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Morphological and molecular identification of *Nematodirus* species (Nematoda, Molineidae) from domestic ruminants in Uzbekistan

H. F. Sobirov*, A. E. Kuchboev*, M. B. Abramatov**

**Institute of Zoology, Academy of Sciences of Uzbekistan, Tashkent, Uzbekistan*
***Termez State University, Termez, Uzbekistan*

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Institute of Zoology Academy of Sciences of Uzbekistan, Bogishamol st., 232b, Tashkent, 100053, Uzbekistan. E-mail: abdurakhimkuchboev@gmail.com

Department of Ecology and Soil Science, Termez State University Barkamol Avlod st., 43, Termez, 190111, Uzbekistan. E-mail: mabramatov@bk.ru

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The species composition and distribution of the genus *Nematodirus* have not been studied in Uzbekistan for a long time, even though nematodes are widespread in the digestive tract of domestic and wild ruminants and are one of the main components of the Molineidae fauna.The article presents the results of a study on the morphometric and molecular analysis of the genus *Nematodirus* Ransom, 1907, nematodes of the digestive system of ruminant ungulates living in the territory of Uzbekistan. In the study, the species of *Nematodirus* identified from domestic sheep and goats can mainly be distinguished by the shape of the male spicules and the structure of the bursa. *Nematodirus abnormalis* can be distinguished from other species because the tip of the spicule is slightly curved and covered with an asymmetrical lanceolate membrane, *N. oiratianus* has a fused tip and a thin tube-like shape, and *N. spathiger* has a slightly curved distal tip of the spicule. Furthermore, based on the nucleotide sequence results of the rDNA ITS-2 region, the *N. abnormalis* samples studied were found to match *Nematodirus* sp. in the GenBank database, while the *N. oiratianus* and *N. spathiger* samples were 98–99% similar to the respective species. Phylogenetic analysis using ITS-2 nucleotide sequences revealed that *N. oiratianus* and *N. abnormalis* are closely related and sister species and *N. spathiger* and *N. helvetianus* are also phylogenetically close. The species *N. abnormalis* was deposited for the first time in the GenBank database.

Keywords: nematode; ribosomal DNA; ITS region; *Nematodirus*; domestic sheep; domestic goat.

Introduction

Nematodes of the genus *Nematodirus* Ransom, 1907, are distributed worldwide, and more than 40 species are known to science (Taylor et al., 2016). Nematode species belonging to this genus are also widespread in ruminants, mainly in Great Britain, Norway, New Zealand, Canada, and the United States (Oliver et al., 2016). *Nematodirus* are an important group of nematodes that parasitize the small intestine of all ruminant ungulates (Anderson, 2000; Hoberg et al., 2005; Abramatov et al., 2022).

Khrustalev (2011) analyzed 28 *Nematodirus* species recorded in the Commonwealth of Independent States (CIS) region and found that 19 of them were true species, 5 were synonyms, and 4 were listed as non-existent or incongruous species. In a subsequent work by this author, he suggested that the species *N. skryabini* Mizkewitsch, 1980 and *N. tarandi* Hadwen, 1922, which are widespread in deer, are morphologically indistinguishable and that *N. skryabini* Mitzkewitsch, 1929 should be reconsidered as a synonym of *N. tarandi* Hadwen, 1922.

Lichtenfels & Pilitt (1983) identified six species of the genus *Nematodirus* that parasitize domestic ruminants in North America through morphological studies. In addition to distinguishing species by the shape, length, and shape of the male bursa, light and scanning electron microscope images were also crucial for identifying them by their cuticular lines. *Nematodirus* males are morphologically distinct from each other by the presence of bursa and spicules on the tail, and by the long and slender spicules with different shapes at the tip (Rashid et al., 2019). The different shapes of the bursa and spicules are necessary for morphological identification of species (Soulsby, 1968; Rickard & Hoberg, 2000).

Alejandro et al. (2017) first identified *N. helvetianus* May, 1921 in cattle in Southern Chile, and provided morphological and morphometric data in their study. Melnychuk et al. (2021) studied the morphometric characteristics of adult male and female nematodes of *N. spathiger* Railliet, 1896 found in sheep in Ukraine. They proposed the use of 40 morphometric characteristics to identify male nematodes. Of these, 11 characteristics were related to the size of the body, 24 characteristics to the size of the tail, and bursa, and 5 characteristics to the size of the spicules and the covering membrane. They proposed the use of 25 morphometric characteristics to help identify female *N. spathiger*.

The identification of *Nematodirus* is determined based on classical morphological characteristics. However, such classical methods often cause difficulties in determining the species of nematodes, especially for larvae and female nematodes. Therefore, in recent years, molecular genetic methods have been widely used in species identification. The ITS-2 region of ribosomal DNA is mainly used for molecular genetic identification of nematode species (Kuchboev et al., 2015; Kuchboev & Kruchken, 2022; Mirzaev et al., 2024). In particular, the ITS-2 region of ribosomal DNA has been used as a useful marker for species identification and differentiation of species of the genus *Nematodirus*. The nucleotide sequence of the ITS region of rDNA of the species *N. battus*, *N. davtiani*, *N. europaeus*, *N. filicollis*, *N. helvetianus*, *N. oiratianus*, *N. spathiger* and *N. rupicaprae* belonging to this genus allows phylogenetic analysis of species and allows precise identification of species (Gasser et al., 1999). Newton et al. (1998) experimentally demonstrated that the nucleotide sequence of the ITS-2 region of four species of the genus *Nematodirus* is useful for precise identification of species. The sequence differences between the nucleotides of *N. spathiger*, *N. helvetianus*, *N. fillicolis*, and *N. battus* were 3.9–24.7% (Newton et al., 1998). The experiment revealed that the most genetically close species of these species are *N. spathiger* and *N. helvetianus*. The results of the study indicate that ITS-2 sequence data can be useful in studying the systematics of nematodes of the Molineoidea family.

In several countries around the world, including China, the complete mitochondrial DNA (mtDNA) genomes of *N. oiratianus* and *N. spathiger* nematode species found in small ruminant animals have been analyzed, with nucleotide sequences of 13,765 and 13,519 bp obtained, respectively. The total mtDNA sequence difference between *N. oiratianus* and *N. spathiger* was 16.29%. Phylogenetic anal-

ysis of *N. oiratianus* and *N. spathiger* samples showed a close phylogenetic relationship with the Dictyocaulidae family (Zhao et al., 2014). Petrigh & Fugassa (2014) obtained an 849 bp identical nucleotide sequence of *Nematodirus* larvae from guanaco llama feces in Argentina's Perito Moreno National Park using the rDNA ITS-2 marker. When compared with species in the NCBI GenBank database, it showed 99% similarity with *N. spathiger* and 97% with *N. helvetianus*. In the study by Alhaboubi et al. (2021), *N. helvetianus* was identified from the digestive system of camels in Iraq. The obtained results, when analyzed with samples from the GenBank database, indicated that these samples were very close to *N. helvetianus*.

Molecular analysis of invasive larvae of the genus *Nematodirus* in sheep raised on natural pastures in the Inner Mongolia Autonomous Region of China revealed that they belonged to the species *Nematodirus oiratianus* and were grouped with *N. oiratianus* in the phylogenetic tree (Liu et al., 2023). Similar studies have shown that nematode eggs found in fecal samples of Australian llamas are *N. spathiger*, *N. filicollis*, *N. helvetianus*, and *N. abnormalis* when examined by molecular genetic methods (Rashid et al., 2019).

In the study of the phylogenetic relationships of species in the genus *Nematodirus*, when the nucleotide sequences of the ITS region of rDNA were analyzed for the species *N. battus*, *N. helvetianus*, *N. spathiger*, and *N. filicollis*, *N. helvetianus* and *N. spathiger* formed the innermost monophyletic group. In subsequent phylogenetic groups, *N. filicollis* and *N. battus* were very close. The results of this study show that the ITS-1 and ITS-2 regions can provide valuable information not only for species identification but also for the phylogenetic analysis of representatives of the genus *Nematodirus* (Audebert et al., 2000). Similar analyses were performed by Nadler et al. (2000) on the sequence of the rDNA 18S, ITS-1, 5.8S, ITS-2 and 28S regions among *N. helvetianus*, *N. spathiger*, *N. filicollis* and *N. battus*. Nineteen changes were detected between the genes of *N. battus* in samples collected from different regions (Nadler et al., 2000).

In addition to the morphological characteristics of the species, the availability of PCR identification methods is important in the diagnosis of diseases. Based on the information given above, it is necessary to conduct experiments on species of *Nematodirus* that have not been studied from the molecular-genetic point of view because it is important in studying their phylogenetic relationships.

The study aims to conduct morphometric characteristics and molecular genetic analysis of the nematodes *N. abnormalis*, *N. spathiger*, and *N. oiratianus* obtained from the small intestine of sheep and goats.

Materials and methods

Samples of the parasitic *Nematodirus* species were collected from domestic animals in the private sector of Jizzakh, Navoiy, Surkhandarya, Kashkadarya, Tashkent, Fergana and Namangan regions of Uzbekistan in 2024. During the study, 80 domestic sheep (*Ovis aries*) and 64 domestic goats (*Capra hircus*) were examined using helminthological methods. The samples from the genus *Nematodirus* were washed with 0.9% NaCl solution and stored in 70% ethanol solution until research (Soulsby, 1968; Ivashkin et al., 1989; Taylor et al., 2016; Rashid et al., 2019; Melnychuk et al., 2021). In total, 1200 adult male and female nematodes of the species were collected, 280 males and 920 females. A total of 112 male and 370 female nematode specimens of the species *N. abnormalis*, 82 male and 250 female specimens of *N. oiratianus*, and 86 male and 300 female specimens of *N. spathiger* were identified. The metric parameters of the adult male and female nematodes were analyzed using photographs of the nematode species taken using Nexcope NE930-FL microscopes in interactive mode using ×5, ×10, ×40 objectives, and ×10 photo eyepiece. Photomicrographs were taken using a digital camera mounted on a NEXCAM-T5 20MP microscope (China) for the identification of parasitic nematode species. Standard deviation (SD) and average values (x) were calculated. The reliability of the differences in mean values for the studied groups of nematodes was determined using the one-way analysis of variance and F-test at a 95.0% confidence level. Mature male and female nematodes found in each animal were analyzed morphologically. In species identification, the characteristics recommended in the literature were applied, especially focusing on the shape and length of the spicules, as well as the shape of the dorsal ray.

In the study, genomic DNA was isolated from *N. abnormalis*, *N. spathiger*, and *N. oiratianus*. DNA was isolated from nematodes using the GeneJET Genomic DNA Purification KIT (Thermo Scientific™, Lithuania) and the manufacturer's protocol. The DNA concentration in genomic DNA samples was determined using a microspectrophotometer (NanoDrop Lite Plus, Thermo Fisher, USA). Polymerase chain reaction was performed using a ProFlex PCR System (China). The nucleotide sequence of the ITS-2 region for PCR was forward NC1: 5′-ACGTCTGGTTCAGGGTTGTT-3′ and reverse NC2: 5′-TTAGTTCTTTTCTCCGCT-3′ primers (Gasser et al., 1993). For the polymerase chain reaction, 0.4 µL dNTP Mix (10 mM), 2 µL buffer (DreamTaq Buffer), 2 µL primers, 0.4 µL polymerase (DreamTaq), 13.2 µL water, and 2 µL DNA sample were added. The PCR was performed for 35 cycles, initial denaturation for 2 min at 95 °C, denaturation for 1 min at 95 °C, annealing for 1 min at 55 °C, synthesis for 1 min at 72 °C, and final synthesis for 5 min at 72 °C (Kuchboev et al., 2020). The PCR products were analyzed by electrophoresis on a 2% gel and the samples were found to contain rDNA fragments of 300 base pairs (bp) nucleotide sequence. The PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, Lithuania) was used for purification of the PCR products.

For the sequencing reaction of the PCR products, 1 µL of Quantum Dye Terminator v.3.1, 2 µL of 5X Sequencing Buffer, 2 µL of purified PCR product, and 4 µL of water were added. The sequencing reaction for the forward primer was performed with an initial denaturation at 95 °C for 1 minute, denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, synthesis at 72 °C for 1 minute, and a final synthesis step at 72 °C for 5 minutes. For the reverse primer, the initial denaturation was at 95 °C for 1 minute, denaturation at 95 °C for 30 seconds, annealing at 54 °C for 30 seconds, synthesis at 72 °C for 1 minute, and the final synthesis step at 72 °C for 5 minutes.

For the phylogenetic analysis of species in the genus *Nematodirus*, the ITS-2 domain data of this genus from the National Center for Biotechnology Information (NCBI, GenBank) database were used. The programs SnapGene Viewer and BioEdit v7.2.0 were used to analyze the obtained sequence data. For the phylogenetic analysis of species in the genus *Nematodirus*, the programs Clustal W2, SeaView 5.0.5, Mega 12, IQ-TREE, FigTree 1.4.4 were used (Tamura et al., 2006).

The nucleotide sequences obtained for the rDNA ITS-2 region of molecular genetic studies were deposited in the National Center for Biotechnology Information (NCBI) gene bank database under the accession numbers *Nematodirus spathiger* – PV135955; PV135956, *N. abnormalis* – PQ725622; PQ725609; PQ725599, and *N. oiratianus* – PV124855; PV133512 (www.ncbi.nlm.nih.gov). In the study of phylogenetic relationships, the out group *Telodorsagia circumcincta* (ON004115) was used to construct a maximum likelihood phylogenetic tree.

Results

*Morphological and morphometric analysis.*Based on our research and literature data, the species were accurately identified according to morphological and morphometric characteristics. This study presents the morphological characteristics and morphometric measurements of *N. abnormalis*, *N. spathiger*, and *N. oiratianus* species found in domestic sheep and goats.

The average body dimensions of *N. abnormalis* May, 1920 male and female nematode specimens were determined. The total body length of the male was 12.5–16.5 mm, with an average length of 15.1 ± 0.9 mm, the esophagus length is 0.462–0.577 mm, with an average length of 0.52 ± 0.02 mm, the head was surrounded by a vesicle, with a length of 0.085–0.105 mm, with an average length of 0.102 ± 0.004 mm, the tip of the spicule was slightly oblique, covered with an asymmetrical lanceolate membrane, with a length of 0.90–1.25 mm, with an average length of 1.10 ± 0.04 mm. The dorsal rays in the bursa were separated from the external dorsal rays laterally, the average length of dorsal ray was 0.061 ± 0.003 mm, the average length of

external dorsal rays was 0.125 ± 0.006 mm, and the tip was reduced. The length of the bursa was in the range of 0.142–0.162 mm, the average length was 0.154 ± 0.01 mm, the width of the bursa was 0.515–0.542 mm, the average length was 0.52 ± 0.02 mm. The average body length of the female was 20.10 ± 1.02 mm, the vesicle surrounds the head, the average vesicle length was 0.105 ± 0.005 mm, the average esophagus length was 0.52 ± 0.02 mm The vulva of the female nematodes was located slightly below the middle of the body. The average length of the area of the vulva was 0.121 ± 0.005 mm. The maximum body width corresponded to the vulva region, and the body tapered from the vulva region onward. The average egg length was 0.215 ± 0.008 mm, and the average egg width length was 0.110 ± 0.004 mm (Table 1, Fig. A, B, C).

Table 1
Morphometric parameters of genus of *Nematodirus* species (n = 10, x ± SD, min–max)

Parameters of nematode	*N. abnormalis*	*N. spathiger*	*N. oiratianus*
male nematodes ♂			
Length of body	15.1 ± 0.9 (12.5–16.5)	15.7 ± 1.0 (12.0–19.0)	17.7 ± 1.10 (13.5–20.0)
Width of body	0.135 ± 0.005 (0.120–0.160	0.137 ± 0.006 (0.120–0.150)	0.151 ± 0.007 (0.125–0.154)
Length of esophagus	0.520 ± 0.020 (0.450–0.570)	0.487 ± 0.024 (0.450–0.520)	0.627 ± 0.030 (0.452–0.624)
Length of vesicle	0.102 ± 0.004 (0.085–0.105)	0.108 ± 0.004 (0.090–0.120)	0.126 ± 0.006 (0.110–0.140)
Length of spicules	1.10 ± 0.04 (0.90–1.25)	0.90 ± 0.04 (0.90–1.20)	1.01 ± 0.04 (0.95–1.10)
Length of bursa	0.154 ± 0.010 (0.142–0.162)	0.161 ± 0.006 (0.130–0.180)	0.161 ± 0.006 (0.152–0.165)
Width of bursa	0.520 ± 0.020 (0.515–0.542)	0.191 ± 0.010 (0.162–0.192)	0.485 ± 0.020 (0.465–0.485)
Length of dorsal ray	0.061 ± 0.003 (0.058–0.064)	0.064 ± 0.030 (0.058–0.065)	0.062 ± 0.030 (0.053–0.064)
Length of external dorsal ray	0.125 ± 0.006 (0.115–0.138)	0.128 ± 0.070 (0.124–0.142)	0.132 ± 0.080 (0.125–0.138)
female nematodes ♀			
Length of body	20.1 ± 1.0 (18.5–24.0)	18.2 ± 0.8 (14.0–22.0)	24.7 ± 0.5 (20.5–28.0)
Length of vesicle	0.105 ± 0.005 (0.090–0.120)	0.109 ± 0.004 (0.100–0.120)	0.126 ± 0.005 (0.120–0.130)
Length of esophagus	0.52 ± 0.02 (0.48–0.58)	0.49 ± 0.02 (0.45–0.57)	0.66 ± 0.03 (0.55–0.75)
Length of the area of vulva	0.121 ± 0.005 (0.115–0.128)	0.118 ± 0.005 (0.110–0.121)	0.126 ± 0.004 (0.115–0.132)
Distance from vulva to anus	6.40 ± 0.23 (6.20–7.60)	5.80 ± 0.24 (5.20–6.40)	7.10 ± 0.25 (6.70–8.20)
Egg length	0.215 ± 0.008 (0.210–0.230)	0.205 ± 0.008 (0.190–0.220)	0.248 ± 0.007 (0.220–0.270)
Egg width	0.110 ± 0.004 (0.950–0.120)	0.103 ± 0.005 (0.095–0.110)	0.125 ± 0.001 (0.100–0.150)

The male nematodes of the species *Nematodirus spathiger* Railliet & Henry, 1909 had the following morphometric characteristics: body length 15.7 ± 1.0 mm, body width 0.137 ± 0.006 mm, esophagus length 0.487 ± 0.024 mm, vesicle length 0.1075 ± 0.0043 mm, spicule length 0.90 ± 0.04 mm, and bursa length 0.1605 ± 0.0064 mm. The average length of dorsal ray 0.064 ± 0.030 mm and the length of external dorsal ray 0.128 ± 0.070 mm. The female *N. spathiger* had a total body length of 18.2 ± 0.8 mm, the vesicle length of 0.109 ± 0.004 mm, and esophagus length of 0.488 ± 0.024 mm, the length of the area of vulva 0.118 ± 0.005 mm, and the distance from vulva to the anus 5.80 ± 0.24 mm (Table 1; Fig. 1D, 1E, 1F).

The average body length of male nematodes of the species *Nematodirus oiratianus* Rajewskaja, 1929 was 17.65 ± 1.10 mm, the average body width was 0.151 ± 0.007 mm. The average spicule length was 1.01 ± 0.04 mm, and the spicules were fused and ended in a thin lanceolate tip. The length of the male nematode esophagus was 0.627 ± 0.030 mm. The width of the head without a vesicle was 0.039 ± 0.002 mm, and the length of the nematode bursa was 0.161 ± 0.006 mm. The average width of bursa is 0.485 ± 0.020 mm, the average length of dorsal ray 0.062 ± 0.030 mm and average length of external dorsal ray 0.132 ± 0.080 mm. The average body length of *N. oiratianus* females is 24.70 ± 0.52 mm, the average body width was 0.151 ± 0.007 mm, and the width of the head with vesicle was 0.061 ± 0.002 mm. The width of the head without vesicles was 0.041 ± 0.002 mm, the average length of the esophagus was 0.655 ± 0.030 mm. The average length of the area of the vulva was 0.126 ± 0.004 mm, the average distance from the vulva to the anus 7.10 ± 0.25 mm (Table 1; Fig. 1G, 1H, 1I).

In the conducted studies, only two parameters of the eggs of this species of nematodes were used, width and length. The eggs of nematodes of the genus *Nematodirus* were distinguished by their large size and prominent blastomeres compared to those of other genera of the Molinidae family (Fig. 2). Also shown was the first stage of larvae (L_1) development from eggs of the genus *Nematodirus* (Fig. 2).

Nematodirus abnormalis, *N. spathiger*, and *N. oiratianus*, which were identified in domestic sheep and goats, are morphologically distinct from each other and can be distinguished mainly by the shape of the spicule and the structure of the bursa. In *N. abnormalis*, the tip of the spicule is slightly curved, covered with an asymmetrical lanceolate membrane, and its length was on average 1.10 ± 0.04 mm. The tip of the spicule of *N. oiratianus* is fused and ends in a thin tube. *N. spathiger* can be distinguished from other species by the slightly curved distal tip of the spicule. The females of *N. abnormalis* and *N. spathiger* are extremely similar in the vulval region of these species, so the males of these nematodes were used for differentiation. The female *N. oiratianus* has a slit-shaped opening at the vulva, located transversely and has two lips, with the anterior lip slightly bent inward in the shape of a beak.

According to the PCR results, 300 bp fragments of rDNA ITS-2 region were isolated from samples of *Nematodirus* species. The identification of *Nematodirus* species was carried out by analyzing the ITS-2 region samples obtained from the GenBank database (Table 1). As a result, when the *N. spathiger* sample was compared with the *N. spathiger* sequences (KC580741; KC998747; KY930440) in the GenBank database using the Blast program, the nucleotide sequence showed 96% similarity.

The next three samples of the *N. abnormalis* species were found to have a 98% nucleotide sequence identity with the *Nematodirus* sp. (HQ844230) in the GenBank database. The *N. abnormalis* species and *Nematodirus* sp. (HQ844230.1) species differed by 6 nucleotide pairs, namely, A-adenine instead of G-guanine at nucleotide 21, A-adenine nucleotide instead of T-thymine at nucleotide 153, T-thymine instead of A-adenine at nucleotide 157, G-guanine instead of C-cytosine at nucleotide 229, A-adenine instead of T-tymine nucleotide 330, and C-cytosine instead of A-adenine at nucleotide 327. The total, *N. abnormalis* (PQ725622) and *Nematodirus* sp. (HQ844230) were found to differ by 6 nucleotides. It should be noted that the nucleotide sequence of the *N. abnormalis* species is not available in the GenBank database. The nucleotide sequence of the ITS-2 region of the morphologically identified *N. abnormalis* species and its three samples was first placed in the NCBI database.

In our study, the sample of *N. oiratianus* (PV124855) showed 98% similarity with the *N. oiratianus* (OP879215) species in the GenBank database. In this case, the *N. oiratianus* (PV124855) sample showed the following nucleotide differences: at nucleotide 191, G-guanine was replaced by A-adenine; at nucleotides 326-329, A-adenine was replaced by C-cytosine; and at nucleotide 327, A-adenine was replaced by T-thymine.

The phylogenetic relationships of the *Nematodirus* species were analyzed by comparing the rDNA ITS-2 sequences of the species *N. spathiger*, *N. oiratianus*, and *N. abnormalis* studied by us and the existing species in the GenBank (NCBI) database (Table 2).

In the study, according to the phylogenetic tree constructed based on the nucleotide sequence of the ITS-2 region, the species *N. battus*, *N. filicollis*, *N. helvetianus*, *N. spathiger*, *N. tarandi*, *N. andersoni*, *N. rupicaprae*, *N. davtiani*, *N. abnormalis*, *Nematodirus* sp. and *N. oiratianus* belonging to the genus *Nematodirus* of the family Molineidae were analyzed and united into 10 distinct monophyletic groups (clades). The species *T. circumcincta* of the genus *Teladorsagia* was used as an outgroup in the study.

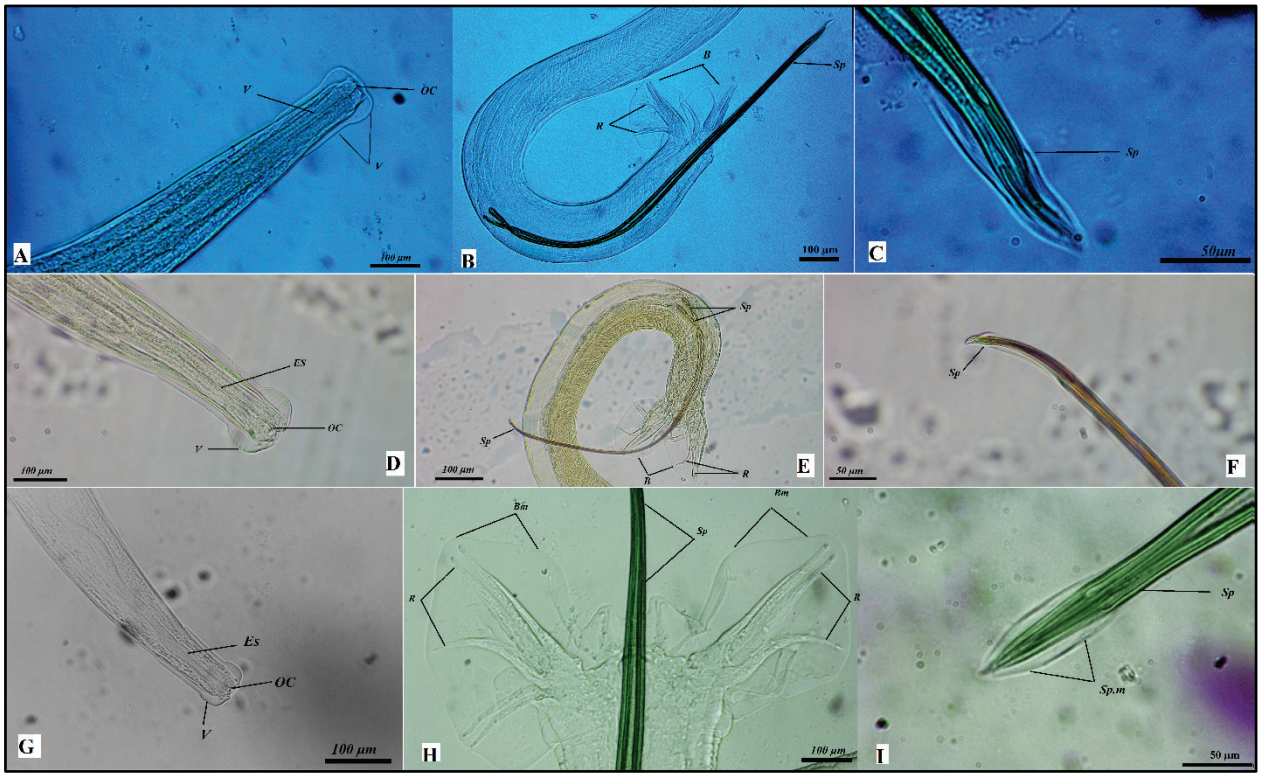

Fig. 1. Microphotography of morphological characteristics of *Nematodirus* species at different magnifications: *A* – head and of ♂ *N. abnormalis*; *B* – tail end of ♂ *N. abnormalis*; *C* – spicule of ♂ *N. abnormalis*; *D* – head end of ♂ *N. spathiger*; *E* – tail end of ♂ *N. spathiger*; *F* – spicule of ♂ *N. spathiger*: *G* – head and of ♂ *N. oiratianus*; *H* – tail end of ♂ *N. oiratianus*; *I* – spicule of ♂ *N. oiratianus*: abbreviation inside the figure; *Es* – esophagus, *Oc* – oral cavity, *V* – vesicle, *Sp* – spicule, *B* – bursa, *Bm* – bursa membrane, *R* – rays, *Sp.m* – spicule membrane

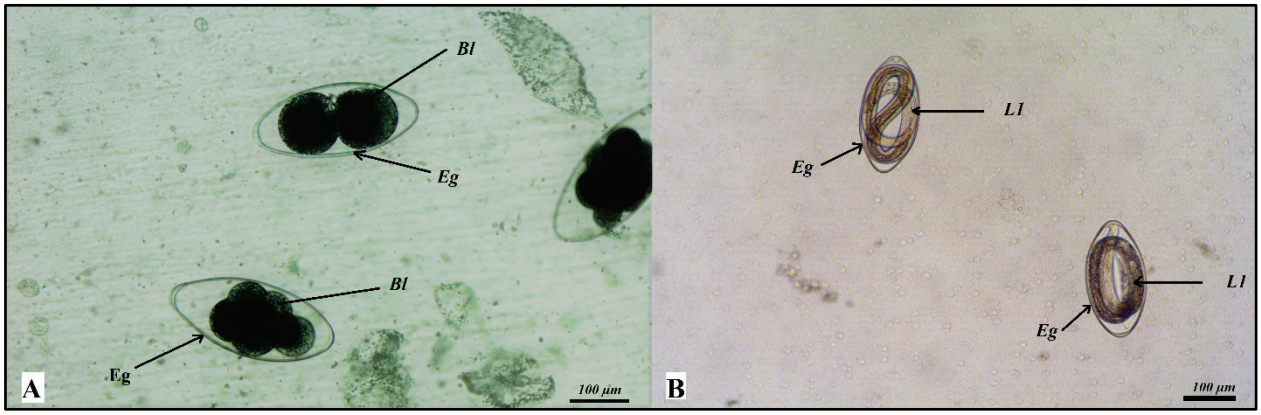

Fig. 2. Microphotography of morphological structure of *Nematodirus* sp. eggs (*A*) and the first stage of larvae development of *Nematodirus* sp. (*B*): *Bl* – blastomere; *Eg* – eggs of *Nematodirus* sp; *L1* – first stage of larvae

Table 2
Sources of sequences representing of *Nematodirus* from the Genbank data base used in comparisons and identification (rDNA ITS2)

No.	Nematode species	GenBank accession, ITS	Host range	Geographic distribution
1.	*Nematodirus battus*	Y14010	sheep	Australia
2.	*N. battus*	AY439023	soay sheep	United Kingdom
3.	*N. battus*	AF194132	sheep	USA
4.	*N. battus*	JF345079	sheep	Ireland
5.	*N. battus*	AF194124	sheep	USA
6.	*N. battus*	AF194123	sheep	Norway
7.	*N. battus*	AF194134	sheep	Canada
8.	*N. battus*	AF194131	sheep	USA
9.	*N. battus*	MZ478657	reindeer	Norway
10.	*N. davtiani*	OP879218	bighorn sheep	Canada
11.	*N. davtiani*	OP879217	bighorn sheep	Canada
12.	*N. davtiani*	OP879216	bighorn sheep	Canada
13.	*N. davtiani alpinus*	AJ239113	chamois	Italy
14.	*N. filicollis*	AY439024	soay sheep	United Kingdom
15.	*N. filicollis*	AF194140	sheep	USA
16.	*N. filicollis*	AF194139	sheep	USA
17.	*N. filicollis*	KC998750	sheep	New Zealand
18.	*N. filicollis*	KC998749	sheep	New Zealand
19.	*N. helvetianus*	AF194142	cattle	USA
20.	*N. helvetianus*	AF194141	cattle	USA
21.	*N. helvetianus*	AF194127	cattle	USA
22.	*N. helvetianus*	JQ828846	cattle	Russia
23.	*N. helvetianus*	KC580751	sheep	China
24.	*N. helvetianus*	KC580752	sheep	China
25.	*N. oiratianus*	AJ239112	chamois	Italy
26.	*N. oiratianus*	KR809574	sheep	China
27.	*N. oiratianus*	OP879214	bighorn sheep	Canada
28.	*N. oiratianus*	OP879215	bighorn sheep	Canada
29.	*N. oiratianus*	HQ389233	sheep	Iran
30.	*N. oiratianus*	KC580732	sheep	China
31.	*N. oiratianus*	MT193658	goat	China

No.	Nematode species	GenBank accession, ITS	Host range	Geographic distribution
32.	*N. tarandi*	MZ478655	reindeer	Norway
33.	*N. tarandi*	MZ478654	reindeer	Norway
34.	*N. tarandi*	MZ478653	reindeer	Norway
35.	*N. tarandi*	MZ478652	reindeer	Norway
36.	*N. tarandi*	MZ478651	reindeer	Norway
37.	*N. andersoni*	OP879220	bighorn sheep	Canada
38.	*N. andersoni*	OP879219	bighorn sheep	Canada
39.	*N. rupicaprae*	AJ239111	chamois	Italy
40.	*Nematodirus sp.*	HQ844230	sheep	China
41.	*N. spathiger*	KC998748	sheep	New Zealand
42.	*N. spathiger*	KY930420	dorcas gazelle	Tunisia
43.	*N. spathiger*	AF194128	sheep	USA
44.	*N. spathiger*	KC998746	sheep	New Zealand
45.	*N. spathiger*	AF194143	sheep	USA
46.	*N. spathiger*	KF305647	guanaco	Argentina
47.	*N. spathiger*	AF194144	sheep	USA
48.	*Teladorsagia circumcincta*	ON004115	sheep	Iraq

In this phylogenetic tree analysis, in the first Clade the samples of *N. oiratianus* from our study clustered with the samples of *N. oiratianus* from the GenBank database into one group (Fig. 3). Their definitive hosts are wild and domestic sheep and goats. In Clade II of the phylogenetic tree, three samples of *N. abnormalis* from our study clustered with a sample of *Nematodirus* sp. (HQ844230) from the GenBank database, forming a group with a high bootstrap value (100%). It should be noted that the species studied as *Nematodirus* sp. by Chinese researchers might be *N. abnormalis*. In the sixth phylogenetic clade, the available *N. tarandi* species from the Genbank database were placed, showing a high bootstrap value (98%). These species have only been recorded in northern reindeer. The III–V clades of the tree contained *N. davtiani*, *N. davtiani alpinus*, *N. rupicaprae N.andersoni*, and which were considered to be close sister species. The definitive hosts for *N. andersoni* and *N. davtiani* were bighorn sheep and for *N. rupicaprae*, wild goat (*Rupicapra rupicapra*). The species in this group produced low bootstrap support (78%). In the VIII clade of the phylogenetic tree, the samples of *N. helvetianus* from the GenBank database were placed. It should be noted that this resulted in a paraphyletic grouping with two subclades. This can be attributed to their host associations: the samples of *N. helvetianus* in the first subclade were found in sheep, while in the second subclade, the species parasitizes cattle.

In our study, the analyzed *Nematodirus spathiger* species, along with the *N. spathiger* samples from the GenBank database, formed the VII clade, which is placed very close to the *N. helvetianus* species. The *Nematodirus spathiger* species has a wide range of definitive hosts, including sheep, llamas, and gazelles. The typical species of the *Nematodirus* genus, *N. filicollis*, is located in clade IX of the phylogenetic tree, close to the outgroup in the phylogenetic tree. The definitive hosts were mainly domestic sheep.

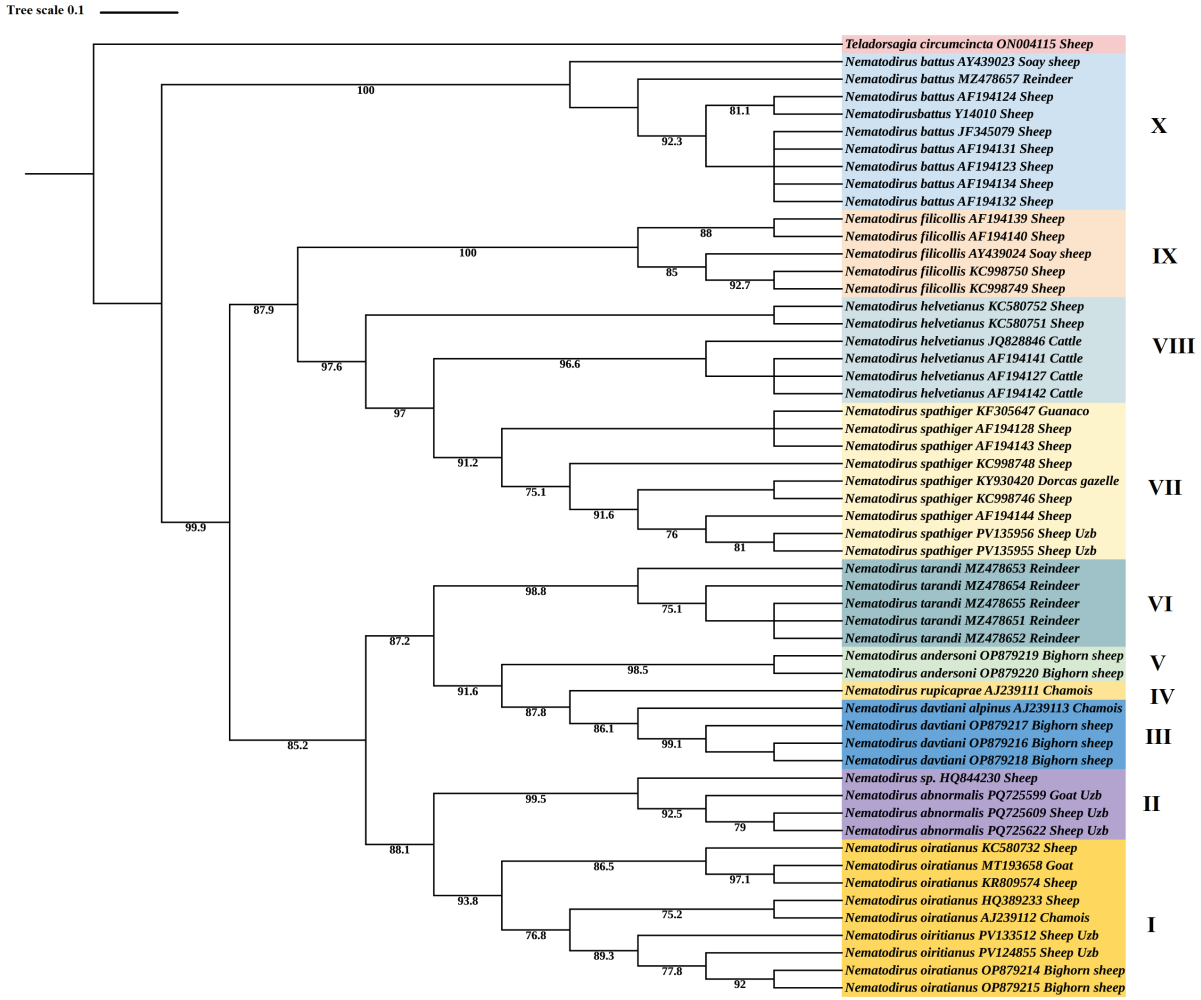

Fig. 3. Phylogenetic tree based on the ITS-2 rRNA gene sequences for genera of the *Nematodirus* by maximum likelihood method

Discussion

Nematodirus species are the main components of the parasite fauna of ungulates, well adapted to environmental conditions. They are parasites of the cranial part of the small intestine of ruminants. The life cycle is direct. The invasion larvae develop within the egg up to the third stage larvae. Species in this genus can be easily identified by the fact that their eggs are twice as large as those of other trichostrongylids. According to the results of morphological studies, more than 40 species of the genus *Nematodirus* are known in the world fauna (Taylor et al., 2016). However, molecular-genetic research has been conducted on more than 10 species.

By cuticular ridge patterns of *N. helvetianus*, *N. oiratianus interruptus*, *N. abnormalis*, and *N. spathiger*, share the characteristics of a more posteriorly distributed pattern of ridges in the cervical region, 18 or more ridges near midbody, smaller dorsal and ventral ridges, a larger number (50–65) of perioral denticles, a longer cephalic expansion, and a smaller bursa with separate dorsal lobes. *N. helvetianus* and *N. oiratianus interruptus* have additional ridges in the cervical and postcervical regions, and are characterized by having more than 18 ridges for most of their length; the males do not have additional ventral ridges in the last quarter. *N. helvetianus* has more ridges (30–36 at midbody) than any of the other species. *N. oiratianus interruptus* can be easily separated from all other species by its discontinuous ridges in the cervical region (Lichtenfels & Pillit, 1983). Morphometric characteristics of sexually mature males and females of *N. spathiger*, obtained from the small intestine of domestic sheep in Ukraine showed that nematodes of this species are morphologically characterized by a thin filiform body, a vesicle at the head end, and a chitinous tooth in a short oral capsule. Differential morphological features of male *N. spathiger* nematodes include the structural features of the spicules, their distal end, as well as the shape and arrangement of the rays of the tail bursa; in females, these are the structural features of the vulva and tail end (Melnychuk et al., 2021). *N. abnormalis* was distinguished from the other species of *Nematodirus* by the twisted and asymmetrical spicule tips of the male and by the discontinuities in the cervical region of the lateral cuticular ridge pairs 2 and 8 (Louw, 1989). According to the results of the morphometric study, male was identified based on the spicule shape, length, morphometric dimensions of the dorsal rays and external-dorsal rays. Males of *N. spathiger* are morphologically characterized by the presence of two long, filamentous spicules. The gubernaculum is absent. Characteristically, the distal parts of spicules are connected by a membrane, which seemingly envelops the spicules and welds them together. The distal end of the connected spicules has the shape of a spatula. Females of *N. spathiger* nematodes are morphologically characterized by the presence of thinner anterior and thicker posterior parts of the body. The tail end bears a well-defined spike. The vulva is located in the posterior part of the body. As for the male of *N. abnormalis*, the dorsal lobe is separated from the lateral by a fairly deep notch. The externo-dorsal rib is thin, with its middle third located almost at the edge of the bursa and somewhat thinner than the distal end. The distal end of the spicules is curved and covered with a membrane in the form of an asymmetrical lancet. The maximum body width in females corresponded to the vulva region, and the body tapered from the vulva region onward. The length of a male nematode of *N. oiratianus* longer than both other nematodes and is 17.65 ± 1.10 mm. The bursa consists of two large lateral lobes and two small dorsal ones. The spicules are equal, long, tubular, slightly expanded at the proximal ends. Starting from the middle third, both spicules are connected by a transparent membrane, which at their free distal end is expanded in the form of a lancet, protruding from the sides. Its anterior end is considerably thinner and longer than the posterior end and is sometimes spirally twisted. The cephalic end is slightly narrowed. Gradually expanding, the body of females reaches its maximum width in the vulva area.

The phylogenetic analysis was performed on this phylogenetic tree based on the available species of the genus *Nematodirus* in the GenBank database. According to the results of the analysis, we can see that the ITS-2 region is a useful marker for analyzing the phylogenetic relationships of *Nematodirus* species based on nucleotide sequences (Newton et al., 1998; Gasser, 1999; Audebert et al., 2000). Sequence data from the ITS1-5.8S-ITS2 regions, 18S and 28S genes of rDNA were used to investigate the sequence diversity of different geographic samples of *N. battus* and to examine the phylogenetic relationships of other *Nematodirus* species. Phylogenetic analysis of these data well supports the relationships between species, with *N. helvetianus* and *N. spathiger* as sister taxa in a clade of these two species and *Nematodirus filicollis*. This tree is consistent with Capinae as the ancestral host, with subsequent variation in the Bovinae host of *N. helvetianus*. The *N. battus* sequences were unique with 19 variable regions among sequences representing the 5 geographic samples and they clustered together as a distinct clade in the phylogenetic tree (Nadler et al., 2000).

In this study, we can see that the *Nematodirus* species formed 10 monophyletic groups. The species *N. abnormalis* clustered in a single monophyletic group with the *Nematodirus* sp. in the phylogenetic tree. It should be noted that the species studied as *Nematodirus* sp. might actually be *N. abnormalis*. The phylogenetic analysis using ITS-2 region nucleotide sequences revealed that *N. oiratianus* and *N. abnormalis* are close species. The *N. spathiger* and *N. helvetianus* species also phylogenetically close. As the result of the study, the obtained nucleotide sequences were uploaded to the GenBank database (NCBI), and the corresponding accession numbers were obtained for *N. spathiger* (PV135955; PV135956), *N. abnormalis* (PQ725622; PQ725609; PQ725599), and *N. oiratianus* (PV124855; PV133512). The samples of the *N. abnormalis* species, studied by us, were uploaded to the GenBank database for the first time.

Conclusion

The studied species of the genus *Nematodirus* differ from each other morphologically, and they can be distinguished mainly by the shape of the spicule and the structure of the bursa in the male individual. In the species *N. abnormalis*, the tip of the spicule is slightly curved, covered with an asymmetrical lanceolate membrane, the tip of the spicule of the species *N. oiratianus* is connected and ends in a thin tube, and the distal tip of the spicule of the species *N. spathiger* has a slightly curved shape. At the same time, the rDNA ITS-2 region of these species allows identification by nucleotide sequence and interspecific and intraspecific phylogenetic analysis. The species *T. circumcincta* of the outgroup *Teladorsagia* genus was used in the phylogenetic tree, and it can be seen from the phylogenetic tree that it originated in the same evolutionary direction as the nematodiruses. In the phylogenetic analysis, the species *N. oiratianus* and *N. abnormalis* studied in our samples were considered to be close species, and their definitive hosts were wild and domestic sheep, goats and chamois. The species *N. helvetianus* is mainly a parasite of cattle and formed the innermost group with the species *N. spathiger*, which is found in sheep, goats and cattle. At the same time, the species *N. helvetianus* formed two subclades with paraphyletic grouping, which can be linked to their definitive hosts. The species *N. andersoni*, *N. rupicaprae*, *N. davtiani alpinus*, and *N. davtiani* were located in the tree and they were considered to be close sister species to each other. The species *N. filicollis* and *N. battus* are found in sheep, goats and other ruminants and are located close to the outer group in the phylogenetic tree. *N. oiritianus* and *N. abnormalis* are very closely related. Three specimens of *N. abnormalis* (PQ725622; PQ725609; PQ725599) were deposited in the GenBank database for the first time. The result of this study confirm that the ITS-2 region is suitable not only for species identification but also for phylogenetic analysis of *Nematodirus* genus representatives.

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