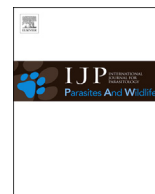




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## First description of *Onchocerca jakutensis* (Nematoda: Filarioidea) in red deer (*Cervus elaphus*) in Switzerland

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### ABSTRACT

Twenty-seven species of the genus *Onchocerca* (Nematoda; Filarioidea) can cause a vector-borne parasitic disease called onchocercosis. Most *Onchocerca* species infect wild and domestic ungulates or the dog, and one species causes river blindness in humans mainly in tropical Africa. The European red deer (*Cervus e. elaphus*) is host to four species, which are transmitted by blackflies (simuliids) or biting midges (ceratopogonids). Two species, *Onchocerca flexuosa* and *Onchocerca jakutensis*, produce subcutaneous nodules, whereas *Onchocerca skrjabini* and *Onchocerca garmsi* live free in the hypodermal serous membranes. During the hunting season, September 2013, red deer ( $n = 25$ ), roe deer (*Capreolus c. capreolus*,  $n = 6$ ) and chamois (*Rupicapra r. rupicapra*,  $n = 7$ ), all shot in the Grisons Region (Switzerland) were investigated for the presence of subcutaneous nodules which were enzymatically digested, and the contained *Onchocerca* worms were identified to species by light and scanning electron microscopy as well as by PCR/sequencing. In addition, microfilariae from skin samples were collected and genetically characterized. Neither nodules nor microfilariae were discovered in the roe deer and chamois. Adult worms were found in 24% of red deer, and all of them were identified as *O. jakutensis*. Two morphologically different microfilariae were obtained from five red deer, and genetic analysis of a skin sample of one red deer indicated the presence of another *Onchocerca* species. This is the first report of *O. jakutensis* in Switzerland with a prevalence in red deer similar to that in neighbouring Germany.

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### 1. Introduction

Onchocercosis is a parasitic disease caused by any of the 27 species of the genus *Onchocerca* which are transmitted by simuliid or ceratopogonid insect vectors (Anderson, 2000). Most *Onchocerca* spp. infect wild and domestic ungulates, while *Onchocerca lupi* Rodonaja, 1967 infects dogs. The parasites cause lesions, inflammation of musculature and joints and lameness in ungulates, or eye disease in dogs (Deplazes et al., 2013). Furthermore, *Onchocerca volvulus* (Leuckart, 1893) Railliet and Henry, 1910 is the causative agent of river blindness of humans, mainly in tropical Africa, with currently over 37 million people being infected (WHO, 2015). Except for river blindness, onchocercosis in humans is rare. Only 25 cases have been described to date (reviewed in Uni et al., 2015),

such as an unusual infection with *Onchocerca jakutensis* in a woman in Austria (Koehsler et al., 2007).

Worldwide seven *Onchocerca* species are known to parasitize Cervidae (Anderson, 2000). Publications from Europe describe four species in red deer (*Cervus elaphus*) in central Europe: *Onchocerca flexuosa* (Wedl, 1856), *O. jakutensis* (Gubanov, 1964) (Syn. *Onchocerca tubingensis* Bain and Schulz-Key, 1974), *Onchocerca skrjabini* (Ruchljadev, 1964) (Syn. *Onchocerca tarsicola* Bain and Schulz-Key, 1974) and *Onchocerca garmsi* Bain and Schulz-Key, 1976 (Schulz-Key et al., 1975). Studies have revealed *Onchocerca* infections in cervids in the Czech Republic and Slovakia (Dyková and Blazek, 1972), Hungary (Meszaros and Sugar, 1974), Yugoslavia (Brglez and Zeleznik, 1976), Romania (Dulceanu and Ghitescu, 1986), Germany (Schulz-Key, 1975), Poland (Demaskiewicz, 1993), Spain (Santin-Durán et al., 2001; San-Miguel et al., 2003) and Italy (Morandi et al., 2011).

Two species, *O. flexuosa* and *O. jakutensis*, produce large subcutaneous nodules up to several centimetres in diameter, whereas *O. skrjabini* and *O. garmsi* live free in the hypodermal serous membranes (Plenge-Bönig et al., 1994). The subcutaneous nodules

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of *O. flexuosa* are located between the shoulder blade and the lumbar region (Brglez et al., 1967), in the region of the sacrum and rarely on the lateral abdomen or proximal parts of the extremities (Plenge-Bönig et al., 1994). The subcutaneous nodules of *O. jakutensis* are found in the region of the external thigh or in the caudal part of the back (Bain and Schulz-Key, 1974). The rare filarial species *O. garmsi* is located in the tissue above the sternum (Schulz-Key et al., 1975). *Onchocerca skrjabini* is found free between the serous membranes above the radio-carpal and tibio-tarsal joints or on the abductor tendon (Bain and Schulz-Key, 1974).

The microfilariae of *O. garmsi* and *O. jakutensis* are present with highest concentrations in the skin above the sternum, and also at the base of the ear. Microfilariae of *O. skrjabini* are predominantly found in the exterior part of the ear. They also appear in the nose region, on the scrotum and on the insides of the thigh (Schulz-Key et al., 1975), whereas preferred sites of *O. flexuosa* microfilariae are the inside of the thigh and the distal parts of the hind leg (Schulz-Key, 1975).

Little is known about the pathogenicity of the different species of *Onchocerca* in red deer. Dyková (1970) described a chronic sclerosing lymphadenitis caused by microfilariae of *O. flexuosa*. Later, she observed a microfilaria-induced myositis of the surrounding musculature and a dystrophic change of the hypodermal tissue (Dyková, 1972). Commichau (1982) reported a polyarthritis caused by an *Onchocerca* species in red deer.

The present investigation elucidates the presence of *Onchocerca* spp. in wild ruminants in Switzerland. The presence as well as the particular species is also of medical interest, as endemic zoonotic onchocercosis should be considered. Furthermore, the morphology of *O. jakutensis* as described by Bain and Schulz-Key (1974) versus Demaskiewicz (1993) differs in some parts. In such situations, DNA analysis has proven helpful and was used e.g. by Morandi et al. (2011) and in a zoonotic case (Koebsler et al., 2007). However, the morphology of the specimens identified in these molecular analyses remains uncertain. A detailed morphological description of the worms recovered here is therefore included in the present investigation to link with the results of DNA analyses.

## 2. Materials and methods

### 2.1. Origin of samples, sample preparation and morphological identification

The skins of 25 free-ranging red deer (*Cervus e. elaphus*), six roe deer (*Capreolus c. capreolus*) and seven chamois (*Rupicapra r. rupicapra*) were investigated for nodules of adult worms during the hunting season, September 2013. The animals were shot in the Grisons Region, Switzerland (Churer Rheintal, Prättigau, Heinzenberg, Albulatal, Hinterrheintal, Lenzerheide, Safiental, Domleschg and Misox). To isolate adult worms, the nodules were incubated for 8 h at 60 °C in subtilisin-enzyme solution (10% [v/v] Enzymim OSA, Bauer Handels GmbH, Adetswil, Switzerland), buffered at pH 8.00 (9888 Titrisol Merck, Germany) and 2 drops of Mollescal-C (Bauer Handels GmbH) per 10 ml. After lysis specimens were fixed and stored in 70% ethanol. For morphological examination, adult worms were cleared in chloro-lactophenol. Drawings were done with a Leitz drawing tube. Scanning electron microscopy was performed at the Center for Microscopy and Image Analysis, University of Zurich using standard procedures. Specimens were dehydrated in 100% ethanol, followed by critical-point-drying and then coated with carbon or platinum.

In addition, 2 × 2 cm skin samples from the abdominal and from the metatarsal regions were collected from all investigated ungulates. The skin samples were treated as described by Schulz-Key (1975), with some modifications: They were cut into small pieces

and incubated in petri dishes for 2–5 h at 37 °C in 20 ml of a saline solution containing 0.9% (w/v) NaCl and 0.1% (v/v) Mollescal-C (BASF, Ludwigshafen, Germany) as a preservative. After the incubation period, the skin shreds were removed, the petri dish set at a slight angle, and 8 droplets of the sediment microscopically investigated for the presence of microfilariae. The rest of the sediment from the samples containing microfilariae were kept frozen in 1.5 ml Eppendorf tubes for genetic analyses. Morphological identification was achieved using the keys given in Bain (1981) and Demaskiewicz (1993).

### 2.2. DNA extraction

About 2 cm long sections of single adult worms (n = 10 from four red deer) were washed 2 to 3 times in phosphate-buffered saline (PBS) at room temperature and transferred into 1.5 ml Eppendorf tubes containing 20–40 µl PBS. To disrupt the cell membranes, the tubes were frozen in liquid nitrogen, thawed in a heat block at 100 °C for 1 min and then rigorously vortexed for 1 min. This step was repeated three times. Worms were then triturated with a tissue homogenizer as described by Wenk et al. (2012). The tubes containing microfilariae were centrifuged for 5 min and the supernatant was reduced to 30 µl. Five µl were again examined for the presence of microfilariae, and the remaining 25 µl were minced in a tissue homogenizer. DNA isolation was done with the Qiagen (Hildesheim, Germany) Qiaamp DNA mini kit according to the tissue protocol. DNA isolations were immediately frozen on dry ice and stored at –18 °C.

### 2.3. PCR

PCR was carried out according to Morales-Hojas et al. (2006), targeting the mitochondrial NADH-dehydrogenase gene. Amplifications were performed using the forward primer ND50vA (5'-TTG GTT GCC TAA GGC TAT GG-3') and the reverse primer ND50vC (5'-CCC CTA GTA AAC AAC AAA CCA CA-3') in a MyCycler thermocycler (Biorad, Cressier, Switzerland). Reactions were performed in a total volume of 20 µl, containing standard Taq-polymerase buffer (BioConcept, Alschwil, Switzerland), 200 µM each dNTP (NBL, UK), 0.4 µM each primer (Microsynth, Balgach, Switzerland), 1 unit Taq-DNA polymerase (BioConcept) and 2 µl of the DNA extract. Amplifications consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C, with a final extension step of 5 min at 72 °C. Amplicons were analysed on 3% agarose gels, stained with GelRed™ (Chemie Brunschwig, Basel, Switzerland) and visualized with UV light. Negative controls containing H<sub>2</sub>O were run to control for contamination.

### 2.4. Sequencing and analysis

Amplicons were sequenced (Synergene, Schlieren, Switzerland), after purification with the minelute PCR purification kit (Qiagen) following the manufacturer's instructions, using the PCR forward primer and 5 µl of the amplicons. Sequences were aligned with Multialin (Corpet, 1988) and then compared with GenBank entries.

## 3. Results

### 3.1. Prevalence of *O. jakutensis*

Of the 38 artiodactyls from the three species examined, *Onchocerca* specimens were found only in red deer. In six of the 25 red deer, subcutaneous nodules (n = 13) (Fig. 1) were collected containing female and male or only female *O. jakutensis* worms as

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