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New findings of *Setaria tundra* and *Setaria cervi* in the red deer (*Cervus elaphus*) in Poland

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Abstract

Our study aimed at examining the phylogenetic position of the newly-found *Setaria* nematodes obtained from the red deer (*Cervus elaphus*) based on sequences of the mitochondrial cytochrome c oxidase subunit 1 (*COX-1*). Alignment and phylogenetic analyses, as well as SEM microscopic analysis, revealed the presence of two *Setaria* species: *S. cervi* and *S. tundra*. *Setaria tundra* was noted in only one individual, a calf of the red deer, while *S. cervi* was observed in three stages, two hinds and one calf of the red deer. According to our knowledge, it is the first case of *S. cervi* in the red deer in Poland confirmed in molecular studies and also the first case of *S. tundra* infection in the red deer.

Introduction

Filarial worms are considered an economic problem and a significant health threat for both humans and animals (Laaksonen *et al*., 2007; Taylor *et al*., 2010). *Setaria* nematodes belong to the Filarioidea superfamily and are parasites of different ungulates. At least four species of this superfamily are present in Europe, including Poland: *Setaria cervi*, *Setaria tundra* (Demiaszkiewicz *et al*., 2015), *Setaria labiatopapillosa* (Demiaszkiewicz *et al*., 2007) and *Setaria equina* (Gawor, 1995).

Different species of mosquitoes, mostly from *Aedes* genus, act as a vector of these parasites (Anderson, 2000), the gradations of which might be linked with climate warming (Genchi *et al*., 2009; Laaksonen *et al*., 2010). Geographical expansion of *Setaria* worms may be indirectly related to wet and warm summers due to the abundance of intermediate hosts but also to the high density of possible definitive hosts as well as wild and domesticated ungulates. The focus of attention has been recently on *S. tundra* as it is widening its range far to the South. However, little is known about the presence of *S. cervi*, another parasite of deer species, in the wild. Although *S. cervi* became a model species for anti-filarial drugs and treatment methods decades ago (Singhal *et al*., 1969), not much is known about its presence in the wild. Known definitive hosts of *S. cervi* are: the moose (*Alces alces*), the red deer (*Cervus elaphus*), the sika deer (*Cervus nippon*), the Asian wapiti (*Cervus elaphus sibirica*), the muntjac (*Cervus muntjac)*, the chital (*Axis axis*), the fallow deer (*Dama dama*) (Yeh, 1959; Wang *et al*., 1990; Anderson, 2000) but also cattle; *Bos taurus* (Baqui and Ansari, 1976; Sundar and D'Souza, 2015) and *Bubalus bubalus* (Almeida *et al*., 1991). Infection with *S. cervi* can lead to pathological changes in the central nervous system in definitive hosts (Blažek and Dykova, 1968; Wang *et al*., 1990). Literature regarding this species in Europe is scant and mostly older than 50 years (Blažek and Dykova, 1968) that is dated before the systematics of *Setaria* worms was established. Moreover, as methods of describing *Setaria* worms based on light microscopy carry a significant risk of misinterpretation (Yeh, 1959), it seems that molecular analysis is essential (Alasaad *et al*., 2012).

Setaria tundra, another filarial nematode, has recently expanded its geographical range by hundreds of kilometres and is known to be a major cause of the mass falling of wild and semi-domesticated reindeer in Fennoscandia (Laaksonen *et al*., 2007, 2009*a*). Since 2010, *S. tundra* has also been reported in Poland (Bednarski *et al*., 2010). Its main host is the roe deer (Kowal *et al*., 2013; Demiaszkiewicz *et al*., 2015; Tomczuk *et al*., 2017) yet the moose can serve as an asymptomatic carrier (Demiaszkiewicz *et al*., 2015). Moreover, microfilariae of *S. tundra* has been detected in *Aedes vexans*, *Ochlerotatus caspius*, *Culex pipiens* and *Culex torretium* mosquitoes in SW and Central Poland (Rydzanicz *et al*., 2016; Masny *et al*., 2013) as well as in Hungary (Kemenesi *et al*., 2015; Zittra *et al*., 2015) and Germany (Czajka *et al*., 2012; Kronefeld *et al*., 2014). According to the literature, the red deer (*C. elaphus*) is considered as a definitive host for only one member of *Setaria* genus – the *S. cervi* (Yeh, 1959; Anderson, 2000).

The main aim of our study was to report for the first time *S. tundra* in the red deer (*C. elaphus*) in Poland. We also examine the phylogenetic position of the newly-found *Setaria* nematodes (*S. tundra* and *S. cervi*) based on sequences of the mitochondrial cytochrome c oxidase subunit 1 (*COX-1*) gene.

Table 1. Precise information on collected *Setaria* worms

Setaria specimen	*Setaria* species	Host species and sex	Location of the *Setaria* worm	Sex of *Setaria*	Length of *Setaria* (mm)
1	*S. cervi*	*C. elpahus*, stag	On the peritoneum	(♀)	91
2	*S. cervi*	*C. elpahus*, hind	On the stomach	(♂)	79
3	*S. cervi*	*C. elpahus*, hind	Under the pericardium	(♀)	51
4	*S. tundra*	*C. elpahus*, calf	On the intestines	(♀)	48
5	*S. tundra*		On the intestines	(♀)	54
6	*S. cervi*		On the stomach	(♀)	89
7	*S. cervi*	*C. elpahus*, stag	On the liver	(♀)	118
8	*S. cervi*		On the liver	(♀)	131
9	*S. cervi*	*C. elpahus*, stag	On the intestines	(♀)	112

Methods

Sample collection

Nematodes were collected from 11 red deer harvested during the seasonal cull close to Opole city and Szczedrzyk village, near Turawskie Lake (SW Poland, 50°41′39.5″N 18°05′38.8″E), between September and December in 2017. The study area comprised of managed forests and fields. Managed forests consist of pine trees (*Pinus sylvestris*) with the addition of deciduous tree species (birch, alder, elm and oak) and are inhabited by a range of wild mammal species indigenous to the region (roe deer, wild boar, fallow deer, hare, red fox, badger, European beaver and many smaller species). Collected *Setaria* nematodes from the red deer were washed with water, sterilized and kept in 70% ethanol until DNA extraction.

Preparation of material for SEM microscopy

The biological material was fixed with 2.5% glutaraldehyde cacodylic buffer and incubated overnight, then washed in 0.1 M cacodylic buffer (pH 7.2). Afterwards, the material was postfixed in 1% OsO_4 in ddH_2O for 3 h and washed three times in ddH_2O. After postfixation samples were dehydrated through a graded series of EtOH (50% – 10 min, 70% – 24 h, 90% – 10 min, 96% – 10 min) and dried on the Critical Point Drying System (POLARON, the UK). Next, dry samples were mounted on aluminium stubs in different positions and coated with gold with the use of a sputter coater (POLARON SC7620, the UK) and were examined in LEO 1430VP scanning electron microscope produced by Zeiss.

DNA extraction, PCR and sequencing of the mitochondrial COX-1 gene

Genomic DNA was extracted from each of the *Setaria* worms using DNeasy Blood & Tissue Kit (Quigen, Hilden, Germany) and stored at −20 °C. Primers and cycling conditions used in this study were described previously (Casiraghi *et al.*, 2001). Detection and genotyping of nematodes were performed by amplification and sequencing of *COX-1* gene fragment (690 bp). *Dirofilaria repens* DNA extracted from the cat was used as a positive control (Bajer *et al.*, 2016). Amplicons were visualized with Midori Green stain (Nippon Genetics Europe GmbH) following electrophoresis in 1.5% agarose gels. Amplicons were purified and sequenced by a private company (Genomed S.A., Poland) in both directions.

Phylogenetic analysis

Obtained nucleotide sequences were analysed using BLAST NCBI and MEGA v. 7.0 software (Kumar *et al.*, 2016) for sequence alignment, species typing and phylogenetic relationships. After testing the data for the best substitution model, phylogenetic trees were obtained using Maximum Likelihood as the tree construction method and Tamura 92 + *G* parameter algorithm as a distance method. By comparison, sequences of *Setaria* spp. obtained from GenBank (https://www.ncbi.nlm.nih.gov) were implemented in the sequence alignment. The stability of inferred phylogenies was assessed by bootstrap analysis of 1000 randomly generated sample trees.

New nucleotide sequences

New nucleotide sequences have been deposited in GenBank NCBI with the accession numbers: MK360913, MK360914 and MK360915.

Results

Red deer examination

Out of 11 examined specimens of the red deer, eight were infested with *Setaria* worms (73%). Intensity of infestation of *Setaria* nematodes ranged from one to three per deer (Table 1). Nine out of 14 of adult *Setaria* worms were located under the peritoneum: on stomach, intestines and liver; one specimen was located directly on a heart muscle under pericardium. Five nematodes were calcified and/or encysted on the surface of the liver or stomach. Only alive worms ($n = 9$) were collected for further molecular and microscopic analysis.

SEM microscopy analysis

Although most studies regarding *Setaria* are supported with light microscopy pictures analysis, it is the images obtained from the electron microscopy which allow for precise morphological analysis. Our first step was to observe crucial morphological features with SEM microscopy.

Pictures taken with SEM microscopy allowed to distinguish males from females as well as pointed out species-specific features of analysed nematodes which are shown in Fig. 1.

The biggest difference between collected species is the shape of bifid projections around the oral opening on the cephalic region. In *S. tundra* there are two, rather small, opposite, clearly separated bifid projections protruding from the oval peribuccal crown (Fig. 1A), while in *S. cervi* the whole peribuccal crown is elevated

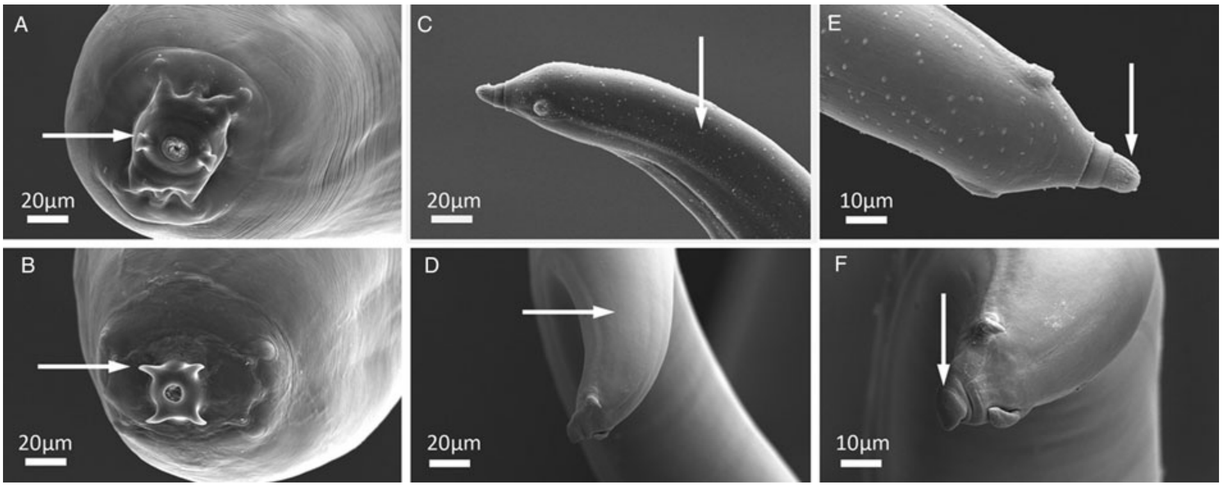

Fig. 1. Key morphological structures of posterior and anterior ends of (♀) of collected *Setaria tundra* (upper row) and *Setaria cervi* (lower row) worms.

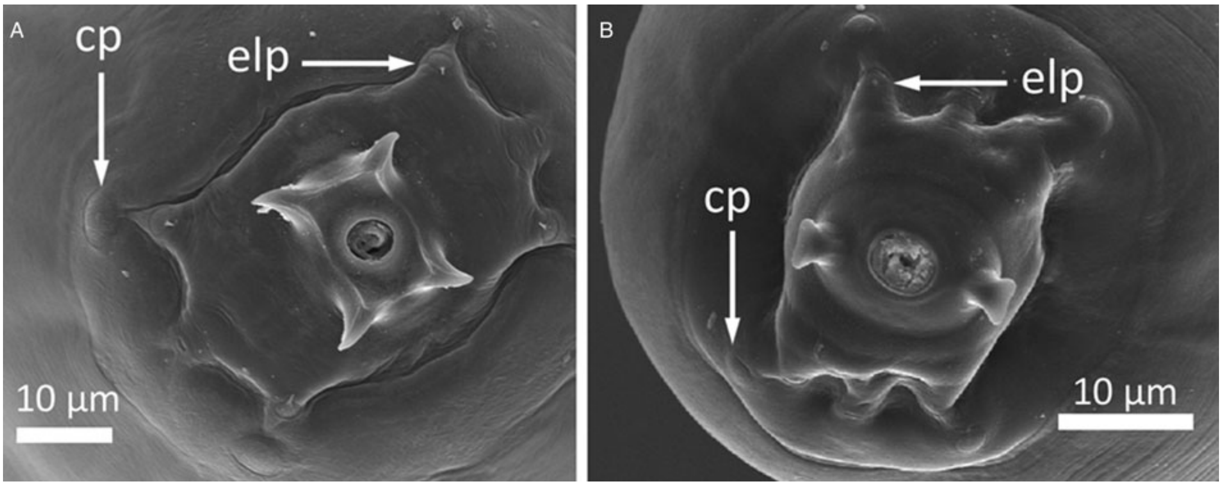

Fig. 2. Posterior ends of (♀) of collected *Setaria cervi* (A) and *Setaria tundra* (B) with similar cephalic papillae (*cp*) and externolabial papillae (*elp*).

and possesses four sharp-ended bifid projections which take a shape of a four-pointed star (Fig. 1B). The next two significant differences are clearly visible on the posterior end. Firstly, the surface of *S. cervi* is smooth (Fig. 1D), while in *S. tundra* there are numerous small papillae from each side (Fig. 1C). Secondly, the bud-like knob at the end of the tail in male *S. tundra* (Fig. 1E) is clearly separated from the rest of the tail by annular narrowing and much different than the one in *S. cervi* which is much shorter, smoother and blunt-ended with caudolateral appendages located closer to the end of the tail (Fig. 1F). Other features, such as four bigger cephalic papillae and four smaller extrenolabial papillae in the cephalic region, are visible in only one of two *S. tundra* specimen, while in *S. cervi* they are visible in all collected specimens. However, they look almost identical in both species (Fig. 2).

Phylogenetic analysis

The 689 bp fragment of the *COX-1* gene was analysed in nine isolates. Seven of nine sequences (78%) were identical and have shown 100% sequences homology with *S. cervi* originally isolated from the red and roe deer in Italy (JF800924) (Fig. 3). The nucleotide identity/similarity of the sequenced *COX-1* fragments of further two isolates (22%) was very high (99.6%) and differed three nucleotides at position 705 (A→T), 708 (T→C) and 921 (G→A) [number corresponds to the nucleotide positions relative to the sequence of the *COX-1* (1647 bp) of *S. digitata* mitochondrion, complete genome (GU138699)]. The nucleotide sequences of these isolates were identical to *S. tundra* found on mosquitoes in Germany (KF692103 and KF692104, respectively) and closely related to other *S. tundra* isolated initially from the roe deer in France (AM749298), Denmark (KU508982) and Spain (KX599455), from the reindeer in Finland (KP760209), as well as from mosquitoes in Hungary (KM45922) and Poland (KM370867) (Fig. 3).

Species of Setaria *detected in the study*

Species typing was performed on the basis of the sequencing of *COX-1* gene fragment (~690 bp) and SEM microscopy analysis. Alignment and phylogenetic analyses, as well as microscopic analysis, revealed the presence of two *Setaria* species: *S. cervi* and *S. tundra*. *Setaria tundra* was noted in only one individual, a calf of the red deer particularly, while *S. cervi* was observed in three stages, two hinds and one calf of the red deer. The coinfection of two *Setaria* species was detected only in the case of the red deer calf (Tab. 1).

Discussion

This is the first time when the results of our study have revealed the *S. tundra* infection in the red deer. The nematodes were identified by the microscopic and molecular analysis. The phylogenetic studies have shown that our isolates were closely related to

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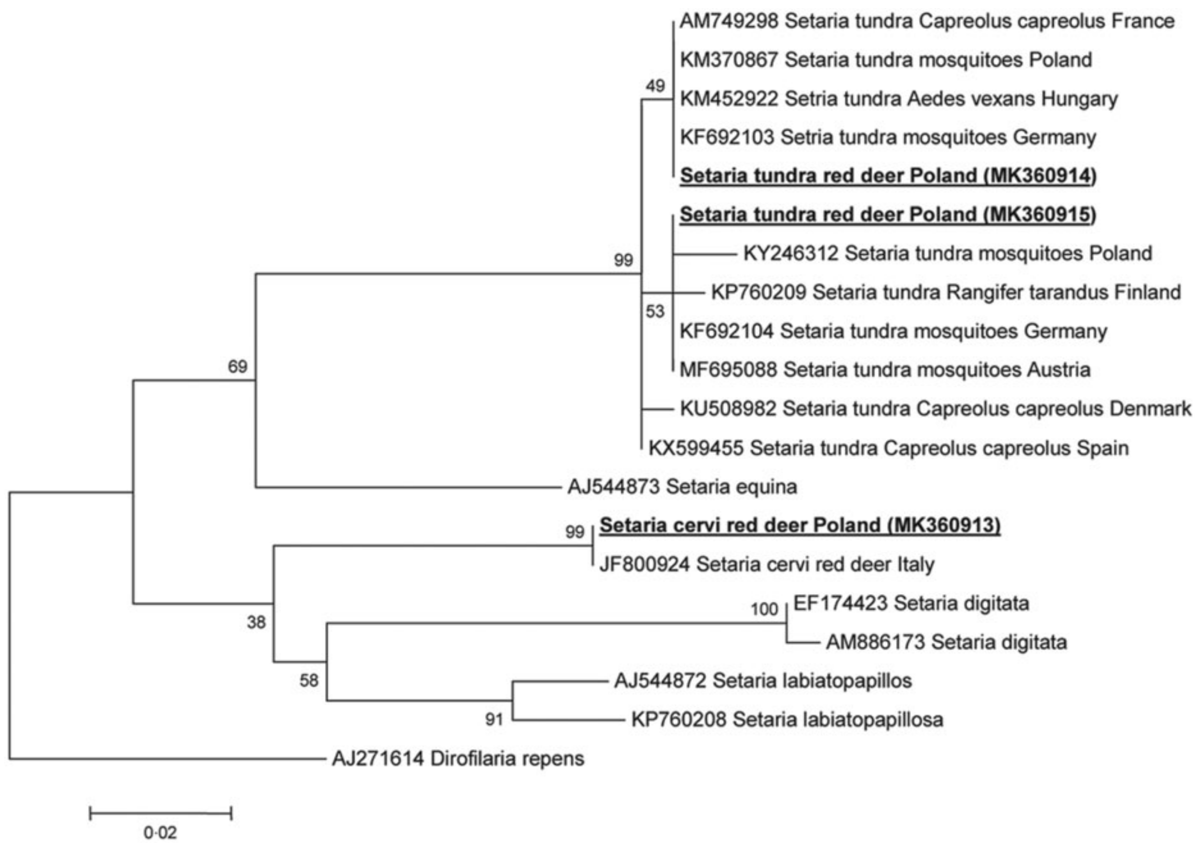

Fig. 3. Phylogenetic tree [maximum likelihood (ML)] of the *Setaria* isolates identified in the red deer from southwest Poland and selected isolates from GenBank, based on a fragment of the *COX-1* gene. The numbers at the nodes of the tree indicate bootstrap values (1000 replicates). The accession number of the newly reported sequence in this study is in bold and underlined.

S. tundra and *S. cervi*, respectively, originally isolated from the roe deer or reindeer as well as from mosquitoes in Europe.

According to recent findings (Favia *et al.*, 2003; Angelone-Alasaad *et al.*, 2016) it is possible that the outbreak route of *S. tundra* was from South to the North of Europe; however, it stands in contraction with the chronology of the detection of this parasite in various parts of Europe. Its main, definitive host is the reindeer (*Rangifer tarandus*) in the North (Laaksonen *et al.*, 2009*a*, 2009*b*), while the roe deer (*Capreolus capreolus*) further South (Enemark *et al.*, 2011). The moose (*A. alces*) can serve as an asymptomatic carrier (Demiaszkiewicz *et al.*, 2015). These were three cervid species known to harbour *S. tundra*. We proved that it has now expanded its host range with the red deer. In Poland, the red deer is found throughout the entire country and, with approximately 286 000 individuals (data for 2017, Agricultural Property Agency, Directorate General of the State Forests and the Polish Hunting Association), represents one of the most numerous game mammals. By the same token, it prevails in most European countries (Burbaitė and Csányi, 2010); thus we can expect further expansion of *S. tundra* in Central Europe.

Although there are more than one hundred publications on anti-filarial drugs, treatment methods and/or filarial antibodies with *S. cervi* as a model species, the literature about its biology and presence in wild hosts as well as any reports supported with molecular data are scarce. Out of 15 accessions available in GenBank, only one has been from Europe so far, and other, except one, come from Asia where two other, similar species occurred: *S. digitata* and *S. labiatopapillosa*. As of today, the literature regarding the presence of *S. cervi* in the wild provides very little information. There are only two sources from Europe regarding *S. cervi* written after Desset's publication (Desset 1966) in which systematics of this species has been established. According to them, we can only conclude that *S. cervi* is present in the Czech Republic and Italy and its only confirmed host is the red deer (Blažek *et al.*, 1968; Alasaad *et al.*, 2012). To the best of our knowledge, it is the first case of *S. cervi* in the red deer in Poland confirmed in molecular studies.

In our study, the intensity of infection with *S. tundra* was similar to other studies and reached maximally two adult worms per one deer. In other studies from Poland, the intensity of *S. tundra* infection reached 1–3 adult worms per one roe deer, with prevalence of 5.6% ($n = 53$) (Tomczuk *et al.*, 2017), while in another study the intensity of infection reached 1–11 adult worms per one roe deer with the prevalence of 9.43% ($n = 53$) (Kowal *et al.*, 2013). There are no comprehensive studies on *S. cervi* in wild hosts to be compared. In our study, a calf of the red deer was infected with both species of *Setaria*, including two adult (V stage) females of *S. tundra*, which proves that the red deer can be a definitive host as well. Nevertheless, the studies in question need to be continued since we were able to examine only 11 red deer specimen so far.

Conclusions

This is the first report of *S. tundra* in the red deer. This finding is consistent with other reports regarding the geographic range of *S. tundra*. Furthermore, we are the first to confirm *S. cervi* infection in the red deer in Poland. However, due to a low number of abducted red deer specimen, studies concerning the presence of *Setaria* nematodes and their species' diversity among game species in Poland should be continued.

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Conflict of interest. None.

Ethical standards. Not applicable.

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