

# Morphological and molecular characterization of *Haemonchus contortus* and *H. placei* (Nematoda: Trichostrongylidae) from Uzbekistan by sequences of the second internal transcribed spacer of ribosomal DNA

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**Abstract.** Nucleotide sequences of the second internal transcribed spacer region (ITS-2) of nuclear ribosomal DNA in *H. contortus* and *H. placei* revealed six (2.6%) nucleotide differences between these two species. The level of the intraspecific difference in ITS-2 in *Haemonchus* was low. A number of morphological differences together with distinctive ITS-2 sequences signatures at the molecular level clearly differentiate *H. placei* from *H. contortus* in the genus *Haemonchus*.

**Keywords:** Nematode; Molecular identification; ITS-2; *Haemonchus contortus*; *Haemonchus placei*.

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## Introduction

Nematodes of the genus *Haemonchus* Cobbold, 1898 parasitize the abomasum of wild and domestic ruminants and are widespread throughout terrestrial ecosystems. Currently, thirteen *Haemonchus* species have been recorded in the world fauna. The ruminants from the families Cervidae, Antilocapridae, Giraffidae, Bovidae, as well as Camelidae, have been recorded as definitive hosts (Skrjabin et al., 1954; Ivashkin et al., 1989; Anderson, 2000; Hoberg et al., 2004). These nematodes are

widespread and cause serious disease (i.e. anemia, loss of appetite, diarrhea, constipation). Losses caused by these diseases to cattle husbandry are significant (Waller and Chandrawathani, 2005).

Until recently, a number of researchers believed that *Haemonchus contortus* (Rudolphi, 1803) parasitize both small and large ruminants (Azimov, 1963; Pryadko, 1962a; 1962b; Pevneva, 1966a; 1966b). However, other scientists (Roberts et al., 1954; Herlich et al., 1958; Daskalov, 1960; 1961; Patyk, 1961;

Lichtenfels et al., 1986; Lichtenfels et al., 1994) believe that two *Haemonchus* species, *H. contortus* and *H. placei*, parasitize these animals. The question of validity of *H. contortus* and *H. placei*, was proved on the basis of detailed morphological and cytological studies.

We find it reasonable to focus attention on this problem and continue the study at the molecular-genetic level. Using molecular genetics methods it is possible to find markers suitable for revealing relations between studied species while describing the structure of the species and dynamics of genetic processes in the populations (Welsh and McClelland, 1990). Studies of genetic variability of nematodes are not only of theoretic, but also of medical-veterinary importance. Of common biological interest are processes of the interrelation of this parasite with the environments, which include not only the complex of natural abiotic factors, but also the organism of the host animal itself.

Our goal was to conduct a morphological study and molecular identification based on ITS-2 spacer of the rDNA of *H. contortus* from sheep and *H. placei* from cattle, obtained from different regions of Uzbekistan, with the purpose of identifying differences between them and obtaining additional data on the rDNA.

## Materials and methods

### *Parasite collection*

Helminthological material from Tashkent, Bukhara, Navoiy, Kashkadarya and Namangan provinces (Uzbekistan) was collected during the dissection of abomasums of sheep and cattle as described by Skrjabin (1928). Nematodes were washed with the physiological solution and the specimens were conserved in 70% ethanol.

Fragments of the second internal transcribed spacer (ITS-2) of rDNA were obtained separately from all males morphologically determined as *H. contortus* from sheep and *H. placei* from cattle. Three male specimens of each of these two species were studied.

### *DNA isolation, amplification and sequencing*

A single specimen of each species was used. The isolation of genomic DNA from nematodes was carried out using the method of lysing buffer containing Tris 50mM, EDTA 20 mM and 10 mkg/ml of proteinase K. 0.5% of sodium dodecyl sulfate (SDS) was added and incubated in the thermostat for 2 hrs at t 55°C. The isolated DNA was kept at 20°C below zero.

The PCR protocol has been perform using the following protocol. The fragments of ITS 2 of rDNA were amplified using the primers set NC1 (forward; 5'-ACGTCTGGTTCAGGGTTGTT-3' and NC2 (reverse; 5'-TTAGTTTCTTTTCTCCGCT-3') (Gasser et al., 1993). The PCR was carried out on the thermocycler (Touchgene Gradient, UK) at the following temperature regime: 92°C – 3 min; 92°C – 15 sec, 55°C – 30 sec, 72°C – 30 sec (35 cycles) and 72°C – 10 min. The reaction was amplified in 50 µl of reaction mixture containing 39 µl ddH<sub>2</sub>O, 5 µl of 10× PCR buffer/ 2 µl 25 mM MgCl<sub>2</sub>, 1,5 µl of each primer, 1,2 µl of 10 mM dNTP, 1 µl Taq-polymerase and 1 µl of genome DNA. The electrophoresis of amplified DNA fragments was conducted in 1,8 % agarose gel in B 1xTAE-buffer and stained in ethidium bromide. The size of the obtained PCR product was identified by comparing it with the fragments of DNA marker Microgel DNA Ladder (Daigger Lab.).

To clone obtained PCR products, we used TOPO TA cloning vector and TOP10 cells *E. coli*. Experiments on cloning and transformation were conducted according to the instruction of the manufacture (Invitrogen, USA). Sequencing was obtained using the Big Dye terminator technology on the equipment of GeneAmp PCR System 9700 with a subsequent capillary electrophoresis ABI 310 Genetic Analyzers (Applied Biosystems, USA).

The analysis of the nucleotide sequences obtained was carried out using the packet of computer software Bioedit, while the alignment and comparison of sequences, using the method of program Clustal W.

Phylogenetic trees were built using the packet of software MEGA (ver. 4.1) using the

bootstrap test of phylogeny method of maximal parsimony. For the phylogenetic analysis we used the species *Haemonchus contortus* (X78803), *H. placei* (X78812), *H. longistipes* (AJ577461) and species *Trichostrongylus colubriformis* (EF427624) were used as outgroups for tree, published in the base GenBank. Nematodes of Uzbekistan were registered Genbank of accession numbers KC503915 (for *H. contortus*) and KC503916 (for *H. placei*).

### Morphological comparisons

Specimens were examined using high magnification light microscopy. Methods described by Skrjabin et al. (1954) and Ivashkin et al. (1989) were used for the study of the species composition and morphology of *Haemonchus* nematodes. To identify morphological criteria of mature *Haemonchus*, we separated the head and tail ends of males and females and made preparations. The collected material was put in flasks, fixed and labeled for storing at the Laboratory of General Parasitology of the Institute of the Pool of Plants and Animals Uzbek Academy of Sciences.

### Results

Obtained data showed that in the nematodes *H. contortus* and *H. placei* the amplified fragments made up 320 pairs of nucleotides. From each specimen of the nematodes *H. contortus* and *H. placei* we isolated fragments ITS-2 with the length of 231 base pairs (bp) (figure 1). Obtained data of nucleotide sequences of the ITS-2 on the three specimens of *H. contortus* and *H. placei* were identical. The content of GC nucleotides in ITS-2 sequences of the nematodes *H. contortus* and *H. placei* was 33 and 33.7%, respectively.

The comparison of the nucleotide sequences of ITS-2 of *H. contortus* and *H. placei* revealed differences in six nucleotides. The differences between the two studied nucleotide sites of these nematodes constituted 2.6%. The comparison of differences in the sequences between *H. contortus* and *H. placei* revealed three nucleotide positions (figure 1: positions 24, 205 and 219), which are represented by a passage between purines (A+G). In position

123 there differences between pyrimidines. Two replaced nucleotides were recorded in two sites (figure 1: 65 and 196) between purines and pyrimidines, representing the double union. The presence of two variable sites is most likely to be the line differentiating the ITS-2 *H. contortus* and *H. placei*.

### Discussion

The results of our study confirm the previously published data by Stevenson et al. (1995), which revealed differences in the structure of ITS-2 specimens of *H. contortus* from sheep in England, Switzerland, China and Australia, and specimens from *H. placei* from cattle from Australia. It is necessary to note that the length of ITS-2 of the objects studied by us coincided with the materials of the authors mentioned above, i.e. our studied resulted in the obtaining of a fragment as long as 231 bp. The study revealed differences in the ITS-2 spacers between *H. contortus* and *H. placei* at the level of 1.3% on three nucleotides only between purines. Besides, the work of the authors indicates possible changes in other positions. It is noteworthy that the replacements of bases in positions 123 and 196 in nucleotide sequences in *H. contortus* when compared with the Australian populations (figure 1).

The level of difference by site ITS-2 within the *Haemonchus* species is low. So, between *H. placei* from Uzbekistan and *H. placei* of bovine animals in Australia (Stevenson et al., 1995), it was 0.43%, while in *H. contortus* and *H. contortus* from respective places it reached 0.86%. The reported differences in our opinion, are only intraspecific.

Figure 2 shows the phylogram obtained on the basis of ITS-2 sequences of two *Haemonchus* varieties and their intraspecific isolates, as well as the closely related genus *Trichostrongylus* from the Trichostrongylidae family.

In the last few years, methods of molecular taxonomy started to be applied for the study of polymorphism. Genetic descriptions of three species, namely *H. longistipes* (camel), *H. placei* (zebra) and *H. contortus* (sheep and goats) were obtained resulting from the study using RAPD markers.

	1	20	40	60
<i>H. contortus</i> *	----- ----- ----- ----- ----- -----			
<i>H. contortus</i> **	AACCATATACTACAATGTGGCTAATTTCAACATTGTTTGTCAAATGGCATTGTCTTTTA			
<i>H. placei</i> *	.....G.....			
<i>H. placei</i> **	.....G.....			
	61	80	100	120
<i>H. contortus</i> *	----- ----- ----- -----			
<i>H. contortus</i> **	GACAATTCCTCATTTCAGTTCAAGAACATATACATGCAACGTGATGTTATGAAATTGTAAC			
<i>H. placei</i> *	.....T.....			
<i>H. placei</i> **	.....			
	121	140	160	180
<i>H. contortus</i> *	----- ----- ----- ----- ----- -----			
<i>H. contortus</i> **	ATTCTGAATGATATGAACATGTTGCCACTATTTGAGTGACTCAGCGAATATTGAGATT			
<i>H. placei</i> *	..C.....			
<i>H. placei</i> **	..C.....			
	181	200	220	231
<i>H. contortus</i> *	----- ----- ----- -----			
<i>H. contortus</i> **	GACTTAGATAGTGACTTGTATGGCGACGATGTTCTTTTATCATTGTATAA			
<i>H. placei</i> *	.....A.....			
<i>H. placei</i> **	.....A.....A.....G.....			

Figure 1. Comparison of the nucleotide sequences data of ITS-2 region of *Haemonchus contortus* and *H. placei* according by Stevenson et al. (1995)\* and our original study\*\*

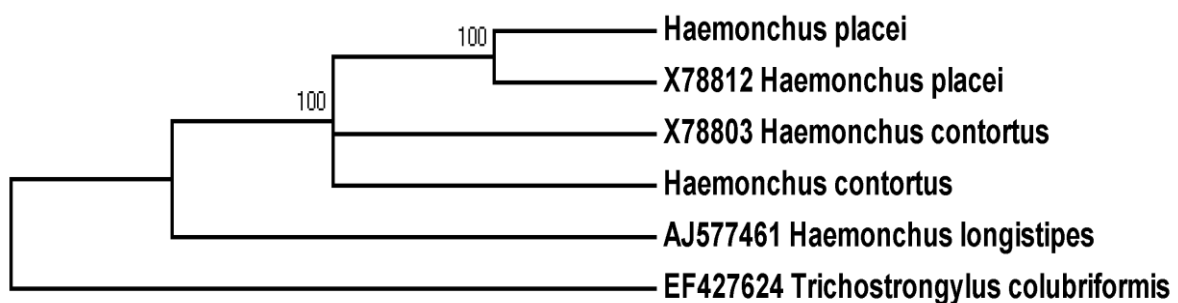


Figure 2. The phylogenetic tree obtained on the basis of sequences of ITS-2 region in two varieties of *Haemonchus* based on our own studies and GenBank data

These species, according to the materials of our studies, were quite isolated, although *H. contortus* and *H. placei* were more closely related to each other (Jacquet et al., 1995). Stevenson et al. (1995) conducted a comparative study of sites of the second interior transcribing spacer (ITS-2) in *H. contortus* and *H. placei* and revealed only three differences in nucleotide sequences of ITS-2. The authors made a conclusion that these

species were independent within the genus *Haemonchus*. To clarify the objectivity of these conclusions we conducted a comparative study of DNA samples of *H. contortus* collected from hosts inhabiting different regions. This comparison, in our view, will enable revealing the level of the intraspecific variability of DNA parts and provide an opportunity to increase the effectiveness of application of molecular

methods for the identification of taxonomy of parasitic nematodes.

Some scientists conducted studies of the mitochondrial DNA of nematodes. So, a low genetic diversity and presence of isolating inter-population barriers in nematodes, parasites of animals, were recorded while studying polymorphism in individual parts of the mitochondrial DNA. A high level of variability was recorded in nematodes parasitizing the intestine of sheep and cattle (Blouin et al., 1995).

The recorded level of polymorphism variation in adults *H. contortus* and *H. placei* is probably the result of the effect of different evolutionary factors affecting the structure of this parasite population at different stages of its ontogeny and host population (Morozova et al., 2002). Of these, the most important factors should be the selection of respective definitive hosts.

The analysis of conducted studies showed that the *Haemonchus* isolated from sheep is different morphologically from those of cattle. Thus, the study revealed morphological differences in the spicules of *H. contortus* and *H. placei*. In *H. contortus* the length of the left spicule reached  $509.9 \pm 7.95 \mu\text{m}$ , while that of the right spicule was  $511.5 \pm 7.91 \mu\text{m}$ . Each spicule had a sharp spike situated at varying distances from the distal end:  $53 \pm 0.72 \mu\text{m}$  and  $22.2 \pm 0.47 \mu\text{m}$  in the right and left spicules, respectively. In *H. placei*, the length of the left spicule was  $539.7 \pm 6.56 \mu\text{m}$ , while that of the right spicule,  $540.2 \pm 6.42 \mu\text{m}$ . The spine is situated at a varying length from the distal end:  $58.94 \pm 0.91 \mu\text{m}$  and  $29.67 \pm 0.74 \mu\text{m}$  in the right and left spicules, respectively.

Besides, spicules in the concave in *H. placei* were curved to the right, while the left spicule was convex from the hook to the end. In *H. contortus* spicules had straight distal ends.

We think that in *H. placei* the spicules were curved slightly to the right and the exterior edge from the hook to the end of the left spicule was convex, whereas in *H. contortus* the spicules were straight and the exterior side from the hook to the end of the left spicule was concave. Regarding the females, in *H. placei*

most of these have a ligulate-form valve and females are registered to have a semi-spherical protuberance at the side of the vulva, while in *H. contortus* females with the semi-spherical protuberance prevail; besides, other types of females as in *H. placei* are encountered. Besides, revealed some differences in the morphology of females. In *H. placei* females ovijectors are longer compared to those of *H. contortus* females –  $540 \pm 7.63 \mu\text{m}$  (=the reservoir of the ovijector with sphincters); the vulva and anus lie farther from the body end. It should be noted that concerning the change in the morphology of exterior female sex organs, the lobes are not a reliable index for identification of the haemonchus varieties.

Thus, we for the first time obtained data on the DNA structure of *Haemonchus* nematodes collected from Uzbekistan. The comparative analysis of ITS-2 fragments, using the ribosomal DNA between *H. contortus* and *H. placei* enabled the recording 2,6% of interspecific differences.

The number of morphological criteria and clear distinctions in the PCR pattern indicate the independence of the species *H. placei* within the genus *Haemonchus*.

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