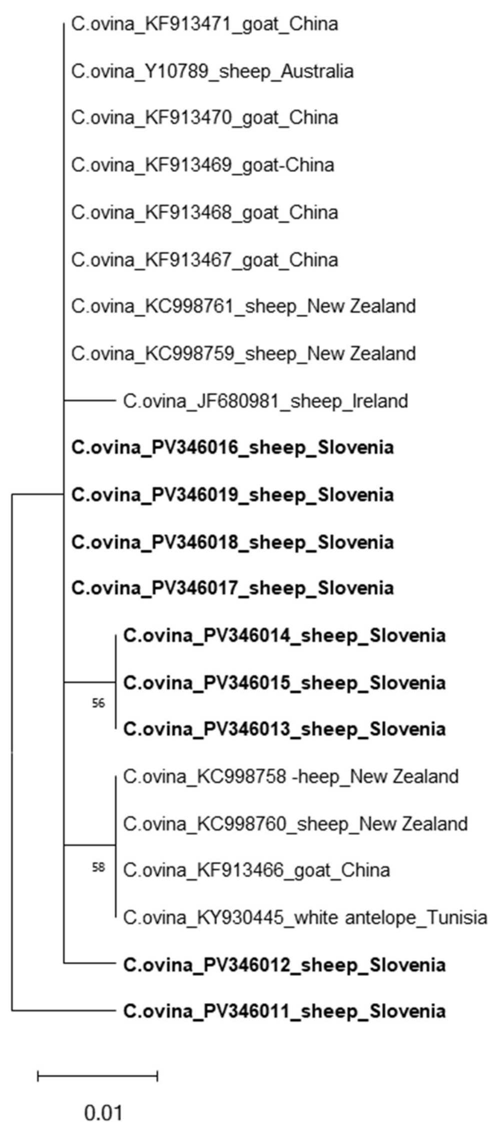

Fig. 10: The phylogenetic comparison of 22 sequences of ITS2 from ***C. ovina*** from GenBank of which nine sequences from Slovenia were determined in this study (marked with bold text). The sequences are named with organism name, GenBank accession number, host and country. The maximum likelihood tree was computed based on the T-92+G model with a bootstrap support of 1000 repetitions.

Conclusions: This study provides preliminary ITS2 sequence data for major gastrointestinal strongyle species in sheep in Slovenia and demonstrates that ITS2 is a suitable marker for species discrimination. Intra-individual variability was generally low and did not compromise species-level identification, while intraspecific variability differed among species but remained within ranges compatible with reliable molecular identification. Clear interspecific differences in sequence length, nucleotide composition, and polymorphic sites enabled consistent differentiation among the five analysed species. The 45 ITS2 sequences deposited in GenBank represent the first such reference dataset for Slovenian strongyle populations and provide a basis for future molecular epidemiology, metabarcoding, and diagnostic studies. Future work should extend sampling to multiple herds and geographic locations, evaluate additional genetic markers, and explore the application of ITS2-based tools for monitoring intraspecific diversity in Slovenian sheep populations.

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Conflict of Interest: The authors declare that there are no conflicts of interest related to the publication of this article.

Data Availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement: Samples were collected post-mortem from animals slaughtered as part of routine commercial slaughter. The animals were not killed specifically for the purposes of this study. Therefore, in accordance with applicable national legislation, no specific ethical approval was required.

Authors Contribution: JP conceived and designed the study. JP performed the experiments. LŠK analysed the data. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

Generative AI Statement: The authors declare that generative AI tools were used only for language editing and improvement of readability. The authors take full responsibility for the content of the manuscript.

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