Identification of nematodes of the genus *Teladorsagia* parasites of ruminants with the help of species-specific markers based on ITS2 rDNA

Anvarbek Ibrokhimov[1,*], *Abdurakhim* Kuchboev[2], *Oybek* Amirov[2], *Boltaboy* Kahorov[3] and *Mirzakamol* Ayubov[4]

[1]Namangan State University, Namangan, 160100, Uzbekistan
[2]Institute of Zoology of the Academy of Sciences of the Republic of Uzbekistan, Tashkent, 100140, Uzbekistan
[3]National University of Uzbekistan, Tashkent, 100174, Uzbekistan
[4]Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan, Tashkent, 100140, Uzbekistan

Abstract. The present study delves into a methodological framework aimed at establishing species-specific markers via the utilization of sequences derived from the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA. This method, in conjunction with polymerase chain reaction (PCR) testing, serves as a diagnostic tool for discerning species belonging to the genus *Teladorsagia* Andreeva et Satubaldin, 1954. These species, constituents of the subfamily *Ostertagiinae* (*Nematoda: Trichostrongylidae*), exhibit wide distribution within the gastrointestinal tracts of ruminants across the geographic expanse of Uzbekistan. The heart of this endeavor is the development of species-specific primers, a pioneering creation in its own right. These primers are crafted using sequences emanating from the ITS2 region of the ribosomal DNA, an innovative approach that facilitates the precise identification of morphospecies within the Teladorsagia genus. Notably, the primers exhibit a nucleotide length of 153 base pairs, an attribute instrumental in their capacity to accurately distinguish and diagnose eggs and larvae of three distinct morphspecies: *T. circumcincta*, *T. trifurcata*, and *T. davtiani*. The potential implications of this method are significant, with ramifications reverberating across the field of veterinary diagnostics. Through the application of these primers, practitioners and researchers alike can effectively ascertain the presence of specific *Teladorsagia* morphospecies in ruminant animals. This holds the promise of not only enhancing diagnostic precision but also contributing to the broader comprehension of the prevalence and distribution of these nematode species within the local ruminant populations.

Keywords. Nematode, Ostertagiinae, Teladorsagia, PCR, rDNA, ITS2, centrifuge, amplification.

* Corresponding author: aibrohimov680@gmail.com

1 Introduction

Currently, parasitic diseases of livestock and other animals of all over the world are considered to be economic importance. In Australia, losses caused by parasitic diseases in sheep and cattle have been estimated at one billion australian dollars [1]. Nematodes are the most common types of parasitic diseases, and the use of anthelmintic drugs against these parasites is not always effective. The use of multiple anthelmintic drugs in animals increases genetic resistance in nematode populations and causes widespread problems [2, 3].

One of the genera widespread in the digestive system of ruminants is *Teladorsagia* Andreeva et Satubaldin belongs to the subfamily Ostertagiinae Lopez-Neira, 1947 1954 (Nematoda: Thichotrongylidae) is widespread in ruminants of Uzbekistan. They are adapted to live as parasites in the udder and intestines of animals, and sometimes the intensity of invasion in one animal organism exceeds thousands of copies [4].

In the detection of parasitic diseases in ruminants , using capralogical methods, parasite species are clarified based on the morphology of the larvae and eggs of parasites. However, this method makes it difficult to accurately diagnose the species, and it also takes a lot of time [5]. To date, in addition to classical methods, research on DNA analysis is widely used to identify nematode species. By studying nucleotides in the internal transcribed spacer 2 (ITS2) regions of nematode ribosomal DNA (rDNA) through PCR, methods were developed to identify nematode eggs, larvae and mature imago at the species level [6].

The primary objective of this research endeavor is to devise a novel method for establishing species-specific markers that leverage the internal transcribed spacer 2 (ITS2) region of ribosomal DNA (rDNA). These markers will be pivotal in the precise diagnosis of nematode species belonging to the Teladorsagia genus [7, 8]. This genus comprises parasitic organisms inhabiting the intricate landscape of the digestive systems in ruminant animals. The proposed diagnostic strategy entails the utilization of polymerase chain reaction (PCR) techniques to validate the efficacy of these species-specific markers [9, 10].

This research initiative is driven by the pressing need for accurate and reliable diagnostic tools within the domain of veterinary science. The intricate interplay between host animals and parasitic nematodes necessitates a meticulous understanding of species diversity and distribution [11]. Through the strategic utilization of species-specific markers harnessed from the ITS2 rDNA region, this study seeks to offer a breakthrough solution to this challenge. The utilization of PCR for testing the efficacy of these markers serves as a pivotal validation step, ensuring their precision and applicability in real-world diagnostic scenarios [12, 13].

The potential implications of this research are far-reaching. Accurate species identification is foundational to effective disease management and treatment strategies in the realm of veterinary medicine [14]. The deployment of species-specific markers, underpinned by molecular techniques, holds the promise of revolutionizing the diagnostic landscape for Teladorsagia nematodes in ruminant populations. By offering a higher level of resolution in species differentiation, this research aims to contribute to a more nuanced understanding of the prevalence, distribution, and potential impact of these parasitic organisms on the digestive systems of ruminant animals.

As this research journey unfolds, it aspires to not only shed light on the intricacies of species differentiation but also to advance the field of veterinary diagnostics. The innovative amalgamation of molecular techniques, rDNA sequencing, and PCR validation stands to redefine the way we approach the identification and characterization of parasitic nematode species [15, 16]. Ultimately, the successful realization of species-specific markers using ITS2 rDNA holds the potential to enhance disease management, animal

health, and the broader understanding of host-parasite interactions in the context of ruminant digestive systems.

2 Materials and Methods

Materials and study area. To carry out this research, the species belonging to the genus *Teladorsagia* and the representatives of morphologically and morphometrically close genera *Marshallagi*a Orloff, 1933, *Haemonchus* Cobb, 1898, *Ostertagia* Ransom, 1907 were collected from Namangan, Syrdaryo, Jizzakh, Samarkand of Republic of Uzbekistan. Complete and incomplete helminthological dissection of the intestines of sheep and cattle slaughtered in poultry houses of Bukhara and Kashkadarya regions was carried out [17] and the collected helminthological samples were fixed in 70% ethanol solution (Figure 1 and Table 1).

Figure 1. Sites of material collection for helminthological sumples for this examination.

The taxonomic affiliation of the species was determined mainly on the basis of the morphology and morphometry of male individuals [18].

*DNA Isolation***:** To extract genomic DNA from nematode samples, 20 µl of NaOH (0.25M) was added to each probe, kept at room temperature for 12 hours, and then heated to 95°C for 3 minutes, samples were added to 10 µl of tris-HCl (0.25 M) and centrifuged at 10,000 rpm for 2 minutes. Samples are removed from the centrifuge, 4 µl of HCl (1:15) is added, vortexed and centrifuged again. Then, 5 µl of triton (2%) is added. Samples were heated at 95°C for 3 minutes and then stored in a freezer at -20°C.

Table 1. List of collected helminthological samples from domestic ruminants of Uzbekistan.

Nematode species	Regions	Hosts
Haemonchus contortus	Namangan, Bukhara	Sheep, cattle
H. placei	Namangan	Sheep
Ostertagia ostertagi	Kashkadarya, Namangan	Sheep, cattle
O. lyrata	Kashkadarya, Namangan	Sheep, cattle
Teladorsagia circumcincta	Jizzakh, Bukhara, Samarkand	Sheep, cattle, sheep
T. davtiani	Jizzakh, Bukhara, Syrdarya	Sheep, sheep, sheep
T. trifurcata	Jizzakh, Bukhara	Sheep, sheep
Marshallagia marshalli	Samarkand, Bukhara, Syrdarya	Sheep, cattle, sheep
M. occidentalis	Samarkand, Bukhara, Tashkent	Sheep, cattle, sheep

*PCR-amplification***:** The ITS2 region of nematode rDNA was amplified using NC1 and NC2 primers used in molecular taxonomy of nucleotide fragments (Gasser et al., 1993). PCR was performed according to the following scheme: 1 – step – DNA denaturation at 95°C for 3 minutes, 2 – step – DNA denaturation at 93°C for 20 seconds, 3 – step – DNA at 55°C for 30 primers sit on the template for seconds, step 4 - elongation at 72°C for 2 minutes, step 5 - chain elongation at 72°C for 10 minutes. From the second to the fourth step, the process was repeated up to 35 times per cycle.

*Gel electrophoresis***:** The presence of DNA fragments in PCR products was determined by electrophoresis on a 1.5% agarose gel at 100 V. The 100 bp DNA ladder were used for DNA fragment size determination. The 250 bp length amplicons were documented using a transilluminator gel-documentation system. Amplicons were isolated from the gel using a set of reagents produced by "Silex M" (Moscow, Russia) following the manufacturer's instructions.

The nucleotide sequence of the rDNA ITS-2 region of species belonging to the genus *Teladorsagia* (https://www.ncbi.nlm.nih.gov/tools/primer-blast) primer sequences were selected for representatives of this generation and these primers were synthesized at SINTOL (Moscow, Russia).

Bioinformatic analyses: The of the results of morphological and morphometric data were statistically analyzed based on different programs [19, 20] and data obtained based on molecularresults were performed based on bioinformatics programs BioEdit, Slustalx, Genedoc, Paup4 and Geneious.

3 Results

Morphological research results: According to the results of the conducted helminthological research, more than 100 permanent and temporary preparations were prepared in laboratory conditions in order to clarify the morphological and morphometric dimensions of representatives of the genus *Teladorsagia*, the morphological and morphometric dimensions of the three species were clarified (Figure 2).

Teladorsagia circumcincta (Stadelmann, 1894)

Morphology. Male - length 10.5-16 mm. The cross section of the body is 0.16-0.21 mm. The length of the esophagus is 0.65-0.82 mm. The bursa is well developed, 0.31-0.59 mm long. The length of two equal spicules is 0.35-0.51 mm. Gubernaculum is racket-shaped, 0.01-0.011 mm long (Figure 2A).

The female is 9.8-17.4 mm long and 0.14-0.21 mm wide. The neural crest is located at a distance of 0.48-0.62 mm from the front part of the body. The length from the top of the

head to the nape of the neck is 0.28-0.65 mm. The vulva is located 1.8-2.1 mm from the end of the tail. The length of the egg is 0.04-0.09 mm.

Teladorsagia trifurcata (Ransom, 1907)

Morphology. Male - 5-10 mm long. The width of the body in front of the bursa is 0.10-0.21 mm. The length of the esophagus is 0.35-0.75 mm. Nerve node at a distance of 0.47-0.82 mm from the head of the body. The bursa is well developed, 0.20-0.47 mm long. The length of the spicule is 0.11-0.35 mm. The length of the gubernaculum is 0.05-0.018 mm (Figure 2B).

The female is 8-15 mm long and 0.11-0.25 mm wide. The neural crest is located at a distance of 0.35-0.77 mm from the front part of the body. The length from the top of the head to the nape of the neck is 0.40-0.95 mm. The vulva is located 1.6-1.9 mm from the end of the tail. The length of the egg is 0.06-0.011 mm.

Teladorsagia davtiani (Andreeva et Satubaldin, 1954)

Morphology. Male - length 10.0-11.5 mm. The width of the body in front of the bursa is 0.12-0.15 mm. The length of the esophagus is 0.063-0.095 mm. Nerve node at a distance of 0.36-0.79 mm from the head of the body. The bursa is well developed, 0.39-0.46 mm long. The length of the spicule is 0.21-0.26 mm. The length of the gubernaculum is 0.107-0.110 mm. (Figure 2C).

The female is 11-14.5 mm long and 0.165-0.195 mm wide. The neural crest is located at a distance of 0.50-0.70 mm from the front part of the body. The length from the top of the head to the nape of the neck is 0.050-0.085 mm. The vulva is located 1.8-2.6 mm from the end of the tail. The length of the egg is 0.05-0.011 mm.

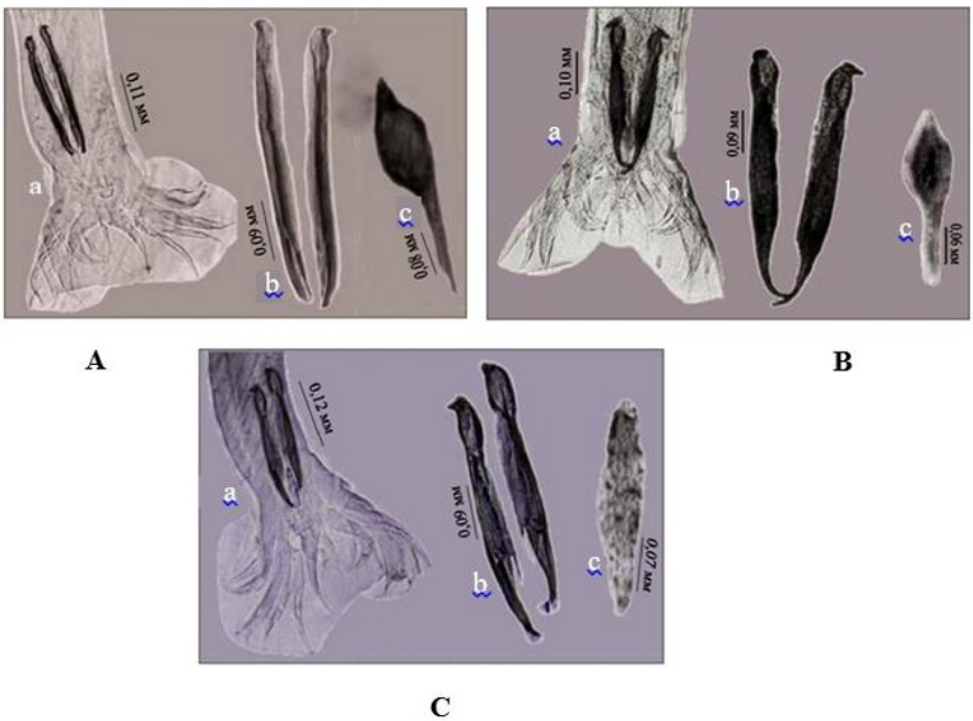

Figure 2. Morphological features of nematodes of the genus *Teladorsagia* Andreeva et Satubaldin, 1954. A - *T. circumcincta*, B - *T. trifurcata*, C - *T. davtiani; Note in the figure:* a- bursa, b-spicule, c - gubernaculum.

As a result of the conducted morphological research, the following was determined on the morphology of male individuals of *Teladorsagia circumcincta*, *T trifurcata* and *T davtiani* species belonging to the genus *Teladorsagia*:

The dorsal rib of *T circumcincta* is rather long and thin, while that of *T davtiani* is thick and relatively short. Gubernaculum *T circumcincta* has an elongated cylindrical shape, while in *T davtiani* it is long and the lower part is thinned and thickened towards the middle part. In *T. circumcincta*, the distal part of the main spicule is truncated and bluntly curved.

In, the distal part of the main spicule ends like a pointed knob, relatively long and thin. In addition, some differences were observed in the length of their body, spicule and gubernaculum.

Primer design for PCR and check them for specificity. We was create the design of specific primers that allow to identify the *Teladorsagia* species from representatives of other genera and based on the results of the molecular study of the rDNA ITS-2 [3] and the data nucleotide sequences from GenBank (NCBI) with use Primer-Blast (Table 2).

Table 2. Description of species-specific primers for the ITS-2 region of the rDNA of nematodes of the genus *Teladorsagia*.

Samples	**Sequences 5` → 3`**	**Lengths**	**Amplicon length**	**Tm**
Tel_1f	ATAACACTGTTTGTCGAATGGCA	23	100	59,18
Tel_2f	ACATGACGGTACGACGGTAG	20	100	55
Tel_3f	ACACTGTTTGTCGAATGGCATTTA	24	100	59,7
Tel_4f	ATAACACTGTTTGTCGAATGGC	22	100	57,5
Tel_5f	TGCAACATGACGGTACGACG	20	100	55
Tel_6f	ATATGCAACATGACGGTACGACGGT	25	153	59,8
Tel_7f	AACATGACGGTACGACGGTAG	22	100	55
Tel_8f	TGGCATTTATCACTTCATTGTGGT	24	100	59,2
Tel_9f	CACTGTTTGTCGAATGGCATTTA	23	100	58,2
Tel_10f	AACATGACGGTACGACGGTA	20	100	58,8

In order to check the newly created primers, the genomic DNA of species belonging to the genus *Telodorsagia* collected from the digestive system of animals in different regions of the Republic of Uzbekistan and were isolated genome DNA from species close to this parasites and were PCR amplified on the basis of these primers.

The PCR was performed using primers designed and primers NC2 and performed in the steps outlined above. The obtained PCR products with scesies-specific primers were checked by electrophoresis in a 1.5% agarose gel with a voltage of 130 V (Figure 3).

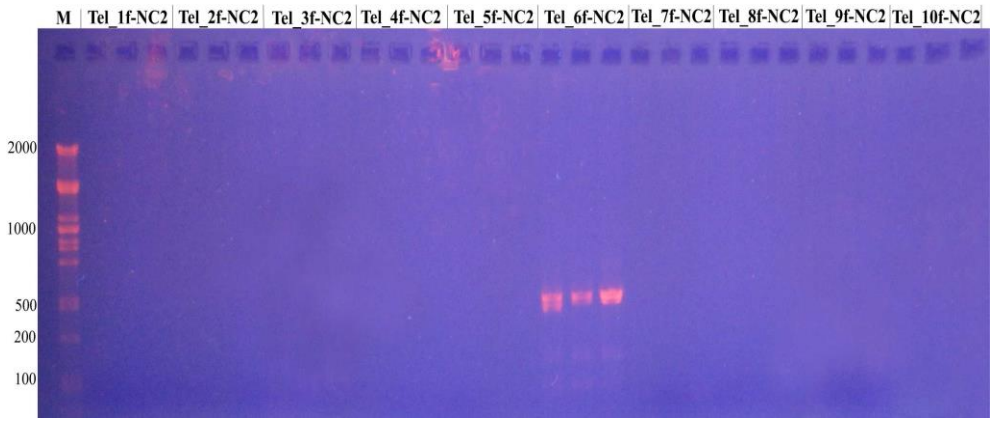

Figure 3. View of the gel electrophoresis of PCR products with primers designed for the three morph species of the genus *Teladorsagia* (6 line primers is Tel_6f-NC2).

According to the electrophoresis results there were DNA bands on the agarose gel with primer Tel_6F (5' ATATGCAACATGACGGTACGACGGT 3') and it was well created for the species of the genus Telodorsagia from other 10 primers (see Figure 3).

To check Tel_6F and NC2 primers was performed PCR from genomic DNA species of *Haemonchus contortus, H. placei, Ostertagia ostertagi, O. lyrata, Marshallagia marshalli, M. Occidentalis, Teladorsagia circumcincta, T. davtiani* and *T. trifurcata* (Figure 4)

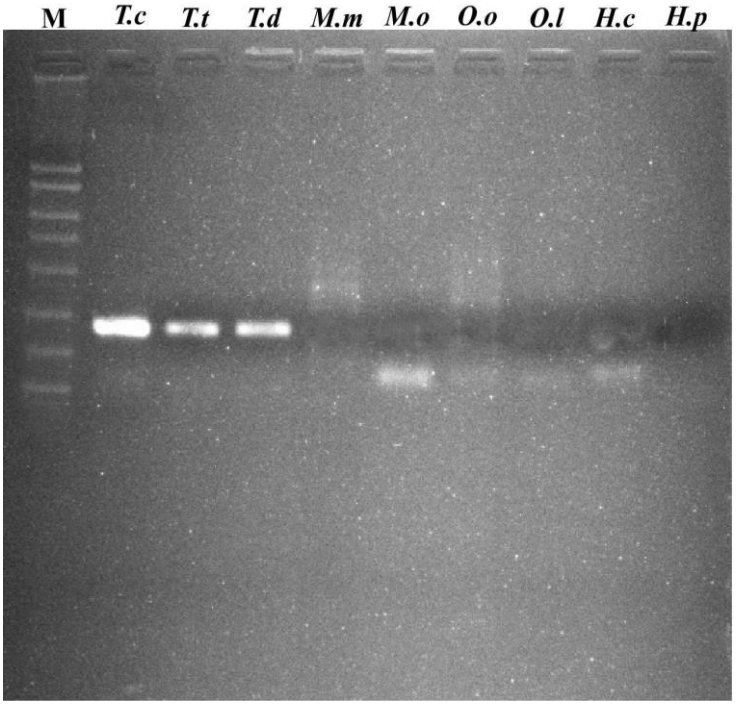

Figure 4. Results of validation of the species-specific primers created for the morphspecies of the genus *Teladorsagia* and other species: Note: *T.c-Teladorsagia circumcincta; T.t-T. trifurcata; T.d-T. davtiani; M.m-Marshallagia marshalli; M.o-M. occidentalis; O.o-Ostertagia ostertagi; O.l-O. lyrata; H.c-Haemonchus contortus; H.p-H. placei.*

According to the PCR results with of the Tel_6F primer was determined to be specific only for morphspecies *T. circumcincta*, *T. davtiani* and *T. trifurcata* of the genus *Teladorsagia*. The obtained PCR products were sequenced and the created primer was placed (Figure 5).

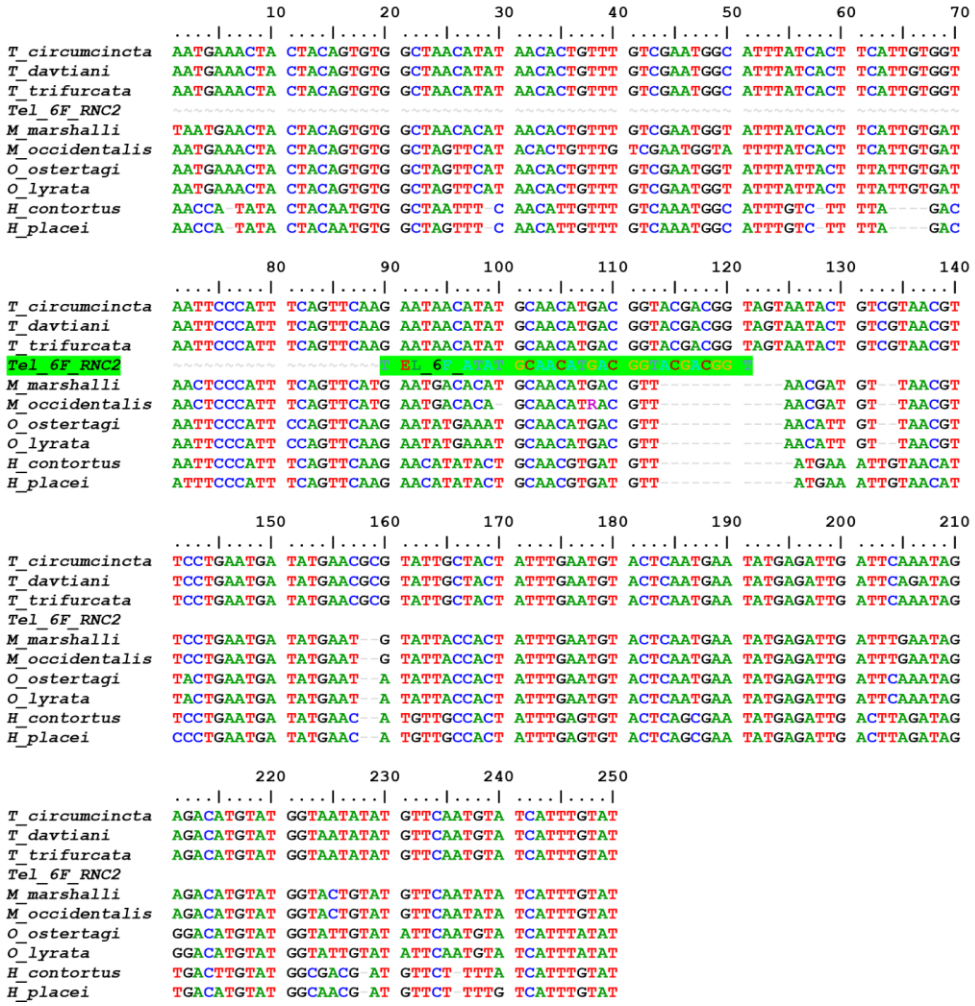

Figure 5. Location of the primer Tel_6f generated for the genus *Teladorsagia* based on sequence material.

As can be seen from Figure 5 above, the nucleotides of the ITS2 region of the rDNA of the species belonging to the genus *Teladorsagia* and the nematode species close to this genus and common in the digestive system of ruminants in our country and the nucleotides of the Tel_6f primer created for the genus *Teladorsagia* were studied. The Tel_6f primer created for species of *T. circumcincta*, *T. trifurcata* and *T. davtiani* of the genus *Teladorsagia* rDNA was found to read 153 bp of nucleotides belonging to the ITS2 region.

4 Discussion

L.A.Stevenson et al. [17] studied the ITS-2 sequences of *T. circumcincta, T. trifurcata* and *T. davtiani* and 2 other members within the same subfamily Ostertagiinae were examined. Although some sequence variation was detected between and within single worms of each taxon of *Teladorsagia*, no unequivocal base differences were detected among their consensus ITS-2 sequence. In comparison, there was 9 % sequence difference between *O. ostertagi* and *O. leptospicularis* and 13-15 % difference between the genera *Teladorsagia* and *Ostertagia*. These findings indicate that T. *circumcincta, T. davtiani* and *T. trifurcata* represent a single species, *T. circumcincta*.

Previously, we also comparative studies of three species *T. circumcincta, T. trifurcata* and *T. davtiani*, which are preliminarily different morphs of the same species, have been carried out. It has been established that there were insignificant differences in morphological sizes and characters between males but of the nucleotide fragments of ITS-2 rDNA of three species no differences were detected [9].

The advantage of DNA-based identification of parasitic nematodes is that it is time-consuming, allowing identification of species at any developmental stage [6-9], however continuing advances have refined the techniques and allowed savings in both time and cost [10]. In addition, the PCR diagnostic method can accurately diagnose parasitic nematodes in ruminants, leading to the correct use of anthelmintic drugs [7].

5 Conclusions

According to the results of the morphological study of species *T. circumcincta, T. trifurcata* and *T. davtiani* belonging to the genus *Teladorsagia,* species significant changes in morphological features and size were noted; According to the results of the molecular study of these species, when the nucleotides of the ITS2 region of the rDNA of the 3 studied species were compared, these species were *T. circumcincta, T. trifurcata* and *T. davtiani* is the basis for considering species as a single species; The Tel_6f primer created for morphspecies of *T. circumcincta, T. trifurcata* and *T. davtiani* was found to read 153 bp of nucleotides belonging to the ITS2 region rDNA and using this primer it will be possible to accurately diagnose the eggs and larvae of the species in the ruminant animals.

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