Parasitology Research (2018) 117:3851–3856
https://doi.org/10.1007/s00436-018-6091-4

ORIGINAL PAPER

Molecular evidence of infection with air sac nematodes in the great tit (*Parus major*) and the captive-bred gyrfalcon (*Falco rusticolus*)

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Received: 16 March 2018 / Accepted: 18 September 2018 / Published online: 22 September 2018

Abstract
Serratospiculiasis is a parasitic disease caused by filariid nematodes of the genus *Serratospiculum* that parasitise the air sacs of various species of falcons, bald eagles and Cooper's hawks around the world. An infection with *Serratospiculum* was recently confirmed in a nonspecific host, the great tit, in Slovakia. Parasitic material from this host was fixed for molecular analysis. Nematode found in the air sacs from a captive-bred gyrfalcon was also stored. Analysis of small subunit (18S) ribosomal DNA (18S rDNA) gene indicated that sequences from *Serratospiculum* sp. and *Serratospiculoides amaculata* were closely related to a reference sequence from *Serratospiculum tendo*, in agreement with morphology. This study is the first to generate molecular data and infer the phylogenetic position of *S. amaculata* as the first representative of the genus *Serratospiculoides*.

Keywords Air sac nematodes · *Serratospiculum* · *Serratospiculoides* · Great tit · 18S rDNA · DNA sequencing

Introduction

Birds can be affected by many species of air sac filariid nematodes of *Diplotriaena*, *Serratospiculum* and *Serratospiculoides* (Spirurida) (Ward and Fairchild 1972; Samour and Naldo 2001; Honisch and Krone 2008). *Serratospiculum* and *Serratospiculoides* are closely related and have similar developmental patterns (Sterner and Cole 2008). Infected arthropods, mainly coprophagous beetles, are presumed to be intermediate insect hosts in the indirect life cycle. Birds become infected by ingesting intermediate or paratenic hosts, such as small birds or mammals (Anderson and Bain 1976; Anderson 2000; Sterner and Cole 2008). Clinical signs in birds infected with these two species include dyspnoea, weight loss, anorexia and lethargy. The occurrence of adult parasites, larvae and embryonated eggs in the air sacs can damage tissues and cause secondary bacterial infections, which can increase the risk of pneumonia or cause airsacculitis, aspergillosis or death of the host (Tarello 2006; Sterner and Cole 2008). Infection with these nematodes may also affect flying performance, such as speed and strength, or predatory effectiveness (Illescas-Gomez et al. 1993; Tarello 2006; Santoro et al. 2010).

Recent studies have reported *Serratospiculum* spp. from European birds of prey in Switzerland, Iceland, Italy and Poland (Kalisińska et al. 2008; Christensen et al. 2015; Santoro et al. 2016; Veiga et al. 2017). Parasitic infections caused by *Serratospiculum* spp. are occurring in various species of the order Falconiformes. Nine species of *Serratospiculum* and two species of *Serratospiculoides* are known to parasitise birds (Sterner and Cole 2008). Data for *Serratospiculum* spp. in birds of prey are relatively abundant, but little information is available for *Serratospiculoides* spp. and *Serratospiculoides amaculata*, which have been reported from a prairie falcon (*Falco mexicanus*) (Hawkins et al. 2001)

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Section Editor: Dr. Georg von Samson-Himmelstjerna

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and other birds of prey in North America (Sterner and Espinosa 1988). Adult specimens of *S. amaculata* in the air sacs of a nonspecific host, the great tit (*Parus major*), have been recently identified in Slovakia based on morphological, clinical and pathological features (Königová et al. 2013). However, this species is currently based on new nomenclature not considered a member of *Serratospiculum* but of *Serratospiculoides* (Sterner and Cole 2008; Anderson et al. 2009). That is why we use the name *Serratospiculoides amaculata* in this study.

Well-described morphological features of nematodes (Skrjabin and Sobolev 1963) combined with molecular data, such as DNA sequences, have recently been used for nematode characterisation. The highly variable, noncoding internal transcribed spacer regions (ITS-1, ITS-2) of ribosomal DNA and mitochondrial cytochrome C oxidase are commonly used for species identification and barcoding. The slowly evolving small subunit ribosomal DNA gene sequences, though, have been used most extensively for studying the phylogenetic relationships of nematodes (Gasser 2001; Honisch and Krone 2008; Ebmer et al. 2017; Vieira et al. 2017). The present study was therefore designed to verify the morphological systematics of *S. amaculata* from the great tit using molecular data and to infer its phylogenetic position based on 18S rDNA sequences.

Material and methods

Parasitic material

An adult female of great tit found dead in 2012 in an Eastern Slovakia's urban area was submitted for necropsy. The air sacs were examined for helminths and few specimens were collected. The identification of the parasites was based on morphological features (Königová et al. 2013). Parasites were washed in saline solution and fixed in 70% ethanol for DNA analyses.

Nematodes were also recovered from the air sacs of a captive-bred gyrfalcon (*Falco rusticolus*) that died in Nad Al Shiba Falcon Hospital, Dubai, United Arab Emirates. Parasites were washed in saline solution and then fixed in 70% ethanol following by clearing in lactophenol solution as described previously (Pritchard and Kruse 1982). Identification of female parasites was based on the morphological features delineating species of the genus *Serratospiculum* (Anderson et al. 2009). Unfortunately, the male specimens were not available; therefore, it was not possible to determine the *Serratospiculum* species morphometrically according to the shape and length of small and long spicules.

DNA isolation, PCR amplification and electrophoresis

Genomic DNA from the *Serratospiculum* sp. sample and from *S. amaculata* sample was isolated using a NucleoSpin Tissue kit (Macherey-Nagel GmbH, Düren, Germany) following the manufacturer's protocol. Six microliters of template gDNA were used in 25-μl PCR reactions. 18S rDNA was amplified using the oligonucleotide primers D-1F and D-1R (Wijova et al. 2006) and 1.25 U GoTaq DNA polymerase (Promega, Madison, USA). PCR was performed under the following thermocycling conditions: denaturation at 95 °C for 9 min; six cycles of 95 °C for 1 min, 44 °C for 1 min and 72 °C for 2 min; 24 cycles of 95 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min; and a final extension at 72 °C for 10 min. PCR products of 18S rDNA stained with ethidium bromide were evaluated by electrophoresis at 90 V for 50 min in a 1% agarose gel in TAE buffer, purified and sequenced as described by Wijova et al. (2006).

Gel purification and sequencing

PCR products were excised from the gel, and the DNA was extracted using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following the manufacturer's instructions. The concentration of the eluted DNA was measured spectrophotometrically (ND 1000, Thermo Fischer Scientific, Waltham, USA), and samples were stored at − 20 °C. Sequencing reactions contained 2 μl of Big Dye reagent (ABI Prism Version 3.1, Applied Biosystems, Foster City, USA), 1 μl of each primer (1/10 of the concentration used in PCR amplification), 2 μl of purified DNA and 5 μl of Milli-Q water. All sequencing reactions were thermocycled and amplified with an initial denaturation at 94 °C for 3 min followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The amplified products were sequenced using a Hitachi ABI Prism 3100 Avant Genetic Analyzer DNA Sequencer (Applied Biosystems, Foster City, USA).

Sequence analysis

Regions corresponding to the PCR primers were removed prior to analysis. For primary identification, the sequences were compared by a Nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the following parameters: database search—all, expected threshold—10, search optimised for BLAST (somewhat similar sequence). Sequence alignment and neighbour-joining analysis were performed using Geneious 11.0.4 (Kearse et al. 2012) with the default settings. Bayesian Inference phylogenetic tree was constructed using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001). Sequences used for the phylogenetic reconstruction were downloaded from the NCBI (Table 1).

Table 1 Species belonging to the orders Spirurida and Oxyurida with corresponding GenBank accession numbers used in the phylogenetic analysis

Species	Family	Accession no.
Serratospiculum tendo	Diplotriaenidae	AY702704
Serratospiculum sp.	Diplotriaenidae	MG792054*
Serratospiculoides amaculata	Diplotriaenidae	MG792055*
Serratospiculoides amaculata	Diplotriaenidae	MG792056*
Serratospiculoides amaculata	Diplotriaenidae	MG792057*
Hadjelia truncata	Habronematidae	JX235976
Cyrnea seurati	Habronematidae	EU004816
Cyrnea leptoptera	Habronematidae	EU004815
Microtetrameres cloacitectus	Habronematidae	EU004814
Synhimantus hamatus	Acuariidae	EU004819
Synhimantus laticeps	Acuariidae	EU004818
Echinuria borealis	Acuariidae	EF180064
Physaloptera apivori	Physalopteridae	EU004817
Physaloptera alata	Physalopteridae	AY702703
Oxyspirura petrowi	Thelaziidae	KF110799
Tetrameres fissispina	Tetrameridae	EF180077
Oxyuris equi	Oxyuridae	EF180062

Accession numbers of new sequences generated in the present study are marked with an asterisk

Results

In this study, DNA from two air sac nematodes was amplified by PCR. One sample was obtained from the gyrfalcon. The nematode was identified as being an adult form of *Serratospiculum* sp. The other sample was obtained from the great tit nonspecific host in Slovakia. A morphological analysis of the male reproductive organs (shape of male spicules) indicated that the species was *S. amaculata* (Königová et al. 2013).

One partial sequence of the 18S rDNA of *Serratospiculum* sp. and three partial sequences of *S. amaculata* were determined from fragments obtained by amplification. All new nematode sequences obtained in our study have been deposited in the GenBank database (Table 1). The 18S rDNA sequences ranged from 201 to 679 bp, consistent with studies of Spirurida nematodes with sizes up to 670 bp (Hamer et al. 2012; Lefoulon et al. 2015; Vieira et al. 2017).

The sequences of *Serratospiculum* sp. and *S. amaculata* 18S rDNA were used for a homology search using BLASTN. The BLAST analysis indicated that the 18S rDNA sequences of *Serratospiculum* sp. were highly homologous to a previously published partial sequence of the spirurid nematode *Serratospiculum tendo* (GenBank accession No. AY702704.1). The sequence was 99% identical to the *S. tendo* sequence. Similarly, the *S. amaculata* sequences had a maximum nucleotide identity of 96% with the partial *S. tendo* sequence, with 100% sequence coverage. The BLAST sequence similarity of all *S. amaculata* sequences ranged from 94 to 96%, with 99–100% coverage. Our molecular analyses found that the *Serratospiculum* sp. and *S. amaculata* 18S rDNA sequences were closely related to the *S. tendo* reference sequence, supporting morphological analysis data.

We inferred the phylogenetic positions of the *Serratospiculum* and *Serratospiculoides* species by comparing their nucleotide sequences with other species. Phylogenetic trees were rooted using *Serratospiculum* sp. sequence (MG792054), *S. amaculata* sequence (MG792055) and sequences of another 12 species obtained from GenBank for the 18 rDNA region, obeying the following criteria: sequences that are somewhat similar to new sequences presented here, taxonomic classification belonging to Spirurida, and which are avian parasites (Vieira et al. 2017). *Oxyuris equi* of the family Oxyuridae (order Oxyurida) was used as an outgroup. A phylogenetic tree for *Serratospiculum* sp. and *S. amaculata* was constructed using neighbour-joining (Fig. 1) and Bayesian Inference (Fig. 2) methods. Both analyses revealed well-supported and almost identical trees. 18S rDNA sequences of *Serratospiculum* sp. and *S. amaculata* were closely related to the *S. tendo* reference sequence, in agreement with our previous results.

Discussion

Morphological identification of nematode species in the order Spirurida is difficult. The air sacs of raptors (Falconiformes) are most often infected with parasites belonging to two nematode genera, i.e., *Diplotriaena* and *Serratospiculum*. Nematodes infecting air sacs belonging to the genus *Serratospiculoides* occur less frequently. All three genera have historically been confused with filariid nematodes based on their long filiform bodies, sexually dimorphic features and presence in air sacs (Sterner and Cole 2008). Skrjabin (1916) described a genus *Serratospiculum*. As morphological characteristics were similar to those of filariids, this genus was placed in the superfamily Filarioidea, family Filariidae and subfamily Setarinae. Later, studies by Chabaud (1964) showed that the species within the genus *Serratospiculum* did not develop microfilariae. Studies conducted by Anderson (1962) confirmed the life cycle and led to reclassification of *Diplotriaena*, *Serratospiculum* and *Serratospiculoides* in the order Spirurida. The new classification placed the genus *Diplotriaena* in the family Diplotriaenidae and subfamily Diplotriaeninae and placed the two genera *Serratospiculum* and *Serratospiculoides* in the subfamily Dicheilonematinae (Anderson 1992).

The number of species within the genera *Serratospiculum* and *Serratospiculoides* remained constant over the last several years. On the basis of length of the spicules, nine species of

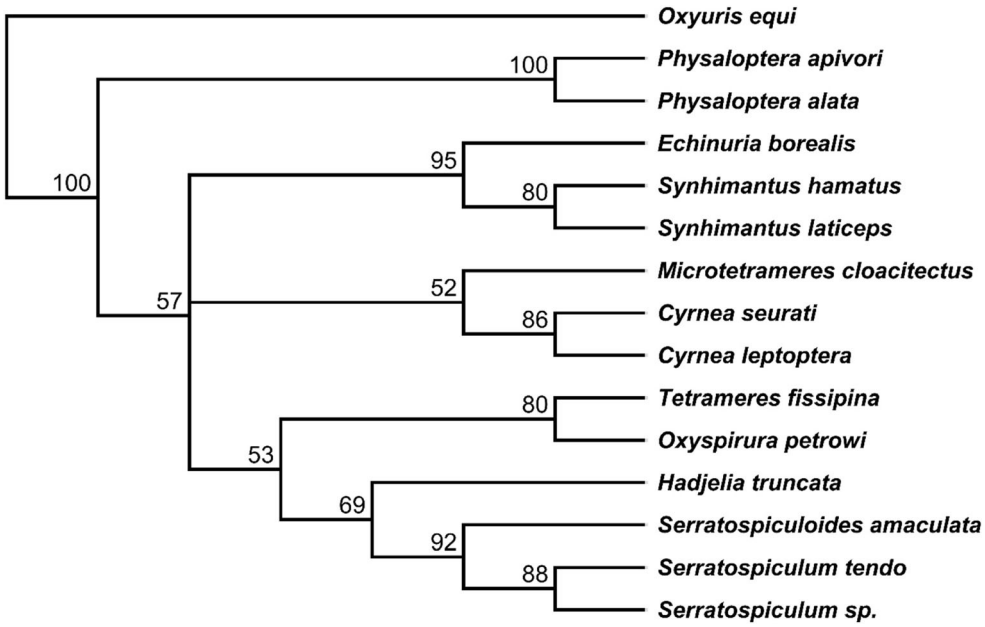

Fig. 1 Phylogenetic tree for *Serratospiculum* sp. and *Serratospiculoides amaculata* constructed based on a neighbour-joining analysis, with *Oxyuris equi* as the outgroup. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

Serratospiculum are recognised from avian hosts in the order Falconiformes (Samour and Naldo 2001). Differences in spicule characteristics led to reclassification of this species as the new genus *Serratospiculoides* (Sonin 1968; Samour and Naldo 2001). There are only two species in this genus, i.e., *S. alii* and *S. amaculata.* Based on above-mentioned reclassification, we use the name *Serratospiculoides amaculata* in great tit, and *Serratospiculum* sp. in gyrfalcon in our study.

Few molecular data, however, are available for the genera *Serratospiculum* and *Serratospiculoides.* We carried out molecular analyses to provide the first data for *S. amaculata*. Our phylogenetic analyses based on 18S rRNA with neighbour-joining (Fig. 1) and Bayesian inference method (Fig. 2) produced trees that agreed with those produced by others. The genera *Serratospiculum* and *Serratospiculoides* were closed to the branch consisting of *Oxyspirura petrowi* (Thelaziidae) and *Tetrameres fissispina* (Tetrameridae) (Vieira et al. 2017). The phylogenetic tree indicated that the genera belonging to the families Acuariidae, Diplotriaenidae, Habronematidae and Tetrameridae were closer to each other and relatively distant to Physalopteridae, in agreement with previous studies (Chabaud and Bain 1994; Vieira et al. 2017).

Only one sequence of 18S rDNA encoding the small ribosomal subunit of *Serratospiculum* species, and no sequences for *S. amaculata*, has previously been deposited in the GenBank database. To the best of our knowledge, this study

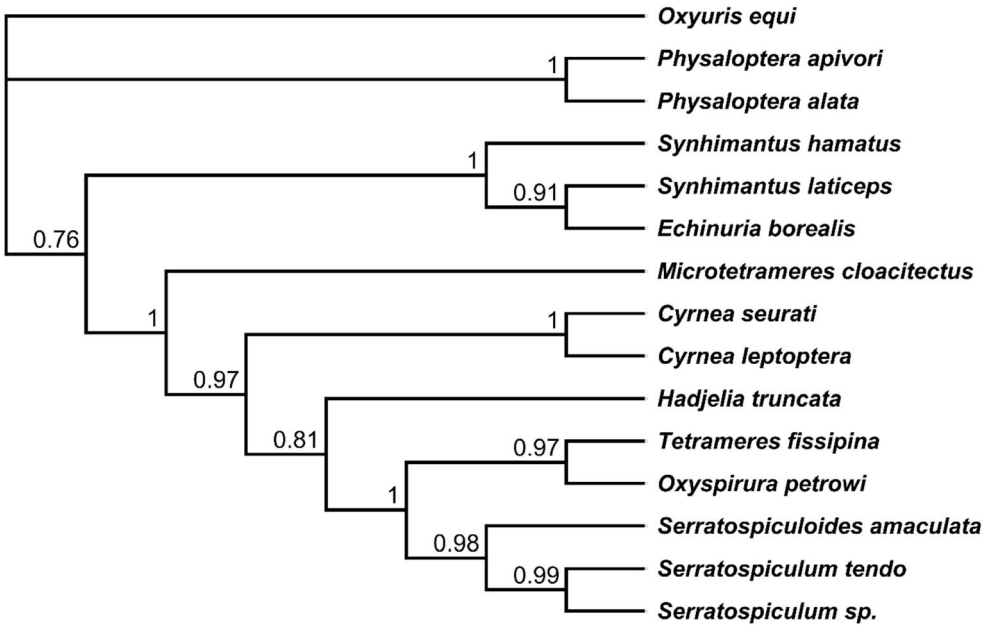

Fig. 2 Phylogenetic tree for *Serratospiculum* sp. and *Serratospiculoides amaculata* constructed based on a Bayesian Inference method, with *Oxyuris equi* as the outgroup. Clade posterior probabilities are indicated at nodes

is the first to generate molecular data and to infer the phylogenetic position of *S. amaculata* as the first representative of the genus and provides the first molecular characterisation of serratospiculiasis in Slovakia.

Acknowledgements We thank Johan Forsman from the Nad Al Shiba Falcon Hospital, Dubai, UAE, for his help with the collection of the worms. We are grateful to Silvia Spišáková for her technical assistance.

Funding information This study was supported by funds from the Slovak Research and Development Agency APVV 14-0169 and scientific grant agency VEGA 2/0120/16.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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