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Detection and molecular characterization of the mosquito-borne filarial nematode *Setaria tundra* in Danish roe deer (*Capreolus capreolus*)

Heidi Larsen Enemark [a, b, *], Antti Oksanen [c], Mariann Chriél [a], Jakob le Fèvre Harslund [a], Ian David Woolsey [b], Mohammad Nafi Solaiman Al-Sabi [a]

[a] Technical University of Denmark, DK-1870, Frederiksberg C, Denmark
[b] Norwegian Veterinary Institute, P.O. Box 750, Sentrum, NO-0106 Oslo, Norway
[c] Finnish Food Safety Authority Evira, Production Animal and Wildlife Health Research Unit (FINPAR), Elektroniikkatie 3, FI-90590, Oulu, Finland

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ABSTRACT

Setaria tundra is a mosquito-borne filarial nematode of cervids in Europe. It has recently been associated with an emerging epidemic disease causing severe morbidity and mortality in reindeer and moose in Finland. Here, we present the first report of *S. tundra* in six roe deer (*Capreolus capreolus*) collected between October 2010 and March 2014 in Denmark. The deer originated from various localities across the country: the eastern part of the Jutland peninsular and four locations on the island Zealand. With the exception of one deer, with parasites residing in a transparent cyst just under the liver capsule, worms (ranging from 2 to >20/deer) were found free in the peritoneal cavity. The worms were identified as *S. tundra* by morphological examination and/or molecular typing of the mitochondrial 12S rRNA and *cox1* genes, which showed 99.1–99.8% identity to previously published *S. tundra* isolates from Europe. Roe deer are generally considered as asymptomatic carriers and their numbers in Denmark have increased significantly in recent decades. In light of climatic changes which result in warmer, more humid weather in Scandinavia greater numbers of mosquitoes and, especially, improved conditions for development of parasite larvae in the mosquito vectors are expected, which may lead to increasing prevalence of *S. tundra*. Monitoring of this vector-borne parasite may thus be needed in order to enhance the knowledge of factors promoting its expansion and prevalence as well as predicting disease outbreaks.
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1. Introduction

Filarial nematodes of the genus *Setaria* comprise more than 40 species of veterinary importance (Sonin, 1977). The life cycle of *Setaria* spp. requires mosquitoes as obligate vectors that take up first stage microfilaria when blood-feeding on infected ungulates (Anderson, 2000). Several mosquito species, mainly of the genus *Aedes,* are potential vectors for the transmission of *Setaria* (Laaksonen et al., 2009). In mosquitoes, the microfilariae develop into the infective third stage larvae that are released during subsequent blood meals (Bain and Babayan, 2003). Scarce information is available on the development and migration of the nematodes in definitive hosts (Anderson, 2000), but *Setaria* spp. generally inhabit the abdominal cavity causing focal areas of mild chronic peritonitis (Rehbinder, 1990).

Setaria tundra was first described in Russian reindeer (Rajewsky, 1929). In Europe, infections with *S. tundra* in roe deer were previously reported from Austria (Kutzer and Hinaidy, 1969), Switzerland (Andrews et al., 1974), Germany (Rehbein et al., 2001), Italy (Favia et al., 2003), and France (Ferri et al., 2009); and recent reports have identified the parasite in Hungary (Kemenesi et al., 2015; Zittra et al., 2015), Spain (Angelone-Alasaad et al., 2016) and Poland (Kowal et al., 2013; Masny et al., 2013).

In the seventies, outbreaks of peritonitis caused by *S. tundra* were reported in Swedish and Norwegian reindeer (Rehbinder et al., 1975) and in Finland, *S. tundra* has been associated with an emerging epidemic disease causing serofibrinous peritonitis, severe morbidity and mortality in reindeer and moose (Laaksonen et al., 2007). The majority of roe deer infections are subclinical,

* Corresponding author. Norwegian Veterinary Institute, P.O. Box 750, Sentrum, NO-0106 Oslo, Norway.
E-mail address: heidi.enemark@vetinst.no (H.L. Enemark).

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hence these ungulates are considered reservoirs of the parasite (Rehbinder et al., 1975; Rehbinder, 1990; Laaksonen et al., 2007). Prior to our study, no findings of *S. tundra* have been reported from Denmark. However, roe deer are widespread throughout the country with an estimated population of 400,000 in 2002 an increase of 250,000 since 1984 (Burbaitė and Csányi, 2009). Here we present several cases collected from various locations over a period of four years. Further, the Danish isolates were compared with previously published European isolates by phylogenetic analyses.

2. Materials and methods

2.1. Case descriptions

The roe deer described in the present study were analysed as part of the general surveillance program of Danish wildlife conducted by the National Veterinary Institute, Technical University of Denmark. The parasitological analyses focused on *Setaria* spp. of which all nematodes were collected and identified morphologically and a subset were further characterized by molecular typing. In contrast, adult lungworms detected by autopsy were not speciated or quantified. Supplementary coprological analyses were carried out to give an impression of the general health and the overall parasitological burden of the animals.

2.1.1. Case 1 (Isolate 2010-1233)

In October 2010 a male fawn was shot near Assentoft, in east Jutland (Fig. 1, A). It was severely emaciated with no fat deposits, but the fur was in good condition despite the presence of several adult and nymphal stages of *Ixodes ricinus*. Post-mortem examination revealed the presence of lungworms and chronic bronchopneumonia, and histological analysis demonstrated perivascular mononuclear cell infiltration of the heart. More than 20 slender, white *S. tundra* worms of approximately 5 cm long were actively swimming in the peritoneal cavity. No macroscopic pathological lesions were observed in the visceral organs. McMaster analysis (Roepstorff and Nansen, 1998) revealed low-grade excretion of *Trichuris* spp. eggs and *Eimeria* spp. oocysts. Further, *Cryptosporidium* spp. (<1000 oocysts per gram) and *Giardia* spp. (>10,000 cysts per gram) were detected by immunofluoroscent staining of faecal smears (Crypto/Giardia Cel Reagent (RR2), Cellabs Pty, Ltd. Brookvale, NSW, Australia).

2.1.2. Case 2 (Isolate 2011-389)

A female fawn was shot in May 2011 near Tappernøje, in southwestern Zealand (Fig. 1, D). The deer was emaciated and had diarrhoea. Its fur was dry and infected with fleas and ticks (*Ixodes ricinus*). The autopsy revealed congested areas in the dorso-caudal parts of the lungs but no lungworms were detected macroscopically. Examination of the intestines demonstrated catarrhal enteritis and watery yellow/green contents. Other visceral organs were normal. A single *S. tundra* worm approximately 8.2 cm long was found free in the peritoneal cavity. Faecal examination detected strongyle eggs (100 eggs/g) and *Eimeria* oocysts (<1000/g) whereas no *Cryptosporidium* oocysts or *Giardia* cysts were observed.

2.1.3. Case 3 (Isolate 2012-1665)

Adult female was hunted in December 2012 in, Rude, northern Zealand (Fig. 1, C). The hunter noticed the presence of several worm-like objects (N = 12) lying underneath the transparent liver capsule (Fig. 2, E) and forwarded only the liver to the National Veterinary Institute for pathological examination. According to the hunter no worms could be found in the peritoneal cavity and the visceral organs appeared normal. The recovered worms were very fragile and could not be isolated *in toto* for morphological analysis, but DNA was successfully extracted for molecular typing.

2.1.4. Cases 4 and 5 (Isolates 2013-410-1 & 2)

These two cases were male fawns approximately one year old, shot in May 2013 near Skælskør, south Zealand (Fig. 1, B). The animals were in good body condition and the autopsy demonstrated no pathological changes in the internal organs of either deer, but worms were detected in the abdominal cavity of both fawns. Single male and female *S. tundra* were found in one deer, and two females and one male were found in the second deer. The worms were approximately 5.5–7.1 cm long. One female worm had tens of thousands microfilariae that were subjected to morphological examination (Fig. 2, D). Ectoparasites were not detected and coprological examination revealed no parasitic eggs/oocysts or larvae.

2.1.5. Case 6 (Isolates 2014-499)

A male fawn was shot due to severe diarrhoea and emaciation in March 2014 in Tølløse, central Zealand (Fig. 1, E). Gross macroscopic examination showed the presence of internal and external parasites including many lice, ticks, and lungworms. Four adult *S. tundra* (6–8 cm) were found free in the peritoneal cavity and identified based on morphology. Coprological examination revealed *Eimeria* spp. oocysts (14,800 oocyst/g), strongyle eggs (830 eggs/g), *Trichuris* spp. eggs (410 eggs/g), and lungworm larvae (not speciated or quantified).

2.2. Detection of Setaria tundra by morphology and molecular analysis

Microscopic examination and identification was performed according to Rajewsky (1929) and Nikander et al. (2007). DNA extraction, PCR amplification and sequencing of fragments of the mitochondrial 12S rRNA and the cytochrome *c* oxidase subunit I (*cox1*) genes was performed on single worms from four of the above cases following standard procedures as described by Casiraghi et al. (2004) and Laaksonen et al. (2007). PCR amplicons were sequenced in both directions using ABI Prism Big Dye Terminator v 3.1 Sequencing Kit (Applied Biosystems, Foster City, CA), and the sequences analysed with Genetic Analyzer 3130 (Applied Biosystems, Appiera Denmark) as per manufacturer's instructions. The generated nucleotide sequences are available in GenBank: Accession numbers KU508979-KU508985.

Consensus 12S rRNA and *cox1* sequences were subjected to BLASTn analysis (http://blast.ncbi.nlm.nih.gov) and compared to all *S. tundra* nucleotide sequences available in current databases. Sequence identities (in %) were calculated by pairwise comparisons. Subsequently, the consensus sequences were aligned with a selected subset of closely related sequences of the genus *Setaria*. Phylogenetic relationships were inferred based on analyses employing the Neighbor-Joining (NJ) method using MEGA7 (Kumar et al., 2016).

3. Results and discussion

The morphology of worms isolated from the peritoneal cavity was identical to *S. tundra* by light microscopy (Fig. 2). This was confirmed by sequences of the 12S rRNA and *cox1* genes, which were 99.1–99.8% identical to previously published *S. tundra* isolates from Germany, France, Italy, Spain and Finland. Phylogenetic trees constructed based on 12S rRNA and *cox1* sequences were similar in topology and therefore only one tree is presented (Fig. 3). Accordingly, the Danish isolates described here were clearly grouped with other isolates of *S. tundra* from Europe in one node that had high bootstrap values for NJ (0.99). However, sequence analysis showed slight variability within the Danish isolates, which was reflected in

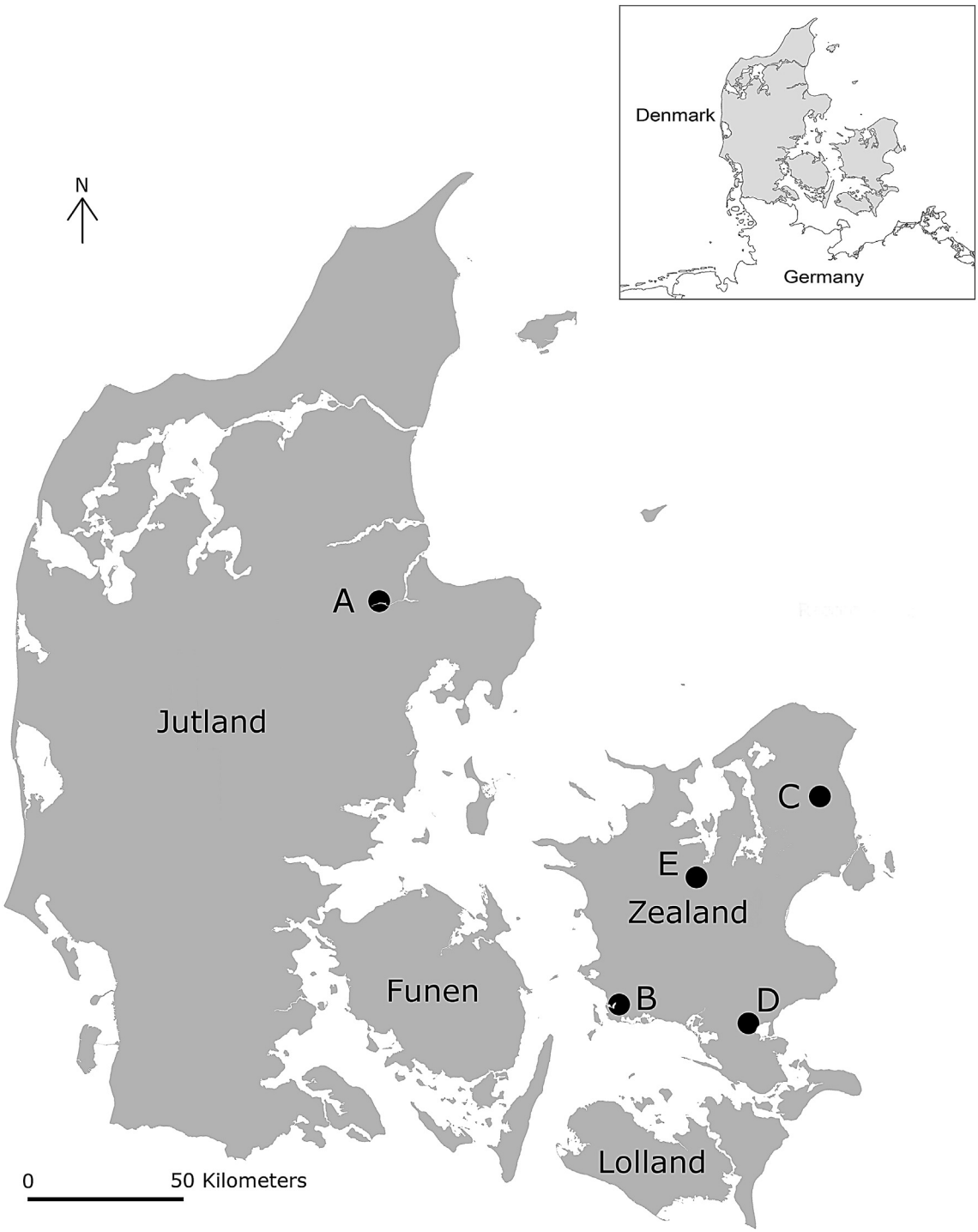

Fig. 1. Geographical origin (black dots) of *Setaria tundra* recovered from six infected roe deer. A: October 2010, B: May 2011, C: December 2012, D: May 2013 (two cases), and E: March 2014.

their topology in the phylogenetic tree. For example, the Danish isolates 2013-410-1 & 2, which geographically originated from Zealand (an island) were more closely related to the isolate 2010-1233 that originated from Jutland (a peninsula) than the other isolate (2012-1665) from Zealand. The Danish isolate 2012-1665, with worms encapsulated in the liver capsule, had 0.5% variation in the *cox1* compared to other Danish isolates, but had a lower level of variability (0.2–0.3%) and was phylogenetically more closely related to other European isolates (Fig. 3). Despite the low bootstrap values, the general topology of the *Setaria* spp. resembles a previously published tree by Alasaad et al. (2012).

This is the first report of *S. tundra* in Denmark. Worms of *Setaria transcaucasica* were earlier recovered from 41 out of 76 roe deer (53.9%) collected from the island Læsø in Denmark (Korsholm, 1988). In that study, the worms recovered from younger animals were encapsulated in different visceral organs and tissues, whereas in older animals the worms were found free in the peritoneal cavity. It is hard to determine if the proposed identification of the parasites

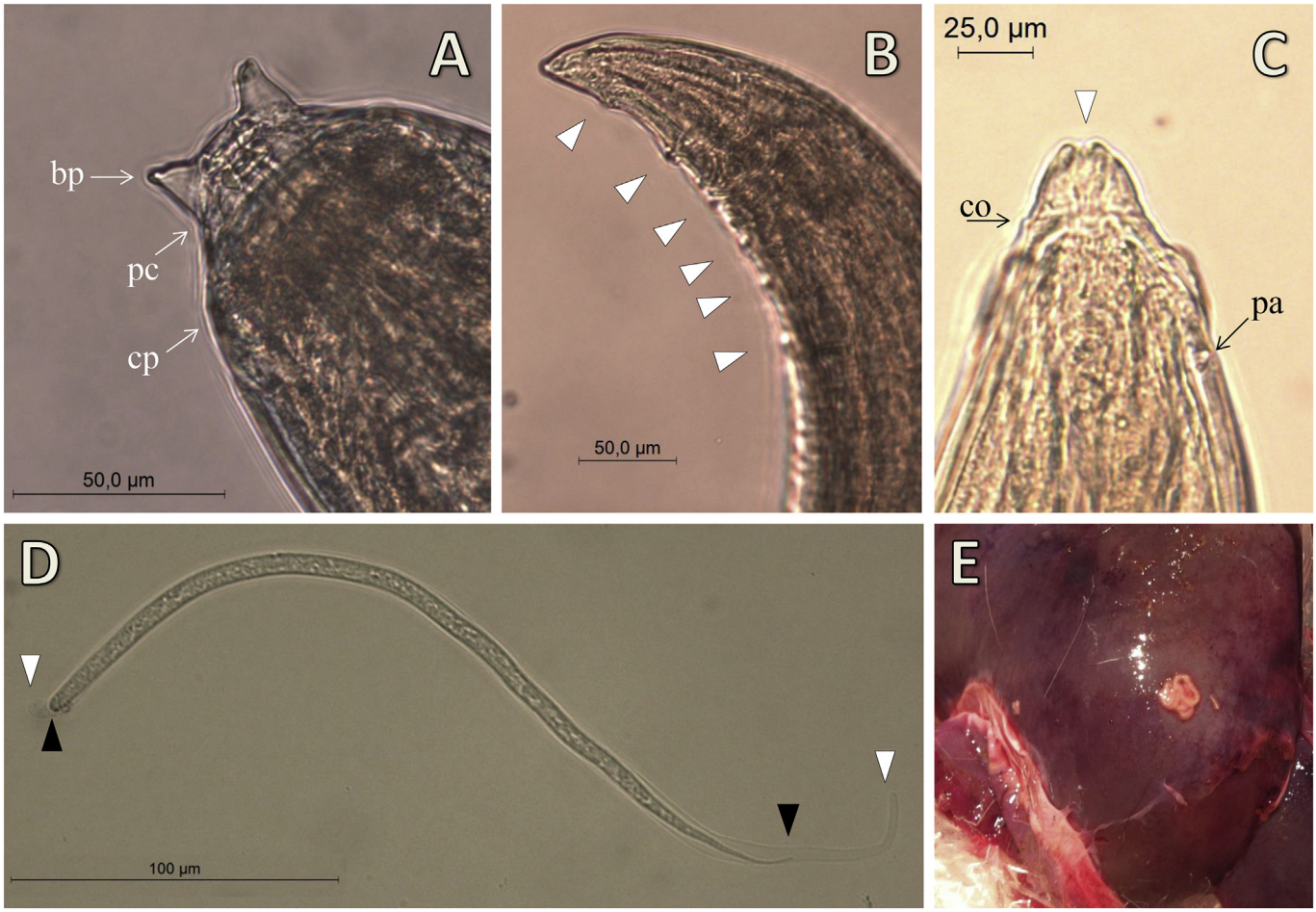

Fig. 2. Morphology of adult worms of *Setaria tundra* (A–C) and microfilaria (D and E) recovered from roe deer in Denmark. A: Cephalic region showing the bifid projections (bp) carried on top of a peribuccal crown (pc) and one of the four cephalic papillae (cp). B: Posterior end of male worm with papillae weakly visible (arrowheads). C: Posterior end of female worm showing a knob at the tip of the tail (arrow head), that possesses longitudinal grooves and pores, a papilla (pa), and a collar composed of a row of bosses (co). D: Microfilaria collected from a female worm. The length of the microfilaria including the sheath (white arrow heads) was approximately 316 µm, whereas the microfilaria was approximately 287 µm, with a blunt anterior end and a tapering posterior end. E: *Setaria tundra* coiled under the liver capsule (case 3). Scale bars indicated for all but figure E.

was valid in the study by Korsholm (1988) because of the similar position of the worms in the subcapsular layer of visceral organs in that study in comparison to the current case 3 (Isolate 2012-1665) and scarce information can be found in the literature about the vectors, mode of transmission or morphology for *S. transcaucasica*. Indeed the taxonomic status of this parasite is highly ambiguous. Thus it is possible that these findings were actually *S. tundra.* Certainly, considering the presence of *S. tundra* to the north and east of the country it is unlikely that the parasites found in the current study represent very recent introductions into Denmark. Nonetheless, the existence of an isolated island with a remarkably high prevalence of a vector-borne filarial nematode may present a model for studying the transmission dynamics of that parasite.

Infections with *S. tundra* are of particular interest to game meat and fur retailers (Rehbinder, 1990; Laaksonen et al., 2007). Reports from Finland linked poor body condition, under-developed fur in winter, dry fur, and reduced mean slaughter weight of reindeer infected with *S. tundra* (Laaksonen et al., 2007). In roe deer, most infections with *S. tundra* have less impact on meat and coat quality but if animals are severely afflicted approximately 152 metric tons of game meat (DVFA, 2012), at an approximate value of 8,8 million Euros could be potentially affected. In Denmark, the annual hunting yield of roe deer is over 125,000 (Asferg, 2012). Infected viscera from reindeer may be condemned at meat inspection, but the carcass is considered fit for human consumption even without heat treating (Laaksonen et al., 2007). Based on the present results we consider the findings of *S. tundra* incidental and not associated with the emaciation, which was observed in three of the six cases (1, 2 & 6). In one animal (case 1) massive excretion of *Giardia* spp. cysts was seen and *Cryptosporidium* spp. oocysts were also detected. Another animal (case 6) was excreting high numbers of gastrointestinal nematodes. Only a single *S. tundra* was uncovered in the third animal (case 2) and no obvious parasitological explanation could be found for the observed enteritis and emaciation. Therefore the clinical signs may have been caused by other non-parasitic pathogens. Due to the low number of animals and the protracted study period nothing can be concluded regarding the general parasitological findings. It is worth noticing though that the zoonotic pathogens *Cryptosporidium* spp. and *Giardia* spp. (García-Presedo et al., 2013) and/or *I. ricinus*, which is an important vector for several zoonotic agents (Kauffmann et al., 2016; Scheid et al., 2016), were detected in all of the emaciated roe deer.

The spatio-temporal distribution and transmission of vector-borne diseases are highly affected by climatic change (Hoberg et al., 2008). Previous outbreaks of setariosis in Scandinavia have been associated with unusually warm weather, and given the right circumstances the parasite has demonstrated its capacity to increase its geographic range considerably (Rehbinder et al., 1975;

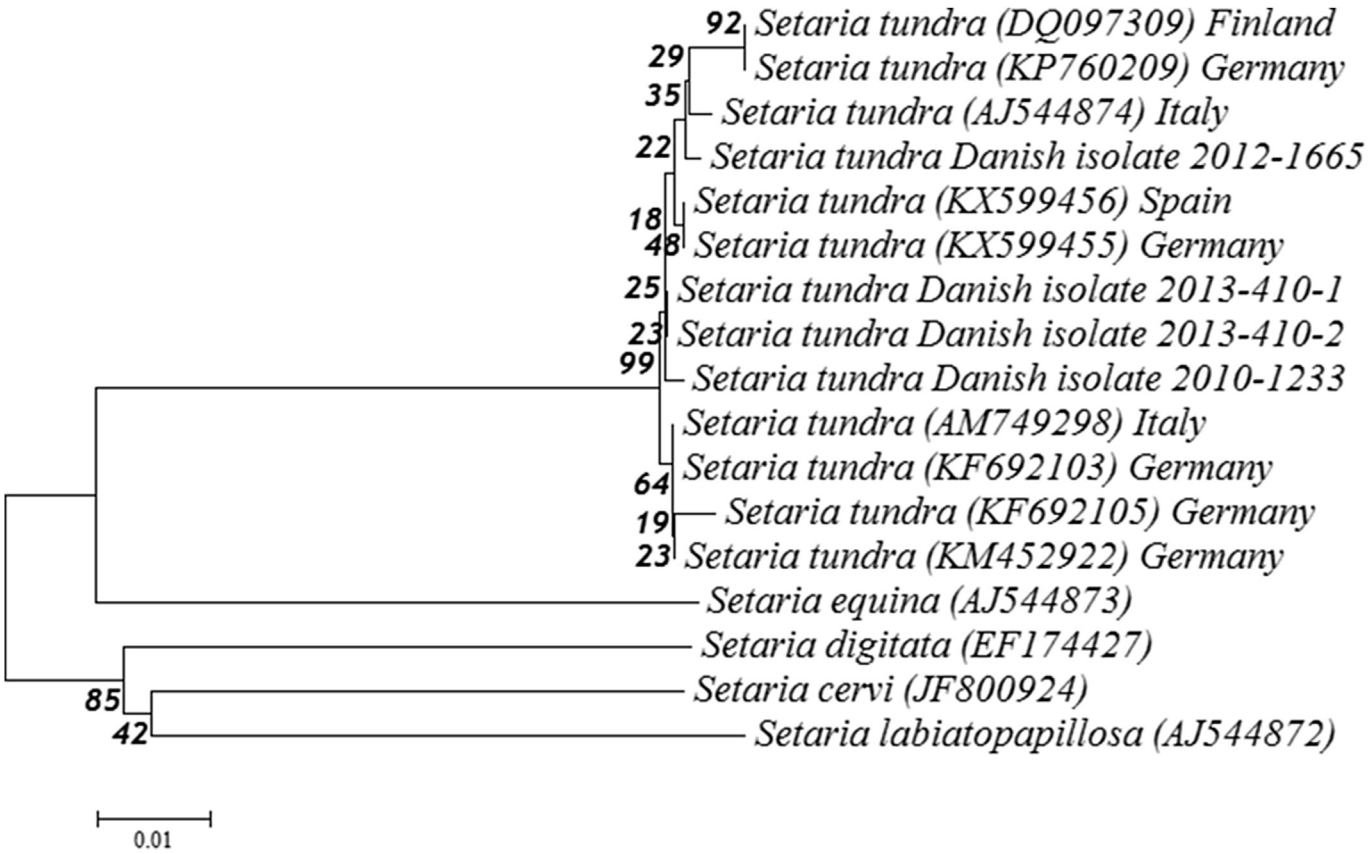

Fig. 3. Neighbor-Joining phylogenetic relationship of four isolates of *Setaria tundra* from distant localities in Denmark. The analysis was based on *cox1* gene sequences (578 bp). Percentage bootstrap support from 1000 replicate samples is indicated at the right of the supported node. Accession numbers for sequences obtained from GenBank are given in parentheses, followed by origin of isolate, only applicable to *S. tundra*. The scale bar indicates distance.

Laaksonen et al., 2009). Additional mosquito species (*Coquillettidia richiardii* and *Ochlerotatus annulipes*) not previously known as *S. tundra* vectors have been observed to carry the parasite in Hungary, potentially increasing the vector species this parasite utilizes and it is likely that *S. tundra* is not vector specific (Laaksonen et al., 2009; Angelone-Alasaad et al., 2016). This expected plasticity in vector species is concerning considering the likelihood that global warming will expand the range of various mosquito species into northern latitudes (Dupouy-Camet, 2016).

While surveillance is based on voluntary submissions to the National Veterinary Laboratory nothing is known about prevalence of *S. tundra* in roe deer, fallow deer (*Dama dama*) and red deer (*Cervus elaphus*) (the three cervid species present in Denmark), although the distant geographical origin of the current cases in conjunction with their relatively diverse molecular characterization is further indicative of a well-established population. This does not preclude, however, increasing prevalence in the future as a response to climate change.

The distribution and abundance of hosts and vectors can explain the spatio-temporal presence of *S. tundra*. The outbreak of setariosis in the 1970s in Scandinavia was reported a few years after the introduction of roe deer into the same area (Haugerud, 1989), which suggest a likely role of roe deer in the dissemination of this parasite (Rehbinder et al., 1975; Laaksonen et al., 2007). The species is considered to be the predominant reservoir in Finland, and in Germany where prevalence ranges from 1.6% in North Rhine-Westphalia to up to 12.3% in northern Bavaria in roe deer (Czajka et al., 2012). With a reported mean daily range of 8.5 ha in roe deer (Jeppesen, 1990) and an open land border between Jutland and Germany, frequent crossings are likely to occur and are an obvious possible route of the parasite into the country. How the parasite was introduced onto the island of Zealand is not clear but the heterogeneous genetic profiles found on the island could indicate multiple introductions. In northern Finland the increased prevalence of *S. tundra* in slaughtered reindeer (Laaksonen et al., 2007) was linked to aggregation of reindeer in herds in mosquito-rich wetlands (Laaksonen et al., 2009). The risk for transmission is highly enhanced when susceptible hosts are aggregated (Opara and Fagbemi, 2008). Given that *S. tundra* is not vector specific, higher rate of transmission of this parasite is accordingly expected in woodlands that are close to water, where ungulates are aggregated in large numbers.

Conflicts of interest

The authors declare no conflicts of interest.

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