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# On The Reproductive Relationships and Molecular Analysis of the *Marshallagia Marshalli* and *M.Occidentalis* (Nematoda: Ostertagiinae)

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**Abstract:** ***The article study the experience of identifying Marshallagia marshalli and M. occidentalis nematodes, which are supposedly constitute two morphologically distinct variants of the species, as well as their molecular identification. In the organism of small cattle infected with M. marshalli, obtained in a mono-invasive state, species belonging to both morphs were found, which indicates that there is no genetic isolation between them. When studying the nucleotide sequence of these morphs, no differences were found between them. Experimental data, as well as the results of molecular work, confirm that M. occidentalis is a minor morph of the species M. marshalli.***

**Keywords:** Nematode, Marshallagia, sheep, goat, morph, major, minor, mitochondrial DNA, PCR, sequence

## 1. Introduction

Nowadays all over the world, more and more large-scale infection of farm animals with various species of parasitic nematodes is an urgent problem of agriculture and hinders the development of animal husbandry, leading to a sharp decline in agricultural production. Thus, infestation of livestock with various parasitic nematodes reduces the productivity of animals by 9-12%, reducing the yield of milk, meat and wool (Amirov, 2017). In this regard, the development of measures to identify and control parasitic nematodes widespread in farm animals is considered as an urgent problem in this field.

Representatives of the Trichostrongids, including *Marshallagia* Orloff, 1933 (Nematoda: Trichostrongylidae) are ruminant parasites and have been found in almost all countries of the world (Fox, 1997). Trichostrongylides reproduce by laying eggs, therefore, at the invasive stage, eggs get into the external environment along with animal excrement. They are considered geohelminths, that is, the larvae develop in the environment, enter the host through water or food. Under favorable conditions, that is, if there is an optimum temperature and humidity for development of the larvae, they will switch to an invasive state within 7-10 days (Oripov et al., 2009).

Males of some species of the subfamily Ostertagiinae Lopez-Neyra, 1947 (in many cases 2) are supposed to differ in morphological features (Drozdz 1965). There are currently 5 polymorphic species of the genus *Marshallagia* (Drozdz 1995). In particular, the *Marshallagia marshalli* is considered as a "major" species, and its "minor" type is *M. occidentalis*.

To address the issue of the polymorphism of the nematode Ostertagiinae, Daskalov (1974), first conducted experiments on the infection of sheep with the method of "cross-infection". According to the study, the author concluded that there is no genetic border between *T. circumcincta*, *T. trifurcata* and *T. davtiani*. It was noted that the male *T. circumcincta* naturally met more often than other morph. Similar studies have been done and Lancaster et al. (1981), Cabaret et al. (1984) and Suarez et Cabaret (1992) other species of ostertagiine.

The aim of the study is to "cross-infection" small cattle with the species of *M. marshalli* in a monoinvasive and identification detected morphs by molecular methods.

## 2. Materials and methods

***Collecting material and conducting experiments***. To perform this work for helminthological methods of *M. marshalli* nematodes were collected in a "monoinvasive" state during the slaughter of domestic sheep in a slaughterhouse located in the city of Tashkent. Eggs were isolated from *M. marshalli* females and transplanted into Petri dishes. To ensure the maturation of eggs in vitro, nutrient medium No. 199 was used and a solution of streptomycin (Streptomycin, 5 mg) was used as an antibiotic. Petri dishes with eggs nematodes were placed in a thermostat at a temperature of 28-32° C, and the larvae hatched from the eggs were fed to an invasive state.

For the experiment, 4 goats and 4 sheep were chosen (they were spared from worms and were outside the invasion). 4 goats and 2 sheep per os were infected by nematode *M. marshalli* larvae, which reached an invasive state. The animals were divided into 4 groups: I-III groups - experimental, and IV group - control (table). On the 35th day of the experiment, that is, when the marshallas reached maturity, one animal from each group was taken for an autopsy. A 70% ethanol solution was used to fix the collected helminthological material. In determining the species, the morphology and morphometry of male individuals were studied (Ivashkin et al., 1989).

***DNA extraction, PCR Amplification, Sequencing and Phylogenetic Analysis***. To isolate DNA from the composition of nematode samples, 20 µl of NaOH (0.25 M) was added to each sample and left at room temperature for

12 hours, then the temperature was increased to 95° C and kept under these conditions for 3 minutes. Samples were diluted with 10 µl of Tris-HCl and altered with a vortex, then centrifuged for 2 minutes. Removing from the centrifuge, 4 µl HCI (1:15) were added and mixed again with a vortex and centrifuged, then 5 µl triton (2%) was added. Aged for 3 minutes at 95° C and stored at -20° C.

Primers (COI-F GGTTGGAGAGTTCTAATCATAAAGA, COI-R CCCAAACATAGTAGCCAACCA) of the mitochondrial DNA (mtDNA) COI gene were used to identify nematodes, which are widely used in molecular taxonomy. Polymerase chain reaction (PCR) was carried out according to the following scheme: at the 1 st stage - DNA denaturation at a temperature of 94 ° C for 5 minutes, at the 2 nd stage - DNA denaturation at a temperature of 94° C for 45 seconds, at the 3 rd stage - loosening the DNA at 55° C for 45 seconds, at the 4th stage - elongation at 72° C for 1 minute and 40 seconds, at the 5th stage - chain elongation at 72° C for 5 minutes. From the second to the fourth stage, the process was repeated cyclically up to 35 times.

The presence of DNA in PCR products was determined by electrophoresis in a 1.0% agarose gel at a voltage of 120 V. For amplification of DNA and separation of DNA from gels, a kit of reagents produced by Sileks M (Moscow, Russia) was used in compliance with the manufacturer's instructions.

DNA sequencing was performed using the ABI PRISM® BigDye ™ Terminator v. Reagent kit. 3.1, the reaction products were recorded in an ABI PRISM 3100-Avant automatic sequencer (Moscow, Russia). The analysis of the obtained nucleotide sequence was carried out using a special computer program Bioedit, Clustal W, DNAstarTM and PAUP4.

Phylogenetic trees were constructed using the Maximum Likelihood (ML) and Neighbor Joining (NJ) methods using the MEGA 6.1 software (Tamura et al., 2011). For all NJ, ML, and ME analyses, the most appropriate nucleotide substitution model was determined, gaps were treated as missing data and internal node support was assessed by bootstrapping over 500 replicates.

## 3. Results

Experimental work carried out showed that at the opening of experimental animal's male specimens of *M. marshalli* and *M. occidentalis* were mainly extracted from their abomasum (table 1).

**Table 1:** Infection of small cattle with nematode larvae of *M. marshalli*

| Group | Experiments | | | Obtained results | |
|---|---|---|---|---|---|
| | Number of animals | Infected with species | Number of larvae | Name of species | Amount of larvae |
| I | 2 goat | *M. marshalli* | 700 | *M. marshalli*<br>*M. occidentalis* | 145<br>14 |
| II | 2 goat | *M. marshalli* | 700 | *M. marshalli*<br>*M. occidentalis* | 153<br>18 |
| III | 2 sheep | *M. marshalli* | 700 | *M. marshalli*<br>*M. occidentalis* | 132<br>8 |
| Control | | | | | |
| IV | 2 sheep | - | - | - | - |

When animals of I, II, III groups were infected with nematodes of *M. marshalli*, the intensity of invasion was 132-153 copies, with nematodes of *M. occidentalis* - 8-18 copies. (Table 1). No nematodes were detected in two sheep from the control group.

Therefore, the results of the experiments indicate the absence of a genetic barrier between species of *M. marshalli* and *M. occidentalis*, that is, *M. marshalli* is a major morph, and *M. occidentalis* is its minor morph. This experiment confirms the hypothesis of the presence of different morphs in one type of ostertagiins (Drozdz 1995).

To confirm the obtained data, we analyzed these two morphs using the molecular method.

For this, 3 samples of *M. marshalli* and *M. occidentalis* were taken, and for the outer group, male nematodes *O.ostertagi* were taken followed by isolation of total DNA. Fragments of the gene for mitochondrial (mt) DNA were isolated from the samples of this nematode.

According to the results of molecular studies, 650 pairs of nucleotides were isolated from mtDNA nematodes *M. marshalli, M. occidentalis* and *O. ostertagi* (Table 2).

**Table 2:** Differences between nucleotide sequences in nematode specimens

| No. | Species | *Marshallagia marshalli* | *Marshallagia occidentalis* | *Ostertagia ostertagi* |
|---|---|---|---|---|
| 1 | *Marshallagia marshalli* | - | - | 3 |
| 2 | *M. occidentalis* | - | - | - |
| 3 | *Ostertagia ostertagi* | 17 | 17 | - |

*Note:* the table shows the differences between the mtDNA nucleotides in the amount of 650 pairs of the COI gene

As can be seen from the table, there are no differences between the *Marshallagia marshalli* and *M. occidentalis* nucleotides. Between nucleotides of *M. marshalli* and *Ostertagia ostertagi*, 17 differences were found, which amounted to 3%. The obtained nucleotide sequences were compared with data from the Genbank database (NCBI). At the same time, on the basis of some data obtained from this database, using the MEGA 5 program, a phylogenetic tree was compiled using the Maximum Likelihood method (Fig.).

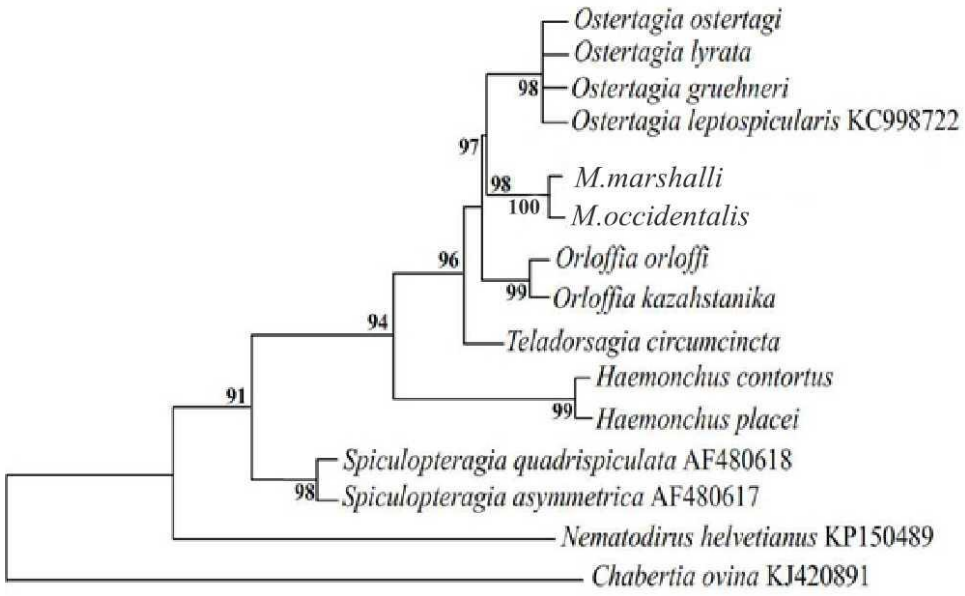


**Figure:** A phylogenetic tree of species of the subfamily Ostertagiinae (500 bootstrap-repeats). Bootstrap support values are listed in the corresponding nodes

Thus, the phylogenetic analysis showed that the representatives of the ostertagiains subfamily form four evolutionary groups: the first group consists of the genus *Ostertagia* Ransom, 1907, the second group - *Marshallagia* Orloff, 1933, the third group - *Orloffia* Drozdz, 1965 and the fourth group - *Teladorsagia* Andreeva et Satubate , 1954. Representatives of these groups are closely related to each other. Thus, the species *Marshallagia marshalli* and *M. occidentalis* have 100 percent bootstrap support, which means that they are representatives of the same species. The next fifth and sixth groups of the phylogenetic tree are representatives of the genus *Haemonchus* and *Spiculopteragia*, compared with other groups of ostertagiains, they have a bootstrap-support value of 91-94%.

In conclusion, it should be noted that the data of the experiment on “cross-infection” confirm the assumption that *M. marshalli* and *M. occidentalis* are always found together, and the results of a comparative molecular study indicate that *M. occidentalis* is a minor morph of *M. marshalli*.

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