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Molecular and morphological detection of *Setaria tundra* in roe deer (*Capreolus capreolus*) from Lithuania

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Abstract: *Setaria* spp. are vector-borne parasitic roundworms commonly found in the abdominal cavities of wild and domesticated ungulates globally. The objective of this study was to conduct a morphological and molecular analysis of *Setaria tundra* Issaitshikoff et Rajewskaya, 1928, collected from roe deer *Capreolus capreolus* Linnaeus in Lithuania. The morphological characteristics of *S. tundra* described and illustrated. The amplification of the 12S rRNA and 18S rRNA genes confirmed the presence of *S. tundra*. This is the first case report of *S. tundra* in roe deer in Lithuania.

Keywords: vector-borne nematode, roundworm, 12S rDNA, 18S rDNA, phylogenetics.

Mature nematodes of the genus *Setaria* Viborg, 1795 are typically found in the abdominal cavities of artiodactyls, hyracoids and equines, while various dipteran species act as intermediate hosts of these parasites (Anderson 2000). This genus consists of 43 species (Anderson 2000, Enemark et al. 2017). In Europe, five species of *Setaria* were identified: *Setaria cervi* (Rudolphi, 1819), *Setaria digitata* von Linstow, 1906, *Setaria equina* (Abildgaard, 1789), *Setaria labiatopapillosa* Alessandrini, 1848 and *Setaria tundra* Issaitshikoff et Rajewskaya, 1928 (see Oloś et al. 2021). These *Setaria* spp. can be distinguished by morphological features (Oloś et al. 2021). However, even slight or contrasting morphological features can be easily misinterpreted, leading to potential misidentification of species (Kitajima et al. 2022). Therefore, a molecular-based analysis is required for reliable species-level identification (Bradbury et al. 2022).

Setaria tundra has been recorded in 15 European countries (Oloś et al. 2021), but there are no data from the remaining European countries, including Lithuania. When considering regional cases closest to Lithuania, *S. tundra* was reported in Poland (Bednarski et al. 2010, Kowal et al. 2013, Demiaszkiewicz et al. 2015). However, when regarding the Baltic region, no data from Latvia and Estonia have been reported (Oloś et al. 2021). Therefore, the aim of this study was to confirm the presence of *S. tundra* in roe deer in Lithuania by morphological and molecular analysis.

In this case study, a single nematode was isolated from the abdominal cavity, attached next to the liver within the peritoneum, of a male roe deer *Capreolus capreolus* (Linnaeus), approximately 3 years old, hunted in May 2021 in Bargailiai, Kėdainiai district, Lithuania (the age of roe deer was determined using a teeth abrasion methodology – White 1974, Baleišis et al. 2002).

The parasite was washed with distilled water to remove dirt and tissue debris and kept in 70% ethanol at -20°C until used for molecular studies. Before molecular examination, the parasite was measured and both ends (anterior and posterior) were photographed using a light microscope (Fig. 1). The worm was identified as a female of *Setaria* sp. based on morphology as characterised by Oloś et al. (2021). The middle part of the helminth was used for molecular analysis, while the anterior and posterior ends were preserved in 70% ethanol at -20°C for further studies as hologenophores.

DNA extraction was performed using the QIAamp® DNA mini Kit (QIAGEN GmbH, Hilden, Germany) following the Quick-Start protocol. A 600 bp fragment of the mitochondrial 12S ribosomal DNA of the parasite was amplified using the primers 12SF and 12SR (Muñoz-García et al. 2018; Table 1). NF50 and BNR1 primers were used to amplify the nuclear 18S rDNA fragment (Pyziel et al. 2017, Muñoz-García et al. 2018; Table 1).

The thermal profile for *Setaria* spp. small subunit (SSU) fragment amplification (both 12S and 18S) involved an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. The amplification of PCR product was evaluated by gel electrophoresis using 1.5% agarose. Amplification products were purified from agarose gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific Baltics JSC, Vilnius, Lithuania).

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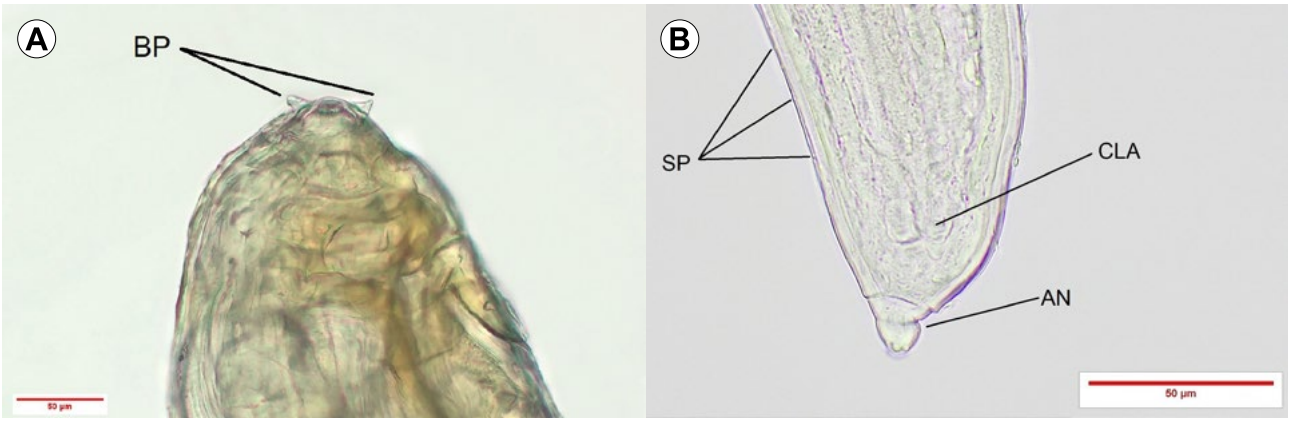

Fig. 1. Female of *Setaria tundra* Issaitshikoff et Rajewskaya, 1928 from roe deer *Capreolus capreolus* Linnaeus in Lithuania. **A** – anterior end; note bifid projections (BP) typical for S. *tundra*; **B** – posterior end; note annular narrowing (AN) and small papillae (SP) typical for S. *tundra*; small caudolateral appendage (CLA).

Table 1. Primers and targeted amplification genes used in this study.

Primer name	Primer sequence	Targeted gene	Reference
12SF 12SR	5′-GTTCCAGAATAATCGGCTA-3′ 5′-ATTGACGGATGTTTGTACC-3′	*12S rRNA*	Muñoz-García et al. 2018
NF50 BNR1	5′-TGAAATGGGAACGGCTCAT-3′ 5′-ACCTACAGATACCTTGT-TACGAC-3′	*18S rRNA*	Pyziel et al. 2017

The prepared samples were sent to Macrogen (Amsterdam, Netherlands) for sequence analysis. All gene sequence analyses and phylogeny tree construction were carried out using Mega-X software. Specific identification of the parasite was made by comparing the sequences with those available for *Setaria* spp. in GenBank.

Sequences for representative sample obtained using different primers in this study were submitted to the GenBank database under the accession numbers PP824648 for *Setaria tundra 12S* SSU rRNA gene sequence and PP819575 for *Setaria tundra 18S* SSU rRNA gene sequence.

The morphological analysis revealed that the helminth found in the abdominal cavity of *C. capreolus* was female of *Setaria tundra*. The parasite was 49.8 mm long. The anterior end had two bifid projections on top of a peribuccal crown (Fig. 1A). The posterior end had bud-like knob with an annular narrowing length of 1.25 mm (Fig. 1B). There were no visible tail wings, only two small, round caudolateral appendices (CLA).

The molecular analysis was performed using sequence analysis of two genes, including 12S rDNA and 18S rDNA. The phylogenetic analysis performed according to partial sequences of the 12S rRNA gene (PP824648) of 442 bp showed 100 % identity to *S. tundra* from Denmark, Italy and Germany (Fig. 2). A total of 13 variable sites were identified within *S. tundra* from Lithuania and other countries (Table 2).

The sequence (PP819575) of 1,147 bp of the 18S rRNA gene formed a separate branch and distinguished from *S. digitata* and other filarial nematodes (Fig. 3). Comparable sequences of *S. tundra* for the amplified part of the 18S rRNA gene were not available in GenBank.

Setaria tundra is found in 15 European countries, as reviewed by Oloś et al. (2021). The nematode parasitises mainly cervids of the subfamily Capreolinae: reindeer *Rangifer tarandus* (Linnaeus), roe deer *C. capreolus* and moose *Alces alces* (Linnaeus) (Kowal et al. 2013), but also it is found in red deer *Cervus elaphus* Linnaeus, in Poland (Oloś et al. 2019) and Slovakia (Lazar et al. 2024).

Our study reports *S. tundra* in Lithuania for the first time. The nearest country to Lithuania where *S. tundra* is registered is Poland, where this species was found in roe deer (Bednarski et al. 2010, Kowal et al. 2013, Tomczuk et al. 2017), moose (Demiaszkiewicz et al. 2015) and in mosquitoes (Masny et al. 2013, 2016, Rydzanicz et al. 2016, 2019). Until now, *Setaria capreoli* Kadenazii, 1957 and *S. labiatopapillosa* in roe deer (Kazlauskas and Arnastauskienė 1969, Kazlauskas and Pužauskas 1974) and *S. equina* in horse (Kazlauskas and Arnastauskienė 1969) have been identified in Lithuania. To our knowledge, *S. tundra* is not known to occur in Latvia and Estonia.

Table 2. Variable sites within the 12S rRNA gene sequences of *Setaria tundra* Issaitshikoff and Rajewskaya, 1928.

Accession No.	Host	Country	22	217	219	229	257	271	272	276	287	339	446	449	450
PP824648	*Capreolus capreolus* Linnaeus	Lithuania	G	G	G	T	A	A	T	G	T	T	T	A	T
KU508979-KU508981	*C. capreolus* Linnaeus	Denmark	.	.	.	.	.	.	.	.	.	.	.	-	-
AM779828	*C. capreolus* Linnaeus	Italy	.	.	.	.	.	.	.	.	.	.	.	-	-
JN228376	*Aedes vexans* Meigen	Germany	.	.	.	.	.	.	.	.	.	.	-	-	-
AM779826	*C. capreolus* Linnaeus	Italy	.	.	.	.	.	.	.	.	C	C	A	T	A
KR676615	*Aedes vexans* Meigen	Hungary	.	A	A	G	T	T	G	.	.	.	.	.	.
KP760355	*Rangifer tarandus* Linnaeus	Finland	A	.	.	.	.	.	.	A	.	.	-	-	-

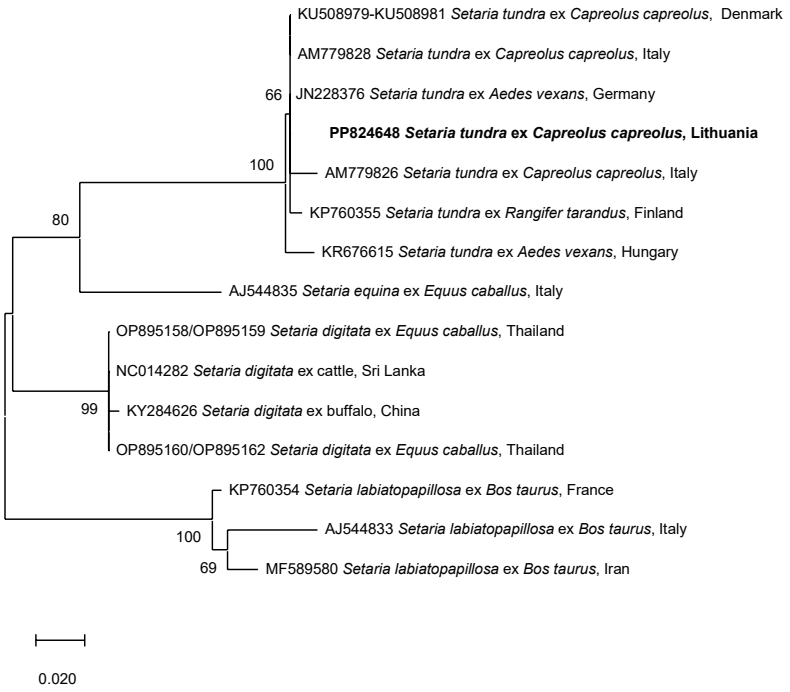

Fig. 2. Maximum-likelihood phylogenetic tree for 12S rRNA partial gene sequences of *Setaria* spp. The phylogenetic tree was constructed using the Tamura – 3 parameter model with a discrete Gamma-distribution (+G) and bootstrap analysis of 1,000 replicates. Numbers on the tree indicate bootstrap support (values < 50% not shown). Samples sequenced in the present study are in bold.

Setaria capreoli was reported in roe deer (Yarvis and Yarvis 1983) and *Setaria transcaucasica* Assadov, 1952 in elk (Järvis 1995) from Estonia. The taxonomic status of *S. capreoli* and *S. transcaucasica* is highly ambiguous, suggesting that these nematodes were most likely *S. tundra* as previously mentioned by other authors (e.g., Demiaszkiewicz et al. 2015, Enemark et al. 2017). This suggests that *S. tundra* may not be a new species in the region and previously dwelled in the Baltic countries, although the nematode was not registered under this name. Furthermore, roe deer *C. capreolus* are commonly recognised as asymptomatic carriers of *S. tundra* (Enemark et al. 2017).

Females of S. *tundra* are about 4.6–7.7 cm in length (Nikander et al. 2007, Demiaszkiewicz et al. 2015). There-

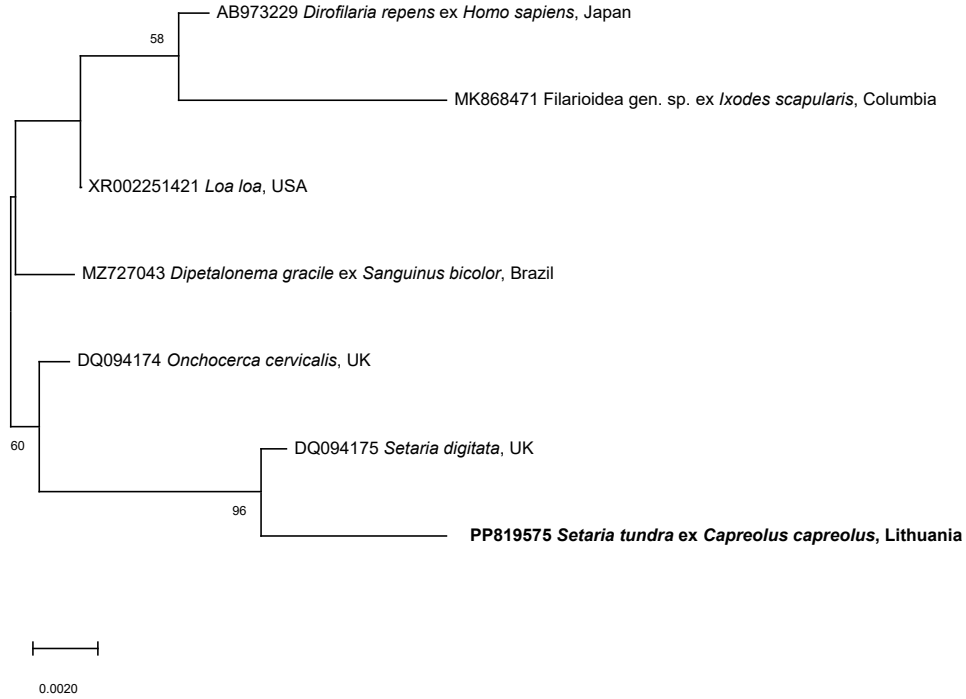

Fig. 3. Maximum-likelihood phylogenetic tree for 18S rRNA partial gene sequences of *Setaria* spp. and related taxa. The phylogenetic tree was constructed using the Tamura – 3 parameter model with a discrete Gamma-distribution (+G) and bootstrap analysis of 1,000 replicates. Numbers on the tree indicate bootstrap support (values < 50% not shown). Samples sequenced in the present study are in bold.

fore, the sample in this study, measuring 5 cm, falls into the reported body length for *S. tundra* females. In contrast, adult females of other *Setaria* species present in Europe have much longer body: *S. cervi* ranges from 11.6 to 14.2 cm, *S. equina* from 10.0 to 15.0 cm, *S. labiatopapillosa* from 12.0 to 15.0 cm and *S. digitata* from 9.0 to 15.6 (Kaufmann 2013, Raju Kumar et al. 2016, Oloś et al. 2019). Moreover, *S. tundra* differs from *S. cervi* by a longer annular narrowing (AN) of the posterior end, which is separated from the rest of the tail (Oloś et al. 2019). In addition, the surface of S. *tundra* is covered with numerous small papillae (SP) from each side (Fig. 2), whereas the surface of *S. cervi* is smooth (Oloś et al. 2019).

In this study, molecular markers such as 12S rDNA and 18S rDNA were selected to clarify the phylogenetic relationship of *S. tundra*. In previous studies, the same genes were used to detect the infection of *S. tundra* (Czajka et al. 2012, Kemenesi et al. 2015, Angelone-Alasaad et al. 2016, Enemark et al. 2017, Čurlík et al. 2023, Šiljegović et al. 2024). The analysis of the obtained sequences revealed that the Lithuanian sample matched other *S. tundra* sequences from European countries available in GenBank.

The 12S rRNA gene sequence obtained in this study was identical to the sequences of *S. tundra* from roe deer in Italy and Denmark, as well as from mosquitoes in Germany (Fig. 2). Meanwhile, a relatively low number of *Setaria* 18S rRNA gene sequences deposited in GenBank limits the scope of extended phylogenetic analysis. Additionally, the *S. tundra* sequences in GenBank correspond to different regions of the 18S rRNA gene. However, *S. tundra* was identified as a distinct branch.

In conclusion, this study reports the first case of the occurrence of *S. tundra* in roe deer *C. capreolus* in Lithuania. However, as this finding was incidental during *a postmortem* examination of roe deer, further studies are needed to determine the true infection rates in Lithuania. These studies should focus on searching for final hosts and vectors.

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