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The molecular and morphometric identification of *Dictyocaulus capreolus* in clinically affected roe deer (*Capreolus capreolus* L.)

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Abstract: The poor state of health and increased mortality rate of young roe deer, as reported by South Moravian hunters, caused by the increasing numbers of adult nematodes in the lungs of roe deer prompted us to identify the parasites using a combination of morphological measurements and a phylogenetic SSU rRNA analysis. The study was conducted in a 294 ha game reserve in South Moravia, Czech Republic. Molecular and morphometric techniques were used to identify adult nematodes collected from the respiratory tracts of nine 4–5 months old roe deer in poor health (low body weight of 3–4 kg, poor haircoat quality, and, in some cases, symptoms of diarrhoea). The morphological identification was based on a combination of adult worm characteristics corresponding to *Dictyocaulus capreolus.* A small subunit rRNA (SSU) partial sequence analysis showed the highest identity scores (99%) corresponding to the sequences of *D. capreolus* from a roe deer (GenBank: AY168859) from Sweden and the outcomes of the phylogenetic analyses resulted in a tree with a high branch support for two groups, with our sequences forming a well-supported clade with *D. capreolus* and *Dictyocaulus* sp. ex *Capreolus capreolus* (FJ589016) and *Dictyocaulus* sp. ex *Rupicapra rupicapra* (FJ589019) sequences from Spain. The examined roe deer have shown symptoms of diarrhoea, anorexia, and respiratory tract inflammation indicating that there might be a connection to the clinical importance of the *Dictyocaulus* infection.

Keywords: lung nematodes; rRNA; respiratory tract; anorexia; diarrhoea

Nematodes of the genus *Dictyocaulus* cause parasitic bronchitis in a wide range of ruminant species. Originally, the species *Dictyocaulus viviparus*, known in cattle, had been reported as a causative agent of semi-domestic and wild cervid parasitoses, including those in roe deer (*Capreolus capreolus*), fallow deer (*Cervus dama*), reindeer (*Rangifer tarandus*), moose (*Alces alces*) and European bison (*Bison bonasus*) (Guildal 1962; Nilsson 1971; Christensson and Rehbinder 1975; Kummeneje 1977; Pyziel 2014). The composition of the genus *Dictyocaulus* in cervids remained controversial through the years. According to Jansen and Borgsteede (1990), lung helminths of cervid hosts were identical to *Dictyocaulus eckerti*. Lungworms of the genus *Dictyocaulus* were then studied using

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molecular tools, mostly based on analyses of the internal transcribed spacer 2 (ITS2) of the nuclear ribosomal RNA (rRNA). Epe et al. (1997) revealed differences in the ribosomal DNA sequences of *Dictyocaulus* lungworms from fallow deer, cattle, sheep, and donkeys. A later study of ITS2 sequences in *Dictyocaulus* species from cattle, roe deer, and moose provided evidence of a new *Dictyocaulus* species in roe deer and moose (Hoglund et al. 1999). The morphology of the new species has been described, and it has been named *D. capreolus* (Gibbons and Hoglund 2002). Phylogenetic studies of the nematodes within the genus *Dictyocaulus* based on analysis of ITS2 and the small subunit rRNA (SSU rRNA) revealed four host species, including three species of Cervidae (moose, reindeer and red deer, *Cervus elaphus*) and one species of Bovidae (musk ox, *Ovibos moschatus*) for *D. eckerti*. Lungworms *D. viviparus* were identified in cattle, *D. filaria* in sheep, and *D. capreolus* in moose and roe deer (Gibbons and Hoglund 2002; Hoglund et al. 2003). Recently, a novel genotype, *D. cervi* from red deer was described based on the unique SSU, ITS2 and mitochondrial cytochrome *c* oxidase subunit 1 sequences and the morphological features different from *D. eckerti* (Pyziel et al. 2017).

Clinical symptoms and pathological changes associated with dictyocaulosis have been widely described in cattle experimentally infected with *D. viviparus*. Among the clinical symptoms, respiratory distress such as polypnoea, coughing, dehydration, anorexia, diarrhoea and constipation, weight loss or poor weight gain were observed; the post-mortem findings included interstitial and subpleural emphysema and bronchopneumonia, bronchiolitis (Rubin and Lucker 1956), peribronchiolitis, as well as bronchial atelectasis and consolidation (Schneider et al. 1991). Infection of *D. cervi* in red deer was associated with interstitial pneumonia, bronchitis and bronchiolitis, moreover, emphysema, atelectasis and lung tissue congestion were also observed (Pyziel et al. 2018). Aguirre et al. (1999) conducted a retrospective epidemiologic study to examine the causes of mortality among wild roe deer in Sweden. The most common parasitic disease found in that study was verminous pneumonia caused primarily by *Varestrongylus capreoli* and *Dictyocaulus* sp. Statistically, the primary infection caused the mortality in 10% and 9% of females and males, respectively. Within Europe, the presence of *D. capreolus* in roe deer was recently reported from Sweden (Gibbons and Hoglund 2002) and Spain (Carreno et al. 2009).

During the past six years, reports from hunters in the Czech Republic's South Moravia Region have noticed an increasing presence of adult nematodes in the lungs of young roe deer that were in a poor state of health and had a higher mortality rate by apparent natural causes. In the present study, using a combination of morphological measurements and a phylogenetic SSU rRNA analysis, we report the determination of the *Dictyocaulus* species found in the roe deer showing clinical symptoms.

MATERIAL AND METHODS

The sampled animals and collection of parasites. During 2016, adult nematodes were collected for the morphological and molecular determination from the respiratory tracts of nine roe deer selected to be hunted due to their worsened health condition. All the animals originated from a 294 ha game reserve near Měnín in South Moravia. The roe deer were 4–5 months old and showed low body weight (3–4 kg), poor haircoat quality and delayed summer haircoat change, and, in some cases, symptoms of diarrhoea. The respiratory tract, including the lungs and trachea, was recovered from each dead animal. The trachea and main bronchi were opened longitudinally and visually examined. The necropsy revealed pathological changes in the lungs, including small apical atelectatic areas with peribronchial inflammation in some animals and ranging up to total bronchopneumonia in others. Adult *Dictyocaulus* nematodes were present in the bronchi and bronchioli in numbers ranging from several individuals to more than 80 nematodes in one animal. The nematodes were collected and stored in 10% formalin for morphological analysis and frozen in phosphate buffered saline at –20 °C until the molecular determination.

Morphological analysis. For the light microscopy examination, the adult nematodes preserved in 10% formalin were cleared for three weeks in a glycerol-ethanol solution by ethanol evaporation, and mounted on slides with a 50% glycerol aqueous solution. All the measurements were performed using an Olympus BX41TF microscope with the aid of an Olympus drawing tube attached to the microscope and based upon the glycerol-mounted specimens. The measurements are pre-

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sented in micrometres unless otherwise indicated. The measured features were: the length of the body, the width at the mid-body, the width of the head, the length of the oesophagus, the width and length of the buccal capsule, the length from the anterior to nerve ring, the anterior to excretory pore. Additional measurements in the males included the length of the spicules and gubernaculum, and in the females, the length of the vulva from the tail tip, the length of the tail, the length from the phasmids anterior to the tail tip, and the length and width of the eggs. Two females and three males were observed and the morphology was compared with the descriptions in the literature. The specimens are deposited at the Department of Pathology and Parasitology, University of Veterinary and Pharmaceutical Sciences, Brno (Czech Republic) under the accession numbers VFUD1–VFUD5.

DNA extraction and amplification. The total DNA from the nine individuals (one individual from each roe deer) was isolated using a NucleoSpin Tissue kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. Part of the SSU rRNA (1107 bp) was amplified using the primers, NC16 and NC2, adopted from Chilton et al. (2003); 25 µl reaction volume containing 12.5 µl of Combi PPP Master Mix (P-Lab, Prague, Czech Republic), 20 pmol of forward and reverse primer, 6.5 µl of polymerase chain reaction (PCR) water, and 2 µl of the DNA (10–20 ng). The cycling conditions were as follow: 94 °C for 5 min, then 30 cycles of 30 s at 94 °C (denaturation), 30 s at 55 °C (annealing) and 1 min at 72 °C (extension), with the final extension at 72 °C for 5 minutes. The PCR products were visualised on a 1.5% agarose gel stained with Good View (Ecoli, Bratislava, Czech Republic). The PCR amplicons were cleaned using ExoSAP-IT (Thermo Fisher Scientific, Waltham, USA) and sequenced commercially by Macrogen (Amsterdam, Netherlands) using the PCR primers.

Phylogenetic analyses. Sequences were assembled and inspected for errors using the Geneious software v5.6.7 (www.geneious.com, Kearse et al. 2012). The contigs were compared to those in the GenBank database in order to find the closest match and assemble a data set. The data set was aligned in the software MAFFT (https://mafft.cbrc.jp) and manually edited in the Geneious software. The most suitable nucleotide substitution model was selected according to the best Akaiko information criteria (AIC) in the jModeltest software v2.1.4 (Posada 2008). The phylogenetic analyses were run in two programs: PHYML (Guindon and Gascuel 2003), using maximum likelihood (ML), and MrBayes v3.2.2 (Ronquist and Huelsenbeck 2003), using Bayesian inference. Bayesian analyses were performed under the GTR + I + G model of nucleotide substitution and were run for 20 million MCMC generations, with four chains and four independent runs. The MCMC chain convergence was checked in AWTY (Nylander et al. 2008). The maximum-likelihood (ML) phylogeny was generated using the PhyML 3.0 software (Guindon and Gascuel 2003). The reliability of the branching patterns within the trees was tested by the bootstrap

Table 1. Small subunit rRNA sequences from the GenBank database used to create the data set. The outgroup sequences are marked with asterisks. The sequences obtained from this study are in bold

GenBank accession number	Species
AJ920351	*Ostertagia leptospicularis**
AJ920352	*Ostertagia ostertagi**
AJ920359	*Tetrabothriostrongylus mackerrasae**
AY168859	*Dictyocaulus capreolus*
AY168860	*Dictyocaulus* sp.
AY168861	*Dictyocaulus filaria*
AY168862	*Dictyocaulus capreolus*
AY168863	*Dictyocaulus eckerti*
AY295808	*Halocercus invaginatus*
AY295814	*Parafilaroides decorus*
AY295817	*Stenurus minor*
AY295818	*Torynurus convolutus*
AY295819	*Skrjabingylus chitwoodorum*
AY295820	*Troglostrongylus wilsoni*
EU086374	*Haemonchus contortus**
FJ589015	*Dictyocaulus* sp.
FJ589016	*Dictyocaulus* sp.
FJ589019	*Dictyocaulus* sp.
GU475121	*Metastrongyloidea* sp.
GU946677	*Troglostrongylus* sp.
GU946678	*Oslerus rostratus*
JX290562	*Troglostrongylus brevior*
KC771250	*Dictyocaulus viviparus*
KM035792	*Oslerus rostratus*
MG833324	***Dictyocaulus capreolus***
MG833325	***Dictyocaulus capreolus***
MG833326	***Dictyocaulus capreolus***

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method with 1000 re-samplings. The trees, thus obtained, were visualised and exported using TreeView v1.4.2 (Page 1996), then adjusted in Inkscape v0.91 (www.inkscape.org). For a full list of the sequences used, see Table 1.

RESULTS

Morphological analysis

The morphological characteristics of the adult *Dictyocaulus* in our study showed an elongated oval mouth opening, dorsoventrally flattened; an oval buccal capsule, dorsoventrally flatened with a thick wall.

Male (n = 3): body 51–52 mm long, 415–420 wide at the mid-body. Head 80–95 wide, oesophagus 1175–1288 long. Buccal capsule 35–48 wide, 12–15 long. Anterior to nerve ring 290–310, anterior to excretory pore 325–355 mm. Spicules 247–269 mm long. Gubernaculum present, 35–70 long.

Female (n = 2): body 51–63 mm long, 442–475 wide at the mid-body. Head 102–115 wide, oesophagus 1150–1550 long. Buccal capsule 52–55 wide, 13–20 long. Anterior to nerve ring 245–370, anterior to excretory pore 355–415. Vulva opens 23.75–26.41 mm from the tail tip. Tail 906–1038 long; phasmids 388–413 anterior to tail tip. Eggs 75–81 × 42–53.

Phylogenetic analysis

In total, we obtained the sequential data from three roe deer (*C. capreolus*) individuals. The sequences obtained for this study were deposited in the GenBank database (accession numbers: MG833324–MG833326). The strongylid SSU sequences available in the GenBank with the closest hit were used to create the data set and, as outgroup sequences, to root the final tree (marked with an asterisk, see Table 1). The total length of the final data set was 1614 bp. Our data showed the highest identity scores (99%) with *Dictyocaulus capreolus* (AY168859) from a roe deer and this result was also confirmed by the phylogenetic analyses (see Figure 1). The final tree is divided into two monophyletic groups (group A and B) with a high branch support (see Figure 1). Group A consists exclusively of the *Dictyocaulus* sp. sequences and the data obtained in this study; group B is formed by the other representatives of the superfamily Metastrongyloidea. Not only did our sequences

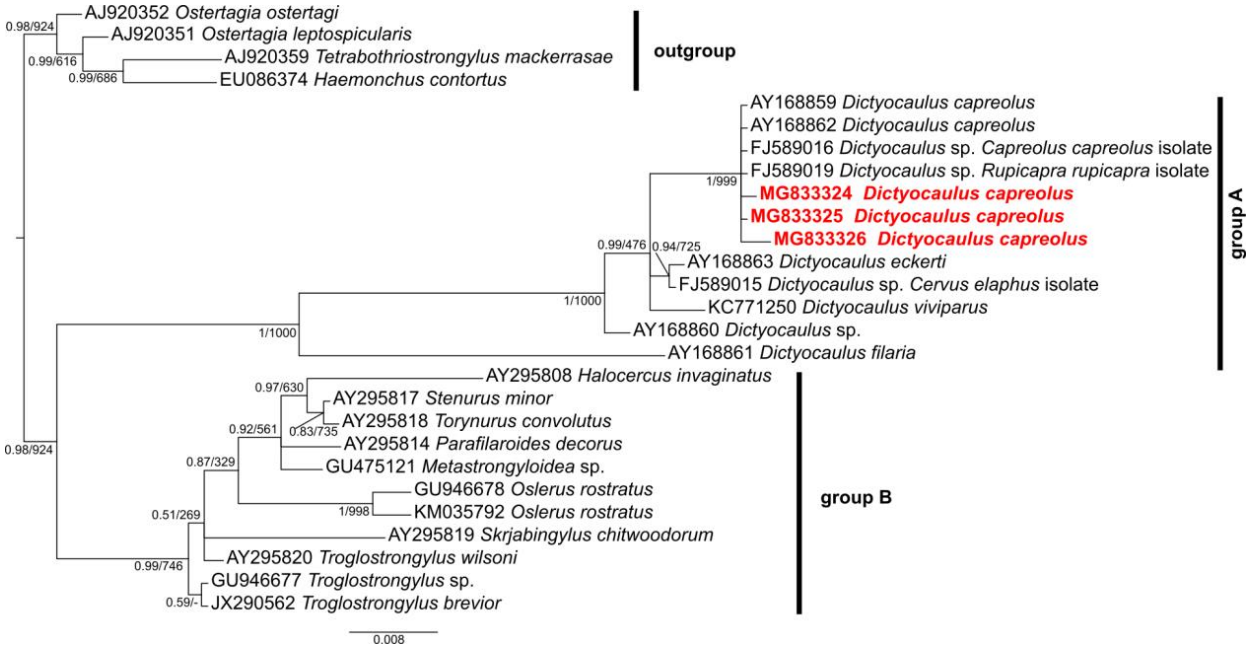

Figure 1. The phylogenetic tree based on the small subunit data of the strongylid nematodes combining posterior probabilities and bootstrap branch supports. The sequences formed into two well-supported monophyletic groups (A, B). Our sequences created a separate branch to the *Dictyocaulus* sp. data with a high bootstrap and posterior probability. Our data are highlighted in red

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form a well-supported clade with the sequences of *D. capreolus* (AY168859 and AY168862) and the sequences of *Dictyocaulus* sp. (FJ589016 and FJ589019), the whole group is highly supported by the posterior probabilities and ML bootstrap.

DISCUSSION

Although the *Dictyocaulus* species identification has historically been based upon the morphological characteristics of adult nematodes or larvae, the application of molecular tools has led to the new species determination and re-description of the host-parasite species relationships. The combination of the adult worm characteristics, including the total body length, the shape of the oral opening, and buccal capsule characteristics is important for the morphological identification of the *Dictyocaulus* species. The elongated oval shape of the specimen's oral opening in the present study differed from the circular opening of *D. viviparus* previously described in roe deer in Central Europe (Enigk and Hildebrandt 1965) and the buccal capsule length differed from those noted for *D. eckerti* (Durette-Desset et al. 1988). Nevertheless, the adult worm morphology from the roe deer in South Moravia corresponded to *D. capreolus* from the roe deer in Sweden (Divina et al. 2000; Gibbons and Hoglund 2002).

As concluded by Divina et al. (2000), however, the morphological characteristics have limitations for identifying the species level and so their use in combination with the molecular approaches is crucial.

In the present study, the SSU rRNA sequence analysis supported the morphological identification of the species as *D. capreolus* since the highest identity scores (99%) corresponded to *D. capreolus* from a roe deer previously described by Hoglund et al. (2003) in Sweden. The phylogenetic analysis in our study showed a well-supported clade of our sequences and the sequences of *Dictyocaulus* sp. from the roe deer and chamois described by Carreno et al. (2009), as well as with the sequences of *D. capreolus* published by Hoglund et al. (2003). The outcomes of the Bayesian and ML analyses were almost identical; hence, the presented tree is based on the Bayesian inference phylogenetic tree topology with branch supports stated for both analysis types – the posterior probabilities for the Bayesian analyses and the bootstrap for the ML. Although there was strong similarity with the results from the phylogenetic analyses, the posterior probabilities of the Bayesian inference gave us a stronger branch support compared to the ML results. The partial sequences of the SSU rRNA proved themselves to constitute a valuable tool for the *Dictyocaulus* taxonomic determination.

The pathological changes, including diarrhoea, anorexia, and respiratory tract inflammation in the examined roe deer, had not previously been described in this host species, but they are in accordance with observations described in cattle infected with *D. viviparus* (Rubin and Lucker 1956) and red deer infected with *D. cervi* (Pyziel et al. 2018). The rather high mortality of the young roe deer observed in the studied area was probably connected to the increase in the lungworm infection caused by *D. capreolus*.

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