**Diplostriaena obtusa** (Nematoda: Filariidae) infection in first-year *Sylvia atricapilla* from Poland – molecular evidence

A. STANICKA1*, K. S. ZAJAC2, M. JEFIMOW3, & M. S. WOJCIECHOWSKI4

1Department of Invertebrate Zoology and Parasitology, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Toruń, Poland, 2Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Kraków, Poland, 3Department of Animal Physiology and Neurobiology, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Toruń, Poland, and 4Department of Vertebrate Zoology and Ecology, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Toruń, Poland

(Received 15 June 2021; accepted 19 October 2021)

**Abstract**

Insectivorous birds are particularly vulnerable to nematodes with heteroxenous life cycles. Although there are many studies on bird filarioids, they mainly focus on economically important or pet bird species, and as a result, the species diversity of these parasites is insufficiently studied. Research on the genus *Diplostriaena* and their hosts is neglected, although they are globally occurring and dangerous parasites with low specificity to the final host. Here we report the prevalence, invasive intensity and species affiliation of the filarial nematodes of the genus *Diplostriaena* in a common passerine – Eurasian blackcap (*Sylvia atricapilla*, L.). In total, 24 first-year individuals of *S. atricapilla* were caught in Toruń (central Poland) at their breeding grounds in July and August 2019, and after 7 months in captivity, they were killed and dissected. Over 20% of dissected birds were infected with *Diplostriaena*, and their air sacs were inhabited by 1 to 18 adult worms. Molecular identification of nematode worms was done using the 18S small subunit rRNA gene, and they were identified as *D. obtusa*. Our study is the first to show the molecular confirmation of the presence of *D. obtusa* in *S. atricapilla*.

**Keywords:** Filarial nematodes, warblers, air sacs, 18S region, prevalence

**Introduction**

Filarial species represent a relatively small group of parasitic nematodes with a significant impact on human and animal health (Morales-Hojas 2009). The most important species from the medical point of view are *Wuchereria bancrofti* and *Brugia* spp. causing lymphatic filariasis (elephantiasis) and *Onchocerca volvulus* responsible for onchocerciasis (river blindness) (Morales-Hojas 2009). However, other zoonotic and truly enzootic filariasis were also identified and described previously (Müller & Wakelin 2002; Otranto et al. 2011; Kenemesi et al. 2015). For example, truly enzootic filariasis is caused by *Setaria* spp., *Acanthocheilonema* spp. or *Eufilaria* spp. in ungulates, rodents and birds, respectively (Ološ et al. 2019; Binkienė et al. 2021; Risch et al. 2021). In most cases, filariases are vector-borne infections transmitted by various insects (mosquitoes, black flies, locusts) and arachnids (ticks) (Namrata et al. 2014; Bravo-Barriga et al. 2016; Akramova et al. 2017; Heym et al. 2019; Aupalee et al. 2020; Tokarz et al. 2020). As a result, arthropod-consuming birds are particularly vulnerable to these parasites. Parasitic worms seem to be more dangerous than other types of parasites, because they may contribute to the death of their avian host. Examples of such parasites include filarial nematodes belonging to the genus *Diplostriaena* Henry & Ozoux, 1909 (Okulewicz & Sitko 2012). Passerine birds ingest the parasite by swallowing an adult Orthoptera infected with the third-stage larvae (L3) (Bain & Vaucher 1973). The larvae migrate...
from the bird intestines through the bile ducts to the liver, where they moult the cuticle. Then the fourth-stage larvae (L4) migrate through the portal vein to the heart, and finally, adult worms settle in the host’s air sacs. Female nematodes lay eggs that are excreted and end up in the trachea, where they are swallowed and then excreted in the faeces (Cawthorn & Anderson 1980). Certain pathogenic effects of Diplostriaena spp. in final hosts have been reported including central nervous system disturbance, marked body mass loss, appetite loss, scarce plumage, subcutaneous emphysema, pneumonia and airsacculitis. The parasite may cause death due to the intensification of the lesions in the host, or sudden death caused by nematode migrations (Keymer 1982; Fasina et al. 2007; Sterner & Cole 2008; Hong et al. 2019). Diplostriaena spp. have been recorded in Africa, America, Asia, and Australia (Mackerras 1962; Olsen & Braun 1971; Fasina et al. 2007; Borji & Razmyar 2011; Okulewicz & Sitko 2012; Fard et al. 2015; Hong et al. 2019; Rentería-Solís et al. 2021). A total of over 70 species have been described (Hong et al. 2019), mainly based on morphological features (Chabaud 1955; Al-Ankari et al. 2003; Okulewicz & Sitko 2012; Soomro et al. 2016; Bernardon et al. 2018; Ceolan Morais et al. 2018). There are only few case reports based on molecular identification of these parasites (Vieira et al. 2017; Hong et al. 2019; Rentería-Solís et al. 2021). Binkiené et al. (2021) pointed out that species diversity of bird filarial nematodes is insufficiently investigated and that research on the filarioïds mainly focus on economically important or pet birds, and only few of were done on wild birds, including the widely distributed warblers, for instance, belonging to the genus Sylvia Scopoli, 1769 (Passeriformes: Sylviidae). Here we report the species affiliation of the filarial nematode of the genus Diplostriaena, its prevalence and the intensity of invasion in Eurasian blackcap Sylvia atricapilla (Linnaeus, 1758) from central Poland.

Supplemented with minerals and vitamins. After completion of experiments, birds were killed by cervical dislocation and immediately dissected. During the necropsy, the presence of parasites was checked only in air sacs, thoracic and abdominal cavities. More precise parasitological post-mortem examinations were not possible because then the bird bodies were subjected to another investigation led by scientists from the Department of Vertebrate Zoology and Ecology, Nicolaus Copernicus University in Toruń, Poland. The research was performed under permits issued by General Directorate for Environmental Protection in Poland (ref. no. DZP-WG.6401.03.144.2018.dl) and by the Local Committee for Ethics in Animal Research in Bydgoszcz, Poland (ref. no. 33/2018).

**Morphological identification of parasites**

Isolated nematodes were counted, their sex was determined (according to Chabaud (1955) and Bernardon et al. (2018)) and body length was measured (to the nearest 1 mm). Then, few nematode specimens were fixed in ethanol (96%) and frozen (−20°C) for subsequent molecular identification. The remaining individuals isolated from S. atricapilla were leached out for a few minutes in 10% KOH and subjected to further morphological identification. Initial genus identification of the nematodes was done based on the morphological traits and morphometry of the specimens according to available images and descriptions (e.g. Chabaud 1955; Vieira et al. 2017; Bernardon et al. 2018) using a light microscope (Axio Lab A1) with various magnification (5x, 10x, and 40x). Images were recorded using a digital camera (Axiocam 105 color, Carl Zeiss) and a computer system running Zen software (version 2.3, blue edition). Due to the different visibility of individual morphological features and the condition of the fixed nematodes, the measurements of individual features were taken from a different number of specimens, but not less than three.

**Materials and methods**

**Sampling and necropsy of birds**

Birds were caught in Toruń (Poland) (53°00′ N, 18° 35′ E) after breeding and at the beginning of autumn migration 2019. Sylvia atricapilla were captured with mist nets and identified at the capture site. Next, the birds were transported to the laboratory where they were kept in captivity for further research purposes for 7 months. During this period S. atricapilla were fed with a mixture of fish feed, hard boiled eggs and DNA extraction, amplification and sequencing

DNA extraction was performed by using NucleoSpin Tissue Kit (Macherey-Nagel, Germany) from adult worms (each originated from another host specimen). A PCR reaction of the 18S small subunit rRNA gene was done by using Nem18SF (5′-CGCGAATRGCTTCATTACAAACG-3′) and Nem18SR (5′-GGGCGGTATCTGATCGCC-3′) primers (Floyd et al. 2005). A PCR reaction of each sample was performed in a 20 µl reaction mixture, consisting of 3.2 µl of template DNA, 10.2 µl of ddH2O, 2 µl of 10x buffer B1 (HOT FIREPol®, Solis BioDyne,
Estonia), 2 µl of 25 mM MgCl₂ (HOT FIREPol®, Solis BioDyne, Estonia), 0.8 µl of each primer, 0.8 µl of 20 mM dNTP (ThermoFisher Scientific, USA), and 0.2 µl of Taq-Polymerase (HOT FIREPol®, Solis BioDyne, Estonia). PCR conditions consisted of 15 min initial denaturation at 95°C; 30 s denaturation at 94°C, followed by 90 s annealing at 55°C, and 90 s elongation at 72°C for 35 cycles; followed by 10 min a final elongation step at 72°C. A 3-µl sample of PCR product was run on a 1.5% agarose gel for 30 min at 100 V to check DNA quality. PCR products were cleaned up by using EPICC Fast (A&A Biotechnology, Poland). A sequencing reaction was performed based on protocols (reaction mixture, conditions) described previously in Zajac and Stec (2020). Sequencing products were cleaned using ExTerminator kit (A&A Biotechnology, Poland) and sequenced in Genomed company (Warsaw, Poland) in both directions. Obtained sequences were deposited in GenBank with the following accession numbers: MW680965, MW680966, MW680967, MW680968.

Data analysis

All obtained sequences were blasted with NCBI BLAST (Altschul et al. 1990) to verify species identification. The sequences that resulted in at least 95% identity in NCBI BLAST to our sequences were chosen for the phylogenetic analysis. To visualize relationships between studied species and other nematodes, selected 18S rRNA sequences of D. obtusa (Accession numbers: MT129507, MT129509, MT129513, MT129515, MT129522-523), D. bargusinica (KX583753, KX545341-347), Serratospiculum tendo (MW168997-999), Gymna seruri (EU004816), Setaria yehi (KT934942), S. digitata (DQ094175), Mastophorus muris (MN086288-290), Onchocerca cervipedis (KT031393), O. cervicais (DQ094174), Rumenfilaria andersoni (KT878979, KT907509, KT885224-225), Dirofilaria repens (AB973229), Dipetalonema yatesi (MW192232-233), Oxystrongylus petroli (LC316613) and W. bancrofti (AY843436) were downloaded from GenBank (Bhandari et al. 2005; Honisch & Krone 2008; Suzuki et al. 2015; Vieira et al. 2017) and together with sequences obtained in this study were aligned in BioEdit 5.0.0 (Hall 1999) with the multiple alignment function of ClustalW (Thompson et al. 1994). The obtained alignment (858 bp length) comprised 39 sequences in total. A sequence of Odostomum cestoides (Trematoda: Azygidae) was used as an outgroup (Accession number: AJ287553; Cribb et al. 200). Uncorrected pairwise distances within D. obtusa were calculated in MEGA v. 7 (Kumar et al. 2016) based on sequences from GenBank and obtained in this study.

Bayesian inference (BI) marginal posterior probabilities were calculated in MrBayes v. 3.2 (Huelsenbeck & Ronquist 2001; Huelsenbeck et al. 2001) with 1 cold and 3 heated Markov chains for 10 million generations and trees were sampled every 1000 generations. In the BI consensus tree, clades recovered with posterior probability (PP) between 0.95 and 1.00 were considered as well supported. The obtained tree was visualized in FigTree v.1.4.3 programme (http://tree.bio.ed.ac.uk/software/figtree).

Results

Parasitological examination

In total, 24 first-year individuals of S. atricapilla were necropsied, and 5 of them (21%) were infected with nematodes. The invasion intensity ranged from 1 to 18 adult worms in the host air sacs (Table I). Two cases of infection involved only one male nematode, while in the remaining cases the infestation included both sexes (Table I).

The nematodes were identified as Diplotriaena sp. based on morphological features presented in Table II. Body length of female and male nematodes were 32–46 mm and 13–25 mm, respectively. The nematodes were characterized by a milky white body with a simple mouth without lips. A pair of chitinous tridents (female: 0.128–0.157 mm, male: 0.123–0.152 mm) was observed at the head end of the male and female worm. On the caudal part of the male body, there was a smaller (0.484–0.660 mm) and a larger spicule (0.63–838 mm). Eggs were oval and smooth measuring 0.046–0.048 × 0.028–0.030 mm (Figure 1).

Molecular examination

The verification in NCBI BLAST resulted in fitting to D. obtusa (MT129522 (Michalski et al. 2021);

Table I. The number of collected filarial nematodes and male to female ratio in Sylota atricapilla from Torun, Poland.

| No. of nematodes collected | Host number | Male | Female | Total | Ratio of male to female |
|----------------------------|-------------|------|--------|-------|-------------------------|
| 1                          | 1           | 0    | 1      | 1     | 1.0                     |
| 2                          | 1           | 0    | 1      | 1     | 1.0                     |
| 3                          | 1           | 3    | 4      | 0.33:1|
| 4                          | 4           | 14   | 18     | 0.29:1|
| 5                          | *           | *    | 1      | *     |                         |

* - Not investigated
Bayesian (BI) phylogenetic tree showed that all sequences obtained in this study were very similar and clustered together with sequences of D. obtusa collected from the barn swallow Hirundo rustica (Passeriformes: Hirundinidae) in the USA, available in GenBank (Accession numbers: MT129507, MT129509, MT129513, MT129515, MT129522-523; Figure 2). In the presented analysis D. obtusa is the most closely related to D. bargusinica (Figure 2). Intraspecific genetic distance within D. obtusa calculated based on sequences that make up the D. obtusa clade (10 sequences in total) was lower than 0.2%. For the comparison, the genetic distance between D. obtusa and D. bargusinica (single haplotype in the dataset) which is the most closely related species to D. obtusa, was 2.02%.

Discussion

Our report is one of a few in the world and the first one in Poland containing molecular confirmation of species identification of the genus Diplotriaena. The GenBank database currently has sequences of the genus Diplotriaena belonging to only three species, namely: D. anthreptis (Hong and Park, unpublished), D. bargusinica (Vieira et al. 2017) and D. obtusa (Rentería-Solís et al. 2021; Michalski et al. 2021). Our research confirms the presence of D. obtusa in S. atricapilla. Using molecular techniques, D. obtusa was also identified in Cyanistes caeruleus (Passeriformes: Paridae) in Germany (Renteria-Solis et al. 2021) as well as in H. rustica and Petrochelidon pyrrhonota (Passeriformes: Hirundinidae) in the United States (Michalski et al. 2021). Until today, there were only limited reports on the occurrence of the genus Diplotriaena in Poland, i.e. D. tridens in S. atricapilla (Oktulewicz & Sitko 2012) and S. borin (Passeriformes: Sylviidae) (Oktulewicz 1982), D. ozouxi in Motacilla flava (Passeriformes: Motacillidae) (Oktulewicz 2013), D. henryi in Parus major (Passeriformes: Paridae) (Oktulewicz 1991) and D. obtusa in

Table II. Measurements of morpho-taxonomic features of nematodes collected in Sylvia atricapilla (± SE) [mm].

| Feature                          | Male               | Female              |
|----------------------------------|--------------------|---------------------|
| Length of body                   | 18.0 (± 0.01)      | 39.9 (± 0.01)       |
| Length of the right spicule (smaller) | 0.573 (± 0.02) | -                   |
| Length of the left spicule (largest) | 0.731 (± 0.04) | -                   |
| Height and width of eggs         | -                  | 0.048 x 0.029 (± 0.001) |
| Length of trident                | 0.139 (± 0.07)     | 0.141 (± 0.08)      |
the parasites. The lack of molecular identification of numerous species of the genus *Diplotriaena* indicates that these parasites are neglected in scientific research.

Research on these parasites is focused on the detection of adult worms in the air sacs or thoracic and abdominal cavities during necropsy of final hosts (Fard *et al.* 2015; Vieira *et al.* 2017; Hong *et al.* 2019; Rentería-Solís *et al.* 2021). Although there are many non-invasive parasitological tests carried out on wild birds (Benedikt 2006; DeGroote & Rodewald 2010; Luedtke *et al.* 2013; Santiago-Alarcon *et al.* 2013; Dadam *et al.* 2019; Bichet *et al.* 2020; Fecchio *et al.* 2020; Popescu *et al.* 2020), in the case of *Diplotriaena* spp. tests based on blood smears may be unreliable due to the periodic presence of microfilariae in the peripheral blood of final hosts (Keymer 1982). In addition, due to the lack of molecular characterization of most of the described filarial species, the examination of the adult worms is essential for their specific identification (Binkienė *et al.* 2021). Nevertheless, we agree that the procedure used by Binkienė *et al.* (2021) to detect different species of filarial nematodes, which involves taking blood samples and testing them in the field, and then collecting only individuals infected with microfilariae for dissection, allows for a better understanding of the system of filarial nematodes and wild birds. Examination of the presence of embryonated eggs in the faeces is another non-invasive diagnostic tool. However, Sterner and Cole (2008) indicated that the investigation is unreliable because other parasites besides air sac worms lay morphologically similar eggs. Additionally, according to our personal observations, the concentration method (flotation in

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**Figure 2.** The Bayesian inference (BI) phylogeny of the selected nematode species constructed based on 18S rRNA sequences. Numbers at nodes indicate Bayesian posterior probability. Bolded and red accession numbers indicate sequences obtained in this study. The scale bar represents 0.03 substitutions per nucleotide position.
saturated MgSO₄ solution) can not detect infection at low intensity, possibly because parasites lay egg periodically. Most research on Diplotriaena spp. was carried out on dead birds found in the field (Fasina et al. 2007; Okulewicz & Sitko 2012; Fard et al. 2015; Hong et al. 2019; Renteria-Solis et al. 2021), so it is difficult to discuss the true prevalence of this parasite in the environment. Here we had this unique opportunity to observe and analyze infestation in birds randomly sampled from the environment, which were killed for other purposes. The prevalence of D. obtusa in S. atricapilla population from the capture site was quite high and reached over 20%. However, it is still difficult to talk about the true prevalence because we investigated the low number of hosts during one season. This is because we used birds captured for other research purposes. Low intensities of infection (number of worms in air sacs), was probably related to the age of birds, although other factors cannot be excluded. For example, the coexistence of other infections can increase or decrease parasitic/pathogen load (Ramsay & Rohr 2021). Okulewicz and Sitko (2012) counted even 138 individuals of D. tridens in a dead host, indicating that the invasion may be the direct cause of the host death. Little is known about the real effect of filarial nematodes on the survival, fitness, or reproductive capacity of their wild bird hosts, as opposed to protozoan parasites e.g. of the genus Haemoproteus (Apicomplexa: Haemoproteidae) or Plasmodium (Apicomplexa: Plasmodiidae) (Martinez-de la Puente et al. 2010; Lachish et al. 2011; Fletcher et al. 2019; Romano et al. 2019). The progress of research on the intermediate hosts of avian parasites is also limited. The majority of studies on the larvae of Diplotriaena spp. in intermediate hosts come from several decades ago, and we are not aware of the insect species used by the parasite or its distribution and prevalence in intermediate host populations (Bain & Vaucher 1973; Cawthon & Anderson 1980; Kabilov 1983; Akramova et al. 2017). Czajka et al. (2012) emphasize that the roles of vectors in the life cycles of many filarial species and their geographical distribution are largely unknown. In contrast, there is a lot of recent research on the presence of avian apicomplexan parasites in the intermediate hosts (Chakarov et al. 2020; Ferreira et al. 2020; Taioe et al. 2020).

Our report highlights how poorly the system between the genus Diplotriaena and both, final and intermediate hosts has been understood so far. It is also the first study showing the molecular confirmation of the presence of D. obtusa in S. atricapilla. Now we can only assume what the real effects of infestation for the host are, how a wide range of host species is used, what is the parasites’ true prevalence and spread in natural populations of the host. We believe that our work will shed light on the ecology of these parasites, and finally extend our knowledge on the genus Diplotriaena.

Acknowledgements
We would like to thank Anna Kowalczewska, Anna Nowak and Anna Przybylska-Piech for their help in the laboratory and in the field.

Funding
This study was supported by National Science Center Grant 2017/25/B/NZ8/00541.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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