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Morphological and molecular characterization of *Haemonchus contortus* isolated from the small ruminants of south Gujarat, India

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Summary

The successful design of strategic control measures against the blood-sucking gastrointestinal nematode, *Haemonchus contortus* in small ruminants can be facilitated by revealing its general features from morphology to the molecular level. In the south Gujarat region of India, a total of 2408 *H. contortus* were collected from 84 slaughtered sheep's abomasum, consisting of 347 males and 2061 females (1:6 ratio) ($p<0.05$). Furthermore, 726 *H. contortus* were collected from 61 goats, comprising 145 males and 581 females (1:4 ratio) ($p<0.05$). The male worms were approximately 12±0.06 mm long, while female worms were about 20±0.09 mm long. The vulvar morphotypes of the female worms were found to be 17.7% linguiform, 76.6 % knobbed/button ($p<0.05$), and 5.7 % smooth type, demonstrating common features of *H. contortus*. The nucleotide sequences of the Internal Transcribed Spacer 1 (ITS-1) of 165 bp or ITS-2 plus of 256 bp were aligned, and it was found that the genotypes of male and female specimens of either sheep or goat origin were identical, with a 100 % match. The present isolates shared >95 % and >94 % homology with published sequences of ITS-1 and ITS-2 plus of *H. contortus*, respectively, with more nucleotide transitions than transversions in the aligned sequences. The reconstructed phylogram of either ITS-1 or ITS-2 plus revealed two major clades, one for *H. contortus* and another for other nematodes, with *Haemonchus placei* showing its proximity with the clade of *H. contortus*. The study established the role of morphological and molecular features in identifying and differentiating *H. contortus* parasite at the local level.
Keywords: Small ruminants; *Haemonchus contortus*; Vulvar morphology; ITS; PCR; Molecular

Introduction

Metazoan parasites have undergone a series of morphological changes in order to survive in the diverse range of ecological niches available to them (Salle *et al*., 2019). The morphology of parasites is essential for their survival in the host and has a significant impact on the pathogenesis and clinical outcomes of the associated diseases (Poulin, 2010). The genetic composition of the parasite is ultimately responsible for determining its morphological features (Cotton *et al*., 2016). Accurate identification of the parasite at the species level is necessary for understanding the epidemiology, population biology, and treatment efficacy of parasitic diseases (Blaxter *et al*., 1998). Genus *Haemonchus* is a significant metazoan parasite of class nematoda that primarily infects ruminants such as sheep, goats, and cattle. The genus consists of several species, including *H. contortus*, *H. placei*, *H. similis*, and *H. longistipes*, which exhibit distinct morphological and genetic characteristics. Identifying the species of *Haemonchus* is difficult due

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to the overlap of morphological characters and genetic variation among populations (Arsenopoulos *et al*., 2021), but is essential to determine their habitat range within the host's gastrointestinal tract. For instance, *H. contortus* inhabits the abomasum, while *H. placei* and *H. similis* occur in the small intestine, and *H. longistipes* resides in the cecum and colon (Soulsby, 1982).

Haemonchus contortus is one of the economically important blood-sucking gastrointestinal nematodes of small ruminants worldwide, primarily in hot and humid conditions (Laha *et al*., 2001; Pal *et al*., 2014; Arsenopoulos *et al*., 2021; Muhammad *et al*., 2021). Its infection occurs through the ingestion of infective third-stage larvae (L3) from contaminated pastures. Once inside the host, the larvae migrate to the abomasum, where they develop into adult worms and begin feeding on the host's blood leading to anemia, hypoproteinemia, weight loss, diarrhoea, reduced productivity, and ultimately death if left untreated. Morphologically, *H. contortus* is characterized by its large size, twisted appearance, and characteristic spicule arrangement in males (Flay *et al*., 2022). Morphological parameters used to study male *Haemonchus* worms include body length (10 – 20 mm), cervical papillae length, spicule length, gubernaculum length, barb length, and cuticular ridges. Female *Haemonchus* worms have a "barber's pole" appearance, with white ovaries and uteri twisted around a red blood-filled intestine, while males are uniformly reddish-brown. Female *Haemonchus* species can be differentiated based on body length (18 – 30 mm), cervical papillae length, number of cuticular ridges, and vulva flap morphology represent the main criteria for identifying and differentiating female parasite species. Additionally, *Haemonchus* has a tooth or lancet in its poorly developed oral cavity, which helps perforate the gastric mucosa and suck blood (Gareh *et al*., 2021). The vulvar flap is a significant morphological feature of the female worm, serving as a morphological marker of ecological adaptation (Gharamah *et al*., 2011a, b; Badawy *et al*., 2015) and a means to understand the biology of *H. contortus* and determine the type of population present in a particular area (Gareh *et al*., 2021). Earlier, the identification and characterization of this parasite relied on morphological features, but more recently, molecular signatures of parasite genes have proven more effective for such studies (Bandyopadhyay *et al*., 2011). The ribosomal DNA (rDNA) cluster, a highly repeated multicopy gene family coding for the structural components of ribosomes, is ubiquitous in nature and contains variable regions flanked by more conserved regions (Hillis & Dixon, 1991). PCR amplification is enhanced by this gene family because many templates are available for initial priming, and primers can be designed to anneal the known conserved regions to amplify across unknown variable regions. This family is interrupted by variable external and internal transcribed spacer (ETS and ITS) regions. The internal transcribed spacer is the most conserved, exhibiting high interspecific sequence divergence and intraspecific sequence homogeneity (Hoste *et al*., 1998; Gasser, 2001; Tan *et al*., 2014). The metazoan parasites possess two internal transcribed spacers, ITS-1 and ITS-2, which occur between the 18S, 5.8S, and 28S coding regions and have emerged as the ideal choice for genetic studies at the species level (Torres-Machorro *et al*., 2010). Therefore, a combined approach of morphological and molecular study is adopted in the present study for the characterization of the *H. contortus*.

Materials and Methods

Parasite collection

The gut contents of 84 sheep and 61 goats were collected from the local abattoir in the Navsari district of south Gujarat, India. The abomasal content was collected by making a long incision along the greater curvature of the abomasum using a surgical blade, and the intestinal contents were collected by opening the small and large intestine with a sharp scissor. The collected contents were mixed with tap water and filtered through a sieve with a 250 µm aperture. The worms were collected from the filtrate by examining a small amount under a microscope in a petri-dish. The collected worms were washed in normal saline followed by phosphate buffered saline (PBS; pH=7.4) with ampicillin (100 mg/ml) and cloxacillin (50 mg/ml) antibiotics to prevent microbial growth. Following the morphological study conducted on the fresh samples, they were subsequently preserved in 70 % ethanol at -20°C for further molecular analysis.

Morphological characterization

The harvested worms were subjected to macroscopic and microscopic examination for morphological characterization. The microscopic examination was conducted by creating a temporary wet mount of the fresh worm specimen on a microscope slide. The nematodes were killed by gentle heat at an optimum temperature of 50°C, which was used for making temporary mounts. Briefly, a drop of water was placed in the center of the slide. The freshly killed nematode specimens were picked and placed in this water drop.

Table 1. Primers used for the amplification of the DNA of the nematode.

Name of gene	Name of primers	Primers sequence	Product length	References
ITS1	Forward	5'-TATGACATGAGCCGTTCGAG-3'	198 bp	Bandyopadhyay *et al*., 2011
	Reverse	5'-TGATCATTAAGGTTCCCCGA-3		
ITS2 plus	Forward	5'-ACGTCTGGTTCAGGGTTGTT-3'	350 bp	Stevenson *et al*., 1995
	Reverse	5'-TTAGTTTCTTTTCCTCCGCT-3'		

A coverslip was positioned to ensure no air bubble remained. These specimens could only be observed under the microscope for a few minutes until the water dried up. Subsequently, the specimen was observed under low magnification, and the magnification was increased to examine specific structures (http://ecoursesonline.iasri.res.in/mod/page/view.php?id=11697). *Haemonchus contortus* was identified based on standard body lengths of females (18 – 30 mm) and males (10 – 20 mm), as well as vulvar morphology, and typical morphological features of the anterior/ posterior region in males (Gareh *et al*., 2021). The vulvar process of female *Haemonchus* spp. was examined under a stereomicroscope and classified into linguiform (with a supra vulvar flap), knobbed/button (with a knob-like vulvar process), or smooth (without any vulvar process) vulvar morphotypes, following the descriptions by Rose (1966) and LeJambre and Whitlock (1968). Within the linguiform morphotype, female *Haemonchus* worms were further subclassified as linguiform A (with one cuticular inflation), linguiform B (without cuticular inflation), linguiform C (with two cuticular inflations), and linguiform I (with one cuticular inflation arising from the linguiform process), according to LeJambre and Whitlock (1968). The morphologically identified *H. contortus* specimens corresponded to the geographical isolate of Navsari, south Gujarat, India.

Molecular characterization

Nucleic acid amplification assay

Genomic DNA was isolated from freshly collected live *H. contortus* by triturating 50 mg of the nematodes in lysis buffer using a sterile pestle and mortar. The protocol for DNA extraction kit (Qiagen, Germany) was followed to extract the DNA, which was then stored at -20°C. Polymerase chain reaction (PCR) was performed to amplify the ITS-1 and ITS-2 plus primer flanking sequence of 198 bp and 350 bp, respectively, from the genomic DNA of the nematodes using the primer pairs listed in Table 1. Each 25 µL of PCR reaction contained 1 µL (25 – 50 ng) of DNA template, 1 µL (10 – 20 pM) of each primer, 12.5 µL of 2x Taq PCR master mix (2x buffer, 0.4 mM deoxynucleotide, and 4 mM $MgCl_2$) of Qiagen, Germany, and 9.5 µL nuclease-free water in a sterile 0.2 mL PCR tube. The standardized amplification conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min, final elongation at 72°C for 10 min, and storage at 4°C for an infinite period. After PCR, 5 – 10 µl of the reaction mixture was analyzed by electrophoresis on a 1 % agarose gel stained with ethidium bromide.

Nucleotide sequence analysis

For the purification of the desired PCR product consisting of ITS-1 and ITS-2 plus fragments of 198 bp and 350 bp, respectively, the gel extraction method was employed. A total volume of 150 µl of the PCR product was electrophoresed on a 1 % agarose gel and visualized under long-range UV light. Once the desired band was identified, it was carefully excised and transferred to a sterile 1.5 ml centrifuge tube. The DNA from the gel slice was then extracted using a gel extraction kit (Qiagen, Germany). The purified DNA was eluted in 30 µl of nuclease-free water. To determine the nucleotide sequence of the gel-extracted product, Sanger's di-deoxy chain termination method was utilized. This involved using the Dye terminator Cycle Sequencing Kit (Applied Biosystems Inc., USA) and an ABI DNA sequencer at the Sequencing Department of Eurofins Genomics India Pvt. Ltd., located in Bangalore-560 066,

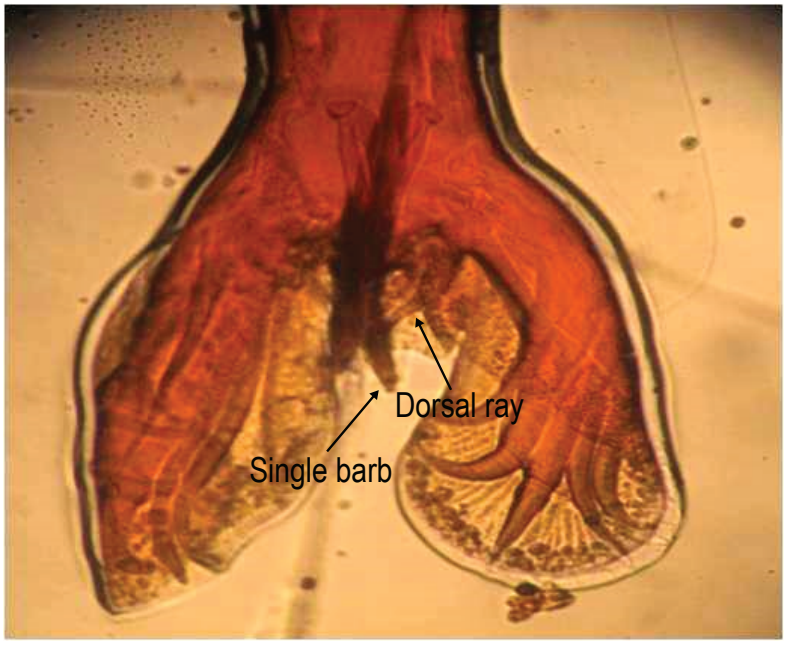


Fig. 1. Bursa of male *H. contortus* showing Y shaped dorsal ray and each spicule with a single barb at its distal tip.

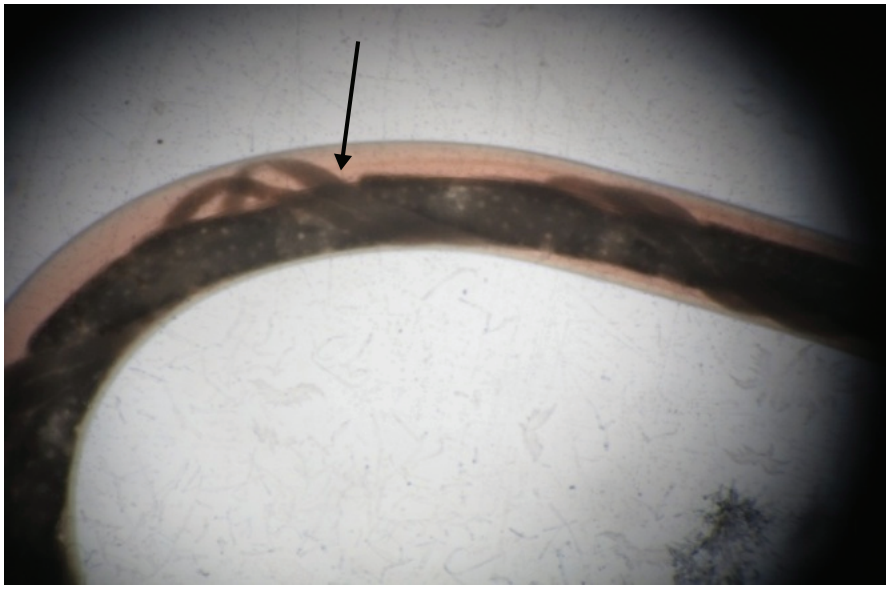


Fig. 2. Intertwining of red intestine and white ovary of *H. contortus* female

India. The newly generated raw sequence data were analyzed using the BioEdit program (version 7.2.6.1), and the chromatogram was inspected to ensure data quality (Sawadpanich *et al*., 2021). Initially, the 5' and 3' ends of the gene sequences were determined by comparing them with previously published sequences. The raw sequences were further analyzed using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST). Blast reports were compiled for subsequent analysis, following parameters such as the maximum score, total score, query coverage, percent identity, and E-value (Expectation value) (Zhang *et al*., 2000). These sequences were then utilized for the reconstruction of the phylogenetic tree.

Phylogenetic analysis

The phylogenetic relationship of the *Haemonchus* species was reconstructed based on ITS sequences of NCBI-GenBank of various nematodes in the Neighbor-Joining (NJ) method (maximum composite likelihood model) (Saitou & Nei, 1987). Multiple sequence alignments were performed using the ClustalW algorithm. Evolutionary analyses were conducted in the Molecular Evolutionary Genetics Analysis (MEGA) 10 (version 10.1.7) with a gap opening penalty of 15 and gap extension penalty of 6.66 in both pairwise or multiple alignments, respectively (Kumar *et al*., 2018). The tree stability was estimated by bootstrap analysis for 1000 replications (Felsenstein, 1985). *Taenia saginata* rDNA sequence of accession no. AY392045.1 was used as outgroup in the tree reconstructions (Tamura & Nei, 1993). All ambiguous positions were removed for each sequence pair (pairwise deletion option). The evolutionary distances were in the units of the number of base substitutions per site, and computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004).

Statistical analysis

Initial descriptive analyses and data comparisons were conducted for various parameters such as the number, length, width, weight of the worm, and vulvar morphotypes using Microsoft Excel (MS Office, 2010). To determine the differences between means, a single factor ANOVA was employed, followed by the application of the Duncan Multiple Range Test (DMRT). Statistical significance was defined as values with $p<0.05$. The OPSTAT software (Sheoran *et al*., 1998) was used to perform the statistical analysis.

Table 2. Distribution pattern of vulvar morphotypes of *H. contortus* in the small ruminants.

Host	Female worms (No.)		Vulvar morphotypes						p value
	Recovered	Examined	Linguiform		Knobbed		Smooth		
			No.	%	No.	%	No.	%	
Goat	2408	2061	342	16.6	1602	77.7	117	5.7	0.045
Sheep	730	581	125	21.5	423	72.8	33	5.7	0.05
Total	3138	2642	467	17.7	2025	76.6	150	5.7	0.05

Note: p value ≤0.05- Significant; p value > 0.05- Non-significant

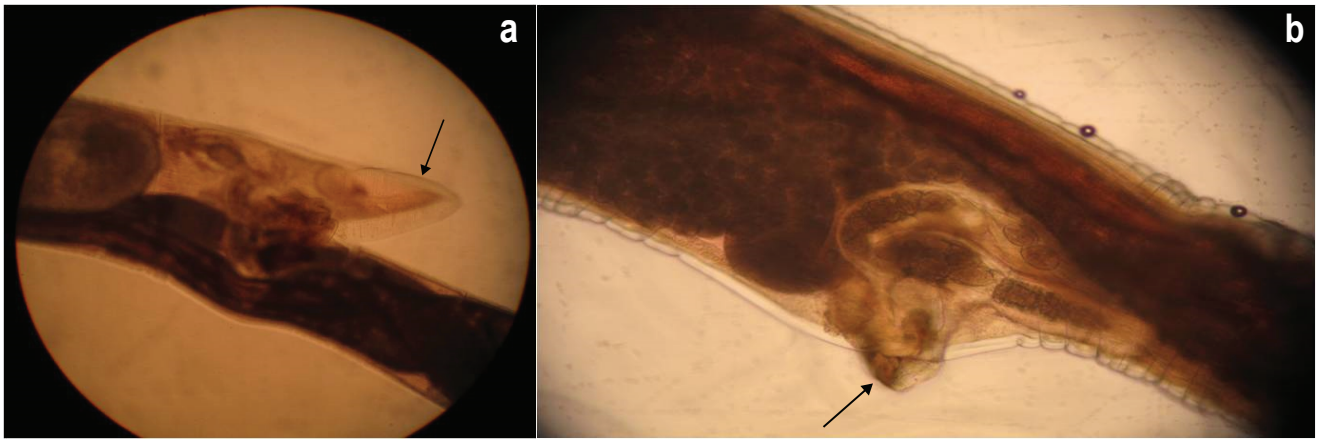


Fig. 3. a: Linguiform type B vulvar flap of *H. contortus* female; b: Linguiform type I vulvar flap of *H. contortus* female

Ethical Approval and/or Informed Consent

The ethical committee approval was not required, as the study was conducted using the gross specimens of the roundworm collected from the gut of slaughtered small ruminants.

Results

Morphological characterization

Upon macroscopic examination, the adult worms were predominantly observed in the fundus region of the abomasum. These worms exhibited a cylindrical-reddish appearance, which was attributed to their blood-feeding behaviour. A total of 347 male and 2061 female worms ($p<0.05$) were collected from goats, with a ratio of 1:6. Similarly, in sheep, 145 male and 581 female worms ($p<0.05$) were collected, with a ratio of 1:4. The proportion of parasites was significantly higher in goats compared to sheep ($p<0.05$). Additionally, there was a significant difference ($p<0.05$) in length between adult male (12.00±0.06 mm) and female (20±0.09 mm) *H. contortus* worms. The average width of the male worm (0.1±0.05 mm) was significantly less than that of the female worm (0.23±0.02 mm) ($p<0.05$). Furthermore, the average weight of the male worm (0.17±0.04 mg) was significantly lower than that of the female worm (0.71±0.04 mg) ($p<0.05$).

The male worm exhibited distinctive morphological features, such as a small buccal cavity with a single dorsal lancet and cervical papillae at the anterior end. The posterior end of the male worm consisted of a bursa with two prominent lateral lobes, along with a small asymmetrical dorsal lobe featuring a Y-shaped dorsal ray. In addition, each spicule of the male worm possessed a single barb at its distal tip (Fig. 1). In contrast, the female worms exhibited a distinctive red and white appearance, primarily attributed to the presence of white ovaries that coiled around the blood-filled intestines (Fig. 2). This visual characteristic can be observed in Figure 2. Furthermore, the female worm's vulva was typically covered by a cuticular vulvar flap, positioned at the anterior limit of the last third of the body. The study identified three vulvar morphotypes: linguiform (17.7 %) (Fig. 3a-b), knobbed/button (76.6 %) (Fig. 4), and smooth (5.7 %) (Fig. 5) (Table 2). Notably, the distribution of the button/knobbed vulvar morphotype was significantly higher

Table 3. Multiple alignment percent identity matrix of ITS-1 of *H. contortus*.

	1	2	3	4	5	6	7	8	9
2	100.0								
3	97.0	97.0							
4	95.8	95.8	98.8						
5	96.3	96.3	98.8	98.8					
6	96.3	96.3	98.8	98.8	100.0				
7	96.3	96.3	98.8	98.8	100.0	100.0			
8	96.4	96.4	99.4	99.4	99.4	99.4	99.4		
9	95.7	95.7	98.8	98.8	98.1	98.1	98.1	99.4	
10	95.2	95.2	98.2	99.4	98.1	98.1	98.1	98.8	98.2

1. 003-Navsari-Male, 2. 001-Navsari-Female, 3. AF044927.1-U.S.A., 4. EU084691.1-U.S.A., 5. JN590059.1-Russia, 6. KJ857556.1-Kolkata, 7. KJ857558.1-Mukteswar, 8. KJ938047.1-Chennai, 9. KP760874.1-Kenya and 10. KX534106.1-China

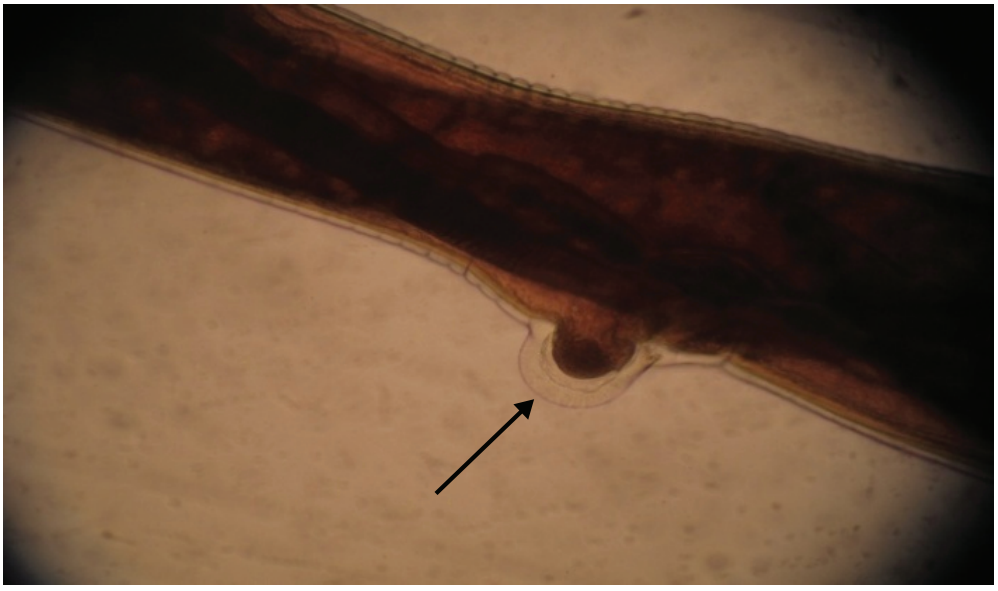


Fig. 4. Knobbed/ button type vulvar flap of *H. contortus* female.

($p<0.05$) in both goats and sheep in the study area (Table 2). Furthermore, among the four subtypes of the linguiform type of the female vulvar flap, two were identified in this study as B (60 %) and I (40 %) (Fig. 3a-b).

Molecular characterization

Nucleic acid amplification assay

The PCR amplification of ITS-1 and ITS-2 plus of genotypic sequence of Navsari isolates of *H. contortus* yielded a fragment of 198 and 350 bp in size, respectively (Fig. 6).

Nucleotide sequence analysis of ITS-1

The trimmed ITS-1 sequence obtained in this study consists of 165 bp, comprising 49 (29 %) Adenine (A), 56 (36 %) Thymine (T), 34 (20 %) Guanine (G), and 26 (15 %) Cytosine (C) bases, with a %GC content of 36.4 %. Furthermore, the alignment of the trimmed ITS-1 sequences showed a complete 100 % identity between the genotypic sequences of both male and female worms, regardless of their morphology and origin from either sheep or goats (Table 3 – 4). There were >95 % homology between present isolates and the published sequences of ITS-1 of

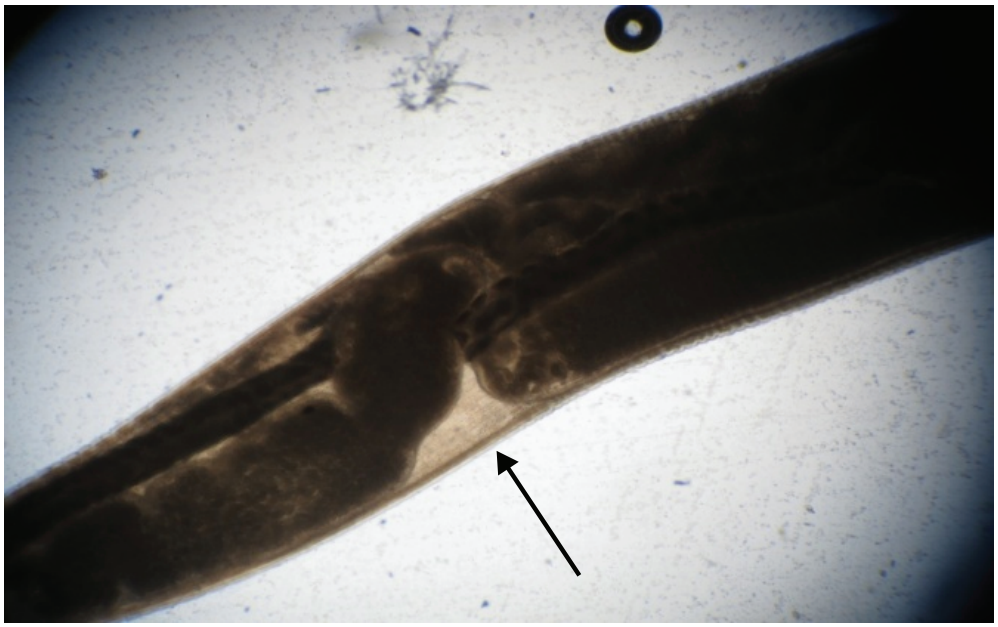


Fig. 5. Smooth type vulvar flap of *H. contortus* female

Table 4. Sequence alignment of ITS-1 of *H. contortus*.

H. contortus retrieved from the NCBI database (Table 3). A single nucleotide polymorphism (SNP) was observed at the 18th position, specifically a T to A transversion, in all sequences except for KP760874.1-Kenya (Table 4). Additionally, at the 66th position, the current sequences, including AF044927.1-U.S.A, showed an A base instead of the typical G base. Similarly, at the 92nd position, the current sequences exhibited an A base instead of a G base. Notably, the 94th position was predominantly characterized by an A base across all sequences. The sequences of *H. contortus*, specifically accession numbers KJ857556.1-Kolkata, KJ857558.1-Mukteswar, and JN590059.1-Russia, displayed a lack of AAGT bases from positions 110-113. Furthermore, a base substitution from C to T was observed in the sequences of accession numbers KX534106.1-China and EU084691.1-U.S.A. At the 3' end of the current sequences, mismatches were observed with an AAA base from positions 159-161 (Table 4). Furthermore, a total of six substitutions (including three transitions and three transversions) were identified across the 165 bp length of the ITS-1 sequence of *H. contortus* when compared to most of the published sequences.

Nucleotide sequence analysis of ITS-2 plus

The trimmed ITS-2 plus sequence obtained in this study comprises 256 bp, and includes 73 (28 %) Adenine (A), 89 (36 %) Thymine (T), 50 (19 %) Guanine (G), and 44 (17 %) Cytosine (C) bases, with a %GC content of 36.7 %. Moreover, the alignment of the trimmed ITS-2 plus sequences revealed a complete 100 % identity between the genotypic sequences of both male and female worms, regardless of their morphology and origin from either sheep or goats (Table 5 – 6). Additionally, a high homology of >94 % was observed between the present isolates and the published sequences of *H. contortus* obtained from the NCBI database (Table 5). In the compared sequences, a T base was observed at the 8th and 86th positions, except in KJ938047.1-Chennai and KP760874.1-Kenya, where a C base was present. Similarly, at the 29th position, a T base was found in all compared sequences, except in KJ857556.1-Kolkata and KP760874.1-Kenya, which contained an A and C base, respectively. Notably, a SNP in the form of a C to T transition was identified at the 33rd position in the Kolkata isolate (KJ857556.1). At the 89th position, all of the compared sequences exhibited an A base, except for MT645506.1-Bangladesh, which contained a C base. Similarly, at the 94th position, a T base was present in all compared sequences, except for KJ857556.1-Kolkata, X78803.1-Australia, KP760874.1-Kenya, and JQ342246.1-Brazil, which contained a C base. Furthermore, at the 99th position, an A base was observed in all compared sequences, except for MT645506.1-Bangladesh, which contained a G base (Table 6). In the Bangladesh isolate (MT645506.1), a SNP was detected in the form of G to C transversion at positions 106th, 152nd, 162nd, and 164th, as well as a T to C transition at position 145th. At position 167th, all compared sequences except for MH481597.1-Australia, X78803.1-Australia, KP101383.1-Thailand, KP760874.1-Kenya, and JQ342246.1-Brazil contained T base instead of A base. The analysis showed that there were more nucleotide transitions than transversions in the aligned sequences as a whole (Table 6).

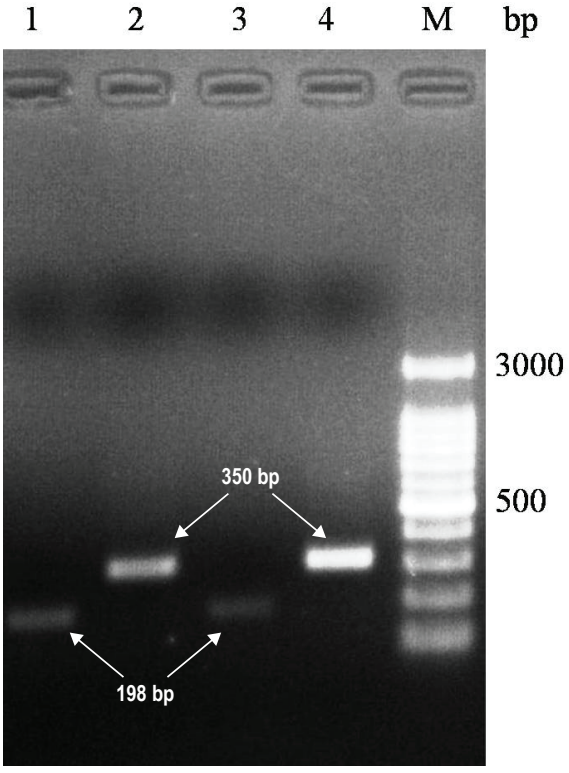


Fig. 6. PCR amplification of ITS-1 of 198 bp (lane 1 and 3) and ITS-2 of 350 bp (lane 2 and 4) genotype sequence of Navsari isolates of *H. contortus*. Lane M: 100 bp DNA ladder.

Phylogenetic analysis based on ITS-1

The phylogenetic analysis of ITS-1, based on 22 nucleotide sequences, resulted in an optimal tree with a sum of branch length equal to 3.19870333. The final dataset of ITS-1 consisted of a total of 1320 positions. The reconstructed phylogram displayed two major clades (Fig. 7). The Navsari genotypes formed a closely-knit cluster within the major clade of *H. contortus*, along with isolates from various locations within the Nematoda class (Fig. 7). Notably, the bootstrapping analysis provided substantial support for the clade encompassing the present and published isolates of the parasites. *Haemonchus placei* (AF044929.1) was found within the clade of *H. contortus*, while *H. longistipes* (MH368674.1 and MH368678.1) occupied a separate position, distinct from the *H. contortus* clade. Furthermore, the nucleotide sequences of *Oesophagostomum columbianum* (MT653093.1), *Trichostrongylus axei* (JQ889794.1), *T. colubriformis* (KC337062.1), *Ostertagia ostertagi* (KR779998.1 and AF044933.1), *Cooperia punctata* (MH267779.1), *C. curticei* (JF680982.1), and *Strongylus vulgaris* (MF489226.1) formed distinct genus-specific clades.

Phylogenetic analysis based on ITS-2 plus

The phylogenetic analysis of ITS-2 plus, based on 25 nucleotide sequences, resulted in an optimal tree with a sum of branch length equal to 2.31431420. The final dataset of ITS-2 plus comprised a total of 1214 positions. The reconstructed phylogram revealed two major clades (Fig. 8). The Navsari genotypes formed a closely-knit cluster within the major clade of *H. contortus*, along with isolates from various locations within the Nematoda class (Fig. 7). Bootstrapping analysis provided significant support for the clade encompassing the present and published isolates of the parasites. *H. placei* (MN709009.1 and KF364627.1) and *H. longistipes* (KU891905.1 and MH374136.1) were found within the major clade of *H. contortus*. Additionally, the nucleotide sequences of *T. axei* (JQ889794.1), *T. colubriformis* (JF680985.1), *Ostertagia ostertagi* (KR779998.1 and AF304561.1), *C. punctata* (MH267779.1), *C. curticei* (JF680982.1), and *Oesophagostomum columbianum* (MT653093.1) occupied different genus-specific clades.

Discussion

The presence of the trichostrongylid nematode, *Haemonchus contortus*, poses a significant challenge for small-scale farmers in resource-limited areas of the tropics, hindering their ability to

Table 5. Multiple alignment percent identity matrix of ITS-2 plus of *H. contortus*.

	1	2	3	4	5	6	7	8	9	10	11	12
2	100.0											
3	100.0	100.0										
4	99.1	99.1	99.0									
5	98.4	98.4	98.4	98.4								
6	99.5	99.5	99.5	98.4	97.8							
7	99.5	99.5	99.5	99.5	97.8	98.9						
8	98.0	98.0	98.0	99.0	97.8	97.3	98.5					
9	100.0	100.0	100.0	99.0	98.4	99.5	99.5	98.0				
10	99.6	99.6	99.5	98.7	97.8	98.9	99.0	97.5	99.5			
11	99.5	99.5	99.5	99.5	97.8	98.9	100.0	98.5	99.5	99.0		
12	96.5	96.5	96.5	95.5	94.6	95.6	96.0	94.5	96.5	96.0	96.0	
13	99.0	99.0	99.0	100.0	98.4	98.4	99.5	99.0	99.0	98.5	99.5	95.5

1. 002-Navsari-Female, 2. 004-Navsari-Male Navsari isolate, 3. EU084691.1-U.S.A., 4. JQ342246.1-Brazil, 5. KJ857556.1-Kolkata, 6. KJ938047.1-Chennai, 7. KP101383.1-Thailand, 8. KP760874.1-Kenya, 9. KX534106.1-China, 10. LC430925.1-Nigeria, 11. MH481597.1-Australia, 12. MT645506.1-Bangladesh and 13. X78803.1-Australia

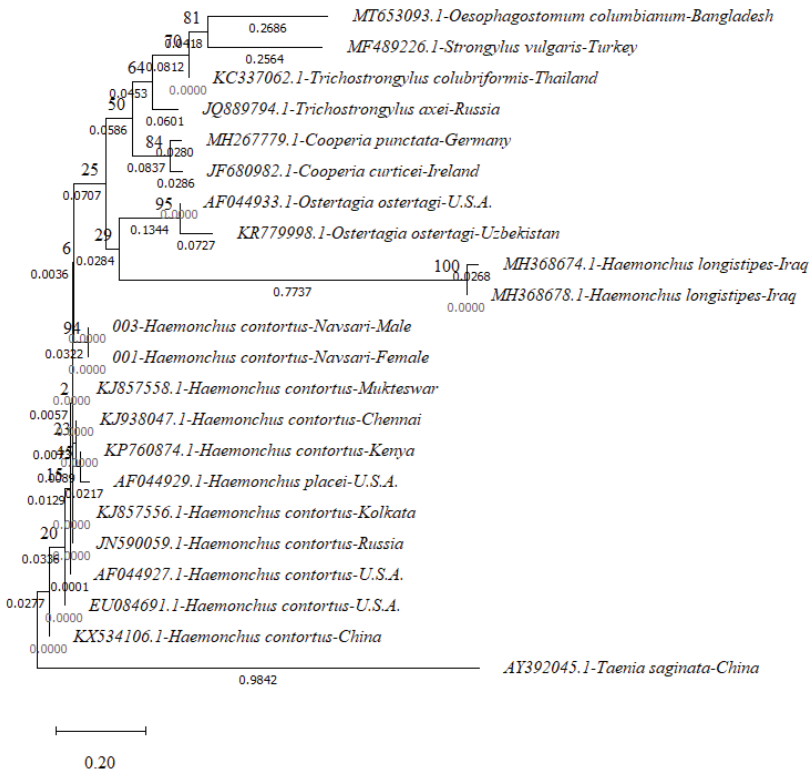
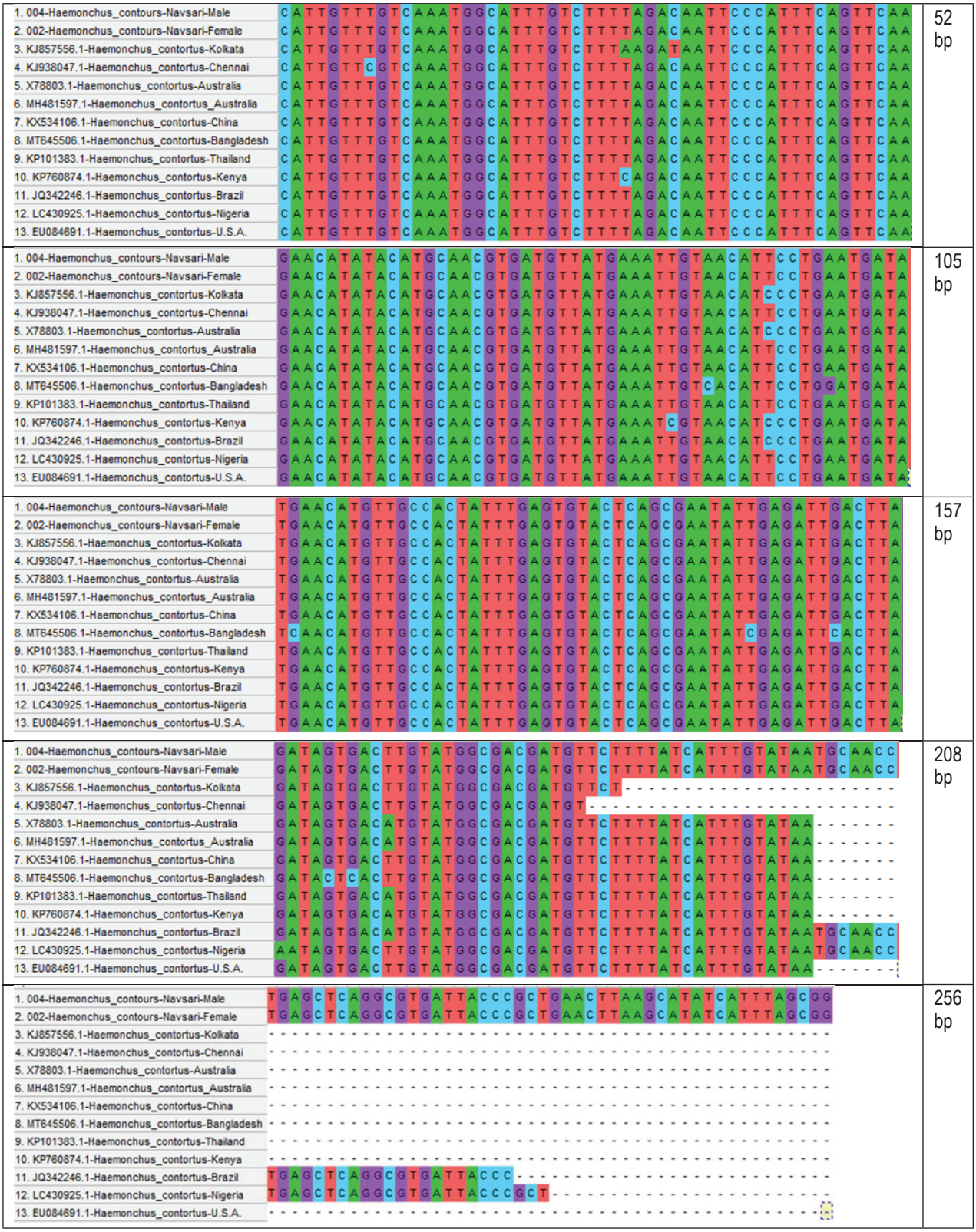


Fig. 7. Phylogenetic relationship of *Haemonchus* along with some other important nematodes based on ITS-1 sequences.

rear profitable small ruminants (McLeod, 2004; Pal *et al*., 2014). Gaining a comprehensive understanding of the host-parasite interaction, genetic diversity, drug resistance, and molecular polymorphisms relies on the exploration of both morphological and molecular characteristics (Cháves-González *et al*., 2022). Such insights are crucial for devising effective strategies to mitigate the impact of this nematode and promote sustainable small ruminant farming practices in tropical regions.

The present study observed a higher prevalence of female worms compared to male worms, with a ratio of 6:1 in goats and 4:1 in sheep. These findings align with the calculated male-to-female ratio of *H. contortus* (1:2.31) reported by Badawy *et al*. (2015) based on their collection of worms from the abomasum of sheep. The analysis of morphometric parameters uncovered a noteworthy difference in the length of adult male and female worms. However, there were no significant variations ($p<0.05$) in length observed among the three vulvar morphotypes, namely linguiform, knobbed/button, and smooth. These findings align with the results reported by Abdel-Hafez *et al*. (2013) and Badawy *et al*. (2015). In contrast, Hunt *et al*. (2008) discovered that linguiform B and knobbed vulvar types were significantly smaller than A and C vulvar types in *H. contortus*. The present study identified the presence of linguiform (17.7 %), knobbed/button (76.6 %), and smooth (5.7 %) vulvar morphotypes. The prevalence of vulvar morphotypes showed minor deviations between sheep and goats but was generally similar ($p>0.05$). The knobbed vulvar flap was encountered as the most predominant type ($p<0.05$). These findings were in accordance with that of Akkari *et al*. (2013a) in North Tunisia and Badawy *et al*. (2015) in Egypt in sheep and goats. However, several other researchers, such as Rahman and Hamid (2007) in Malaysia, Thomas *et al*. (2007) in Ethiopia, Kumsa *et al*. (2008) in Eastern Ethiopia, Gharamah *et al*. (2011b) in Malaysia, Abdel-Hafez *et al*. (2013) in Egypt, Demissie *et al*. (2013) in Ethiopia, Vadlejch *et al*. (2014) in Czech Republic, and Ndosi *et al*. (2023) in Tanzania noted the predominance of linguiform vulvar morphotype. Gharamah *et al*. (2011b) in Yemen showed an equal distribution pattern each of 42 % of linguiform and knobbed vulvar types. The presence of only two linguiform vulvar flap subtypes: B (60.0 %) and I (40.0 %) was the uniqueness of the present study, the observation strongly differs from the report of Thomas *et al*. (2007), Akkari *et al*. (2013a) and Abdel-Hafez *et al*. (2013) who noted the distribution of all four linguiform subtypes of A, C, B and I. Vulvar flap polymorphism of female *H. contortus* has a great taxonomic importance and consider as a phenotypic marker for physical adaptation and diversity. The Internal Transcribed Spacer (ITS) DNA is positioned between the genes for small-subunit ribosomal RNA (rRNA) and large subunit rRNA on the chromosome (Hillis & Dixon, 1991). Eukaryotes possess two ITS regions: ITS-1, found between the 18S and 5.8S rRNA genes, and ITS-2, located between the 5.8S and 28S genes in animal rRNA sequences. While the ITS DNA demonstrates

Table 6. Sequence alignment of ITS-2 plus of *H. contortus*.



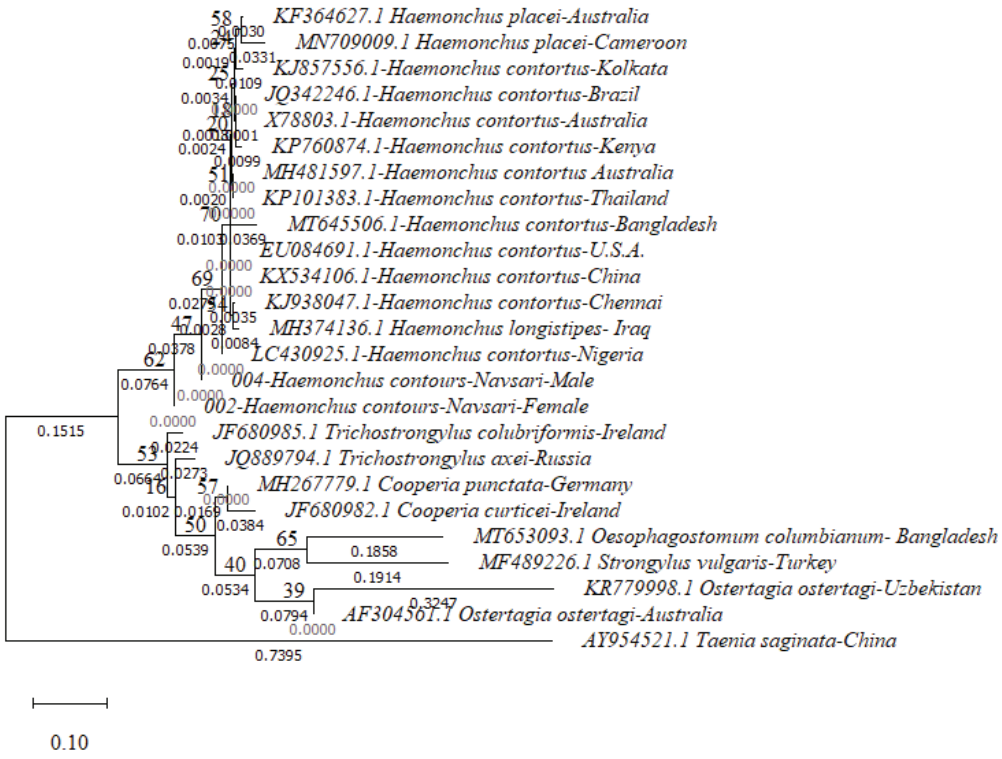


Fig. 8. Phylogenetic relationship of *Haemonchus* along with some other important nematodes based on ITS-2 plus sequences.

a high degree of conservation, it also contains regions with greater variability (Cerutti *et al*., 2010). As a result, it is extensively employed in molecular and phylogenetic studies, as well as for identifying species of helminth parasites (Bandyopadhyay *et al*., 2011; Abramatov *et al*., 2013; Akkari *et al*., 2013b; Yin *et al*., 2013; Mangkit *et al*., 2014; Vadlejch *et al*., 2014; Badawy *et al*., 2015; Meshgi *et al*., 2015).

The examination of the ITS sequences from *H. contortus* in small ruminants within the studied region yielded a single genotype/haplotype. It is noteworthy that area-specific variations in the ITS-1 (Bandyopadhyay *et al*., 2011) and ITS-2 (Abramatov *et al*., 2013; Yin *et al*., 2013; Mangkit *et al*., 2014; Vadlejch *et al*., 2014) have been frequently documented by researchers worldwide. In our study, the ITS sequences of male and female *H. contortus* exhibited a high level of similarity, aligning with the findings of Bandyopadhyay *et al*. (2011) and Vadlejch *et al*. (2014). In this study, the GC content was determined to be 36.4 % for ITS-1 and 36.7 % for ITS-2 plus, which closely resembled findings from previous investigations. For instance, a study conducted in Thailand reported a GC content of 32.9 % for ITS-2 in goats and sheep (Mangkit *et al*., 2014). Similarly, research conducted in Australia, the United Kingdom, Switzerland, and China reported a GC content of 33 % for ITS-2 in sheep (Stevenson *et al*., 1995). Additionally, a study encompassing goats and sheep from Malaysia and Yemen reported a GC content of 33.4 % for ITS-2 (Gharamah *et al*., 2012). In Tunisia, the GC content was found to be 33 % for sheep and cattle, and 36 % for goats in their ITS-2 sequences (Akkari *et al*., 2013b). Finally, a study in Uzbekistan reported a GC content of 33 % for sheep ITS-2 (Abramatov *et al*., 2013).

In our current study, upon aligning the trimmed ITS-1 and ITS-2 plus sequences, it was observed that the male and female genotypic sequences from both sheep and goat origins were 100 % identical. This uniformity could be attributed to significant gene flow occurring among various ruminant hosts, particularly in intensively managed flocks. Furthermore, when comparing the Navsari isolate's ribosomal DNA sequences in the ITS-1 and ITS-2 regions with published *H. contortus* sequences, sequence polymorphism was evident. Multiple nucleotide transitions and transversions were observed at various positions within the aligned ITS sequences. These point mutations have the potential to serve as genetic markers for distinguishing between different isolates. Notably, our findings indicated the presence of intraspecies variation in *H. contortus* across distinct geographical regions. This high level of sequence homology at the species level aligns with the observations made by Bandyopadhyay *et al*. (2011), Akkari *et al*. (2013b), and Yin *et al*. (2013). Multiple transitions and transversions were identified at various positions within the aligned sequences of both ITS-1 (165 bp) and ITS-2 plus (256 bp) in *H. contortus*. Akkari *et al*. (2013b) reported the occurrence of 10 substitutions in a 231 bp segment of *H. contortus* from goats in Tunisia. Similarly, Gasser *et al*. (1998) observed 12 nucleotide variations in ITS-2, including 4 transitions, 5 transversions, 1 insertion, and 2 deletions.

The reconstructed phylograms based on ITS-1 and ITS-2 plus sequences revealed the presence of two major clades, with the current Navsari genotypes falling within the major clade of *H. contortus*. Within both phylograms, *Haemonchus placei* sequences (AF044927.1-U.S.A., MN709009.1, and KF364627.1) occupied a position in the major clade of *H. contortus*, indicating a minimal level of intraspecific sequence difference in the ITS-1 and ITS-2 plus regions. The proximity between these two species was also supported by previous studies conducted by Jacquiet *et al*. (1995), Stevenson *et al*. (1995), and Abramatov *et al*. (2013). In the ITS-1 phylogram, *Haemonchus longistipes* was positioned outside the clade of *H. contortus* (MH368674.1 and MH368678.1), while in the ITS-2 plus phylogram, it fell within the clade of *H. contortus* (MH374136.1). Kandil *et al*. (2018) noted limited homology between *H. longistipes* and *H. contortus*, with *H. longistipes* being the genetically distinct taxon based on mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) gene analysis. The characterization of ITS regions in other *H. contortus* isolates from India will provide insights into the extent of polymorphism exhibited by these sequences. Such analysis will facilitate the accurate genetic characterization of *H. contortus* isolates in our country and aid in distinguishing gastrointestinal nematodes that infect small ruminants. In conclusion, this study has confirmed the significance of both morphological and molecular characteristics in the identification and differentiation of the *H. contortus* parasite at the local level. While morphological identification serves as a preliminary step, molecular identification utilizing ITS sequences is crucial for species-specific identification of *Haemonchus* isolates. Sequence and phylogenetic analysis of ITSs provide valuable insights into the true taxonomic classification of different genotypes, enabling the development of precise strategies for parasite detection. Ultimately, these findings hold practical implications for designing more effective control strategies aimed at managing *H. contortus* infections.

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Author Contributions

Conceptualization, B.D., N.K. J.B.S. and I.H.K.; Biological sample collection and performing laboratory test, B.D. and M.M.J.; Data analysis and manuscript writing, B.D. and N.K.; Review and editing, N.K. and J.B.S. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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