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Large lungworms (Nematoda: Dictyocaulidae) recovered from the European bison may represent a new nematode subspecies

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ABSTRACT

Although the *Dictyocaulus* lungworm, the agent of dictyocaulosis, is one of parasitological threats to European bison, its systematic position remains unclear. The aim of the present study was to evaluate the morphological features of the lungworm and the pathological lesions it induces, and to analyse mitochondrial (*mt*) genetic markers for systematic and molecular epidemiological studies. The morphological findings indicate that *Dictyocaulus* lungworms of European bison can be distinguished from those of cattle on the basis of differences in buccal capsule wall length, total body length, and spicules length in males, all of which were significantly longer in those of European bison. Nucleotide diversity calculated from pairwise sequence alignments of partial cytochrome *c* oxidase subunit 1 (*cox*1), cytochrome B (*cyt*B) and *NADH* dehydrogenase subunit 5 (*nad*5) of specimens from cattle and European bison varied from 1.7% for *nad*5, 2.1% for *cyt*B, to 3.7% for *cox*1 gene. Thus, among the lungworms of European bison and cattle, *nad*5 and *cyt*B were the most conserved proteins, whereas *cox*1 was the most diverse. The *mt cyt*B marker gene may be a suitable candidate for distinguishing between the two genotypes, as *nad*5 demonstrated the greatest within-genus sequence variation. The lung tissue of infected European bison manifests signs of verminous pneumonia characterized by interstitial pneumonia, bronchitis and bronchiolitis. Therefore, it appears that European bison and cattle are infected with slightly diverged, morphologically-different, genotypes of *D. viviparus*, indicating they belong to two separate worm populations. We propose, therefore, that the lungworm of European bison should be classified as *D. viviparus* subsp. *bisontis*.

1. Introduction

Free-roaming populations of wild ruminants enrich the biodiversity of heavily-urbanized landscapes of European countries. A unique bovid inhabiting Poland is the European bison, *Bison bonasus*: the largest terrestrial mammal in Europe; however, it is considered vulnerable to extinction by the International Union for Conservation of Nature - Red List of Threatened Species (IUCN, 2020). The European bison also

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suffers from a loss of genetic variation, and is hence more vulnerable to pathogens due to inbreeding depression and the depletion of variation in the genes responsible for immunity against parasites (Keller and Waller, 2002; Radwan et al., 2010). Dictyocaulosis is a condition affecting various ungulate hosts, characterized by symptoms of bronchitis, with infection leading fatal outcomes in heavily-infected individuals (Demiaszkiewicz et al., 2009; Pyziel et al., 2018a). In order to understand the risk for cross-transmission between free-roaming European bison and cattle, and to the control the spread of dictyocaulosis, it is essential to have the potential to correctly identify lungworm species. However, although some initial studies on European bison-derived lungworms have been published, their systematic position remains unclear (Pyziel, 2014, 2018; Pyziel et al., 2017).

The small subunit (SSU) rDNA sequence of *D. viviparus* from European bison has been found to be generally identical to those from cattle; however, the two groups were found to diverge at the internal transcribed spacer (ITS2) region of the ribosomal RNA-array (Pyziel, 2014). Additionally, the *mt* cytochrome *c* oxidase subunit 1 (*cox*1) nucleotide sequence demonstrates high intraspecific diversity between the two strains of *D. viviparus* (Pyziel et al., 2017). However, the *mt* cytochrome *c* oxidase subunit 3 (*cox*3) gene was found as an inefficient *mt* marker for systematic study on *Dictyocaulus* spp. (Pyziel et al., 2018b).

The present study examines the morphological features of European bison lungworm, evaluates its impact on the lung tissue of the host, and identifies the most efficient *mt* genetic markers for systematic and molecular epidemiological studies.

2. Materials and methods

2.1. Specimen collection

A total of 30 male and 35 female adult *Dictyocaulus* lungworms were isolated from the respiratory tract of 15 free-roaming European bison. All European bison had been euthanized for various health reasons in Białowieża Primeval Forest ($n = 8$) and Borecka Forest ($n = 7$) (northeast Poland) during the years 2017–2019. Lethal control is an element of health monitoring conducted annually. Individuals of weak condition with general or specific signs of disease (e.g. necrotic inflammation of the prepuce) were selected. The formal aspects of conducted annual lethal control was described in Klich et al. (2020). The respiratory tracts were taken exclusively from animals infected by *Dictyocaulus* sp. The trachea, bronchi, and bronchioles were cut open and immerses into beakers filled with tap water, the sediment was investigated under a stereomicroscope and worms were recovered with the use of dissecting needles (Pyziel et al., 2017). Lungworms from each animal were stored separately, preserved in 70% ethanol and then transported to the Swedish University of Agricultural Sciences for morphological and molecular investigations.

2.2. Morphological study

Specimens were cleared in lactophenol and mounted in glycerin jelly on slides following Pyziel et al. (2017). Selected morphological features were measured: body length, buccal capsule (BC), buccal capsule wall (BCW), head, cephalic vesicle, esophagus of males and females; copulatory bursa, gubernaculum, spicules of male reproductive system; vestibules, anterior sphincter and infundibulum, posterior sphincter and infundibulum, mature and immature eggs in the uterus, tail and phasmids of the female reproductive system. Additionally, location of nerve ring and excretory pore from anterior extremity were evaluated, as well as location of vulva opening from tail-tip and body width at vulva opening in females. Photomicrographs of the features were taken using an Opta-Tech Lab40 light microscope (× 40 - × 1000 magnification) and the OptaView IS-PL Opta-Tech software package (Opta-Tech, Warsaw, Poland). Specimens intended for scanning electron microscopy (SEM) were dehydrated in increasing concentrations of ethanol and stored in acetone (Eisenback, 1985). The dehydrated specimens were then subjected to critical-point drying with liquid CO_2, their proximal endings were cut and mounted on a SEM mounting stub with double-coated tape, sputter-coated with gold, and examined with the use of a LEO 1430VP scanning electron microscope.

2.3. DNA extraction, amplification and sequencing

Depending on individual intensity of infection, 1–5 male worms from each examined European bison were used for molecular analyses. Genomic DNA was extracted individually from 48 adult male lungworms (preserved in 70% ethanol) using a Nucleospin tissue DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Partial regions of mitochondrial (*mt*) *cox*1, *NADH* dehydrogenase subunit 5 (*nad*5), and cytochrome B (*cyt*B) of the worms were amplified by PCR using the following sets of primers: LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) + HC02198 (5′-TAA ACT ATC GGG TGA CCA AAA AAT CA-3′) for *cox*1 (Folmer et al., 1994); OP424 (5′-ATG ACT AAT CGT ATT GGG GA-3′) + OP425 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) for *nad*5 (Höglund et al., 2006); cytB_F (5′-TGA AAA RGT TAA GAT RRT TGG GAC-3′) + cytB_R (5′-TTA GGA ATA GCA CGC AAA ATA GC-3′) for *cyt*B (present study). The primers were designed with the use of FastPCR software, version 5.4 (Primer Digital, Helsinki, Finland).

PCR was performed in a 2720 thermal cycler (Applied Biosystems, Foster City, California) in a volume of 50 µl. Each 50 µl PCR reaction contained 20 µl of Molecular Biology Reagent Water (Sigma-Aldrich, USA), 25 µl Quant-Bio's AccuStart™ II PCR ToughMix® (× 2 concentration) (Quantabio, Beverly, USA), 1 µl GelTrack Loading Dye (× 50 concentration) (Quantabio, Beverly, USA), 1 µl forward primer (20 mM), 1 µl reverse primer (20 mM), and 2 µl of template DNA. The conditions for PCR were as follows: 94 °C for 2 min to denature the DNA, with 35 cycles at 94 °C for 40 s (*cox*1 and *nad*5) or for 45 s (*cyt*B), 57 °C for 45 s (*cox*1 and *nad*5) or 56 °C for 60 s (*cyt*B), and 72 °C for 30 s (*cox*1, *nad*5) or for 45 s (*cyt*B); with a final extension of 5 min (*cox*1, *nad*5) or 10 min (*cyt*B) at 72 °C to ensure complete amplifications. The PCR product was purified with the use of the Nucleospin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany), eluted with 30 µl of Molecular Biology Reagent Water (Sigma-Aldrich, USA) and sequenced in both directions by Macrogen Europe (Amsterdam, the Netherlands) with the use of primers used for amplification (5 mM). The sequences were then assembled into contigs using CodonCode Aligner version 8.0 (CodonCode Corporation, Massachusetts, USA). The generated sequences were then compared with those from the same regions in *D. viviparus* of cattle, and those downloaded from GeneBank (see below). The accession numbers are listed in Table 1.

Table 1
List of taxa included in the molecular analysis using combined sequence data of mitochondrial *cox*1+ *cyt*B + *nad*5.

Species	Host	*cox*1	*cyt*B	*nad*5
		GenBank	GenBank	GenBank
Dictocaulus viviparus	*Bos taurus*	JX519460.1	JX519460.1	JX519460.1
Dictocaulus viviparus	*Bos taurus*	AP017683.1	AP017683.1	AP017683.1
Dictocaulus viviparus	*Bison bonasus*	KM359417.1	KM503300.1	KM359431.1
Dictocaulus viviparus	*Bison bonasus*	MN656991.1	MN503301.1	MT157226.1/ MN656992.1
Dictyocaulus eckerti	*Cervus elaphus*	NC_019809.1	NC_019809.1	NC_019809.1
Angiostrongylus cantonensis	*Rattus norvegicus*	AP017672.1	AP017672.1	AP017672.1

2.4. Phylogenetic analyses

Phylogenetic analysis of *Dictyocaulus* spp. taxa was based on aligned amino acid sequences of the combined partial *cox*1 (654 bp), *cyt*B (714 bp) and *nad*5 (351 bp) nucleotide sequences with the use of the GTR + G (*cox*1 and *cyt*B) and GTR + I (*nad*5) models of evolution (Table 1). The GTR model was chosen on the basis of JModelTest 2.1.10 (Guindon and Gascuel, 2003; Darriba et al., 2012) using AIC criterion. Phylogenetic tree was constructed using Bayesian inference (BI) analysis with MrBayes version 3.2. (Huelsenbeck and Ronquist, 2001).

2.5. Histopathological examination of the lungs

The lungs were initially checked for the lungworms directly during dissection conducted under field conditions. Fifteen lungs with parasitic infection were macroscopically examined, packed into plastic bags and transported immediately to the laboratory.

Samples of pulmonary tissue surrounding parasites were fixed in 10% buffered formalin and submitted for histopathological investigation as described in Pyziel et al. (2018a). The samples were dehydrated through graded series of ethanol and cleared in xylene, then embedded in paraffin wax, cut into sections (4 μm), stained with hematoxylin and eosin (HE), and processed for light microscope examination (Olympus BX43; Olympus, Japan).

2.6. Statistical analysis

The measurements for total body length, BCW length, BCW width, and spicule length of European bison-derived lungworms taken during this study were compared with those of *D. viviparus* of cattle presented in Divina et al. (2000) with regard to their mean, standard deviation and number of samples. The females and males were separated into groups and Welch *t*-test was used to compare those from the European bison with those from the cattle. It was assumed that the data from the cattle-derived lungworms obtained by Divina et al. (2000) met the assumptions for the *t*-test, as they had been analyzed using a general linear model. The assumptions regarding the data for *Dictyocaulus* lungworm isolated from European bison were also checked. Probability values less than 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Morphological study

Buccal capsule wall (BCW) of investigated European bison-derived specimens was oval, flattened dorsoventrally with an elongated oval oral opening (Fig. 1A). European bison-derived BCWs were thin (Fig. 1B). A single ring of four symmetrical submedian cephalic papillae and two lateral amphids were observed in the head region (Fig. 1C). Cervical papillae were absent, and a nerve ring was present (Fig. 1A and B). European bison-derived lungworms were longer than the cattle-derived *D. viviparus* (Table 2), with the difference being statistically significant for both sexes ($p < 0.001$ for males and $p = 0.029$ for females). In addition, the BCW was significantly longer in both the male and female European bison lungworm ($p < 0.001$) (Table 3); however, while the BCWs of males isolated from European bison were significantly narrower than those from cattle ($p < 0.001$), no such statistically significant difference was observed for the females ($p = 0.176$) (Table 3). The European bison and cattle-derived lungworms differed with regard to head width; specifically, the European bison lungworms presented higher minimum and maximum values (Table 3). The female reproductive system of the European bison lungworm was didelphic-amphidelphic (Fig. 2A). As the anterior/posterior sphincter and infundibulum were only slightly separated from each other (Fig. 2B), their measurements are given together (Table 4). The female body ended in a tail with two phasmids at its mid-length (Fig. 2C). The male body ended in a bell-shaped copulatory bursa (Fig. 3A and B). A pair of dark brown spicules and gubernaculum was observed in each male (Fig. 3C). A small transparent membrane around the tip of each spicule was clearly visible (Fig. 3C). The spicules of European bison-derived lungworms were significantly longer ($p < 0.001$) than those isolated from cattle (Table 5).

3.2. DNA sequences

Partial nucleotide sequences of *mt cox*1, *nad*5, and *cyt*B genes of European bison-derived lungworms were obtained from 15 animals in the Białowieża Primeval and Borecka Forests. Beside one nucleotide difference in a partial sequence of *mt nad*5, no diversity was found between the lungworm sequences obtained from these geographical regions: the remaining sequences of the *mt* genes were identical. Therefore, only single nucleotide sequences of the *cox*1 and *cyt*B genes of European bison worms was submitted from both locations; in contrast, three diverse sequences of *nad*5 were submitted to GenBank.

Nucleotide diversity was calculated from pairwise sequence alignments of partial *cox*1, *cyt*B and *nad*5 of *D. viviparus* from cattle (GenBank: JX519460.1; AP017683.1) and from European bison (GenBank: KM359417.1 + KM503300.1 + KM359431.1; MN656991.1 + MN503301.1 + MT157226.1; MN656991.1 + MN503301.1 + MN656992.1, respectively). These results varied from 1.7% for *nad*5 (6 nucleotides of difference), and 2.1% for *cyt*B (15 nucleotides of

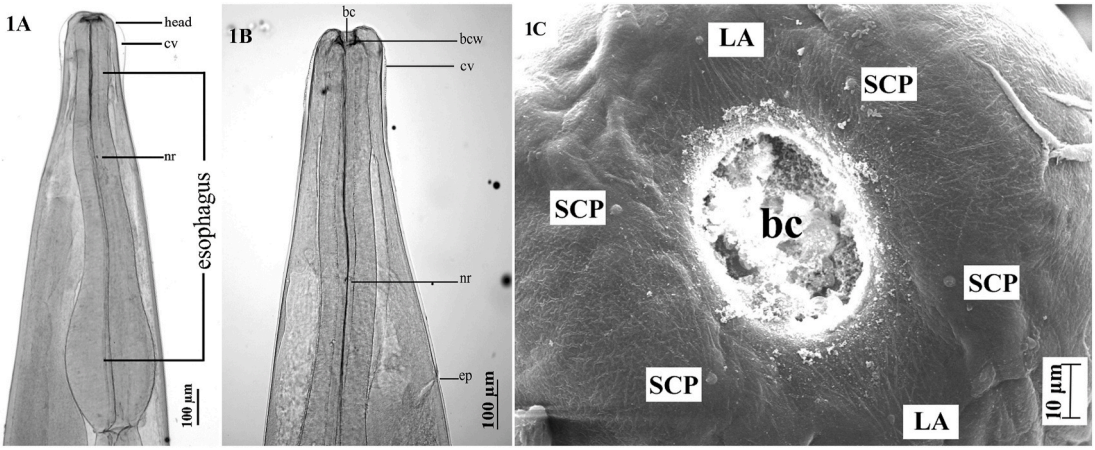

Fig. 1. *Dictyocaulus viviparus* of European bison, anterior end. **(A)** Male, anterior end in optical section, showing head, cephalic vesicle (cv), esophagus, nerve ring (nr), lateral view. **(B)** Female, anterior end in optical section, showing buccal capsule (bc), buccal capsule wall (bcw), cephalic vesicle (cv), nerve ring (nr), and excretory pore (ep), lateral view. **(C)** Cephalic region, scanning electron microscopy, showing two lateral amphids (LA) and four submedian papillae (SCP).

Table 2
The length characterization of morphological features of European bison-derived *Dictyocaulus viviparus* in comparison with cattle-derived lungworm data following Divina et al. (2000).

FEATURE		HOST					
		Cattle (*Bos taurus*)		European bison (*Bison bonasus*)		Cattle vs. European bison	
		male	famale	male	female	male	female
Body[a]	Range	14 - 55	14 - 76	20.1–69.5	16.8–83.7	$p < 0.001$	
	Sample size (*n*)	$n = 37$	$n = 89$	$n = 30$	$n = 35$		$p = 0.029$
	Mean ± standard deviation ($X \pm SD$)	38 ± 2	48 ± 1	47.5 ± 11.9	54.4 ± 16.6		
Cephalic vesicle	Range	–	–	144.9–378	106.7–283	–	
	Sample size (*n*)			$n = 28$	$n = 9$		–
	Mean ± standard deviation ($X \pm SD$)			242.9 ± 60.2	196.9 ± 67.3		
Buccal capsule	Range	–	–	34.1–62	37.8–66	–	
	Sample size (*n*)			$n = 11$	$n = 17$		–
	Mean ± standard deviation ($X \pm SD$)			50 ± 7.9	50.6 ± 7.4		
Buccal capsule wall	Range	9.9–20.9	6.8–30.9	37 - 53	48.3–84	$p < 0.001$	
	Sample size (*n*)	$n = 35$	$n = 77$	$n = 11$	$n = 20$		$p < 0.001$
	Mean ± standard deviation ($X \pm SD$)	16.2 ± 0.6	16.1 ± 0.4	44.7 ± 4.9	67.1 ± 10.2		
Esophagus	Range	808–992		931.4–1546	975.5–1562	–	
	Sample size (*n*)		896 - 1040	$n = 26$	$n = 25$		–
	Mean ± standard deviation ($X \pm SD$)			1281 ± 162.6	1308 ± 172.1		
Anterior to nerve ring	Range	308–398	235–379	359–592.1	224–612.1	–	
	Sample size (*n*)	–	–	$n = 24$	$n = 26$		–
	Mean ± standard deviation ($X \pm SD$)	–	–	479.4 ± 63.2	474.6 ± 84.3		
Anterior to excretory pore	Range	412–526	393–493	454 - 771	414 - 828		
	Sample size (*n*)	–	–	$n = 18$	$n = 19$	–	–
	Mean ± standard deviation ($X \pm SD$)	–	–	656.9 ± 86.2	597.2 ± 107.6		

[a] All dimensions are given in μm except body length, given in mm.

Table 3
The width characterization of morphological features of European bison-derived *Dictyocaulus viviparus* in comparison with cattle-derived lungworm data following Divina et al. (2000).

FEATURE[a]		HOST					
		Cattle (*Bos taurus*)		European bison (*Bison bonasus*)		Cattle vs. European bison	
		male	famale	male	female	male	female
Head	Range	74–91	86–104	97.9–166	93.6–191.4	–	–
	Sample size (*n*)			$n = 21$	$n = 23$		
	Mean ± standard deviation ($X \pm SD$)			143.9 ± 17.1	144.6 ± 26.1		
Cephalic vesicle	Range	–	–	10 - 42	8 - 31	–	–
	Sample size (*n*)			$n = 30$	$n = 9$		
	Mean ± standard deviation ($X \pm SD$)			23.9 ± 8.2	17.5 ± 8.3		
Buccal capsule	Range	–	–	28 - 39	22–48.3	–	–
	Sample size (*n*)			$n = 11$	$n = 16$		
	Mean ± standard deviation ($X \pm SD$)			35.3 ± 3	31.6 ± 7.2		
Buccal capsule wall	Range	3.9–15.8	2–16.6	4 - 8	4.8–14	$p < 0.001$	$p = 0.176$
	Sample size (*n*)	$n = 35$	$n = 77$	$n = 11$	$n = 24$		*Non significant*
	Mean ± standard deviation ($X \pm SD$)	8.1 ± 0.4	8.8 ± 0.2	5.8 ± 1.3	8.2 ± 2.1		
Esophagus max.	Range	–	–	132.4–365	136.7–287	–	–
	Sample size (*n*)			$n = 25$	$n = 24$		
	Mean ± standard deviation ($X \pm SD$)			247.4 ± 63.1	216 ± 41		

[a] All dimensions are given in μm.

difference) to 3.7% for the *cox*1 marker gene (24 nucleotides of difference) (Table 6). Thus, *cox*1 demonstrated the greatest sequence variation, whereas *cyt*B and *nad*5 were more conserved. At the same time, *nad*5 was the most diverse gene within European bison-derived worms (0.6%), with up to two nucleotides of difference (Table 6). Additionally, in the analysis of *mt* sequence variation between *D. viviparus* (GenBank: JX519460.1; AP017683) and *D.* sp. *cf. eckerti* (NC_019809.1) the highest level of diversity (18.2%–18.5%) was found for the *nad*5 region, with the *cox*1 region being more conserved (11.3%–11.6%) (Table 6).

3.3. Phylogenetic reconstruction

Bayesian analysis (BI) of *mt* sequence data (*cox*1 + *nad*5 + *cyt*B) with *Angiostrongylus cantonensis* (GenBank: AP017672.1) as an outgroup revealed two strongly supported clades (Fig. 4). One cluster of *Dictyocaulus* lungworms comprised two subclades: one including taxa of European bison from Białowieża Primeval Forest (GenBank: KM359417.1 + KM359431.1 + MN503300.1) with their sister taxa from Borecka Forest - Borki (GenBank: MN656991.1 + MN656992.1/ MT157226.1 + MN503301.1). Another clade included *D. viviparus* of cattle (GenBank: JX519460.1; AP017683.1). A third clade included *D.* sp. *cf.eckerti* of red deer (GenBank: NC_019809.1).

3.4. Macroscopic examination of the lungs

In all cases the lungs were pale pink, airy, pasty and soft. In two cases the lungs were also focally congested. Lungworms were found in bronchi and bronchioles of each examined lung, mostly in caudal and cranial lung lobes. They were covered with mucus. The range of infection was 12–160 adult lungworms per host.

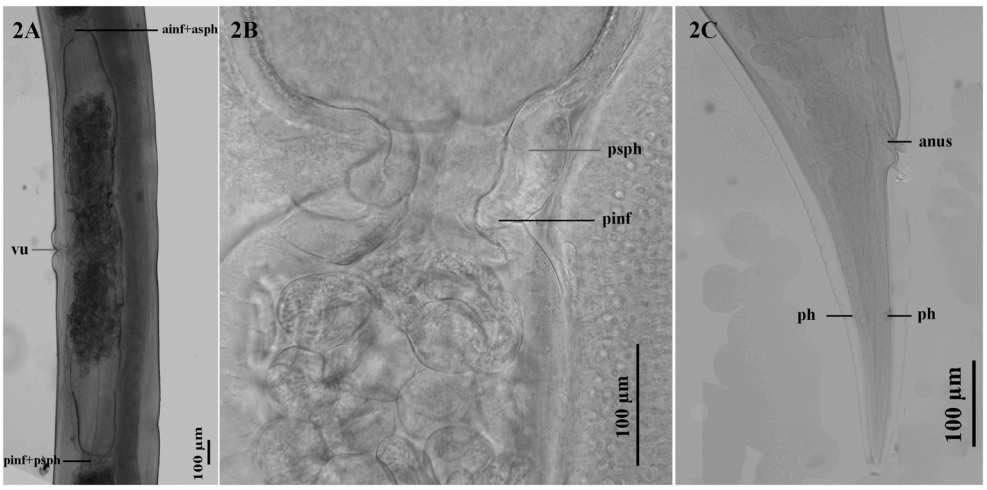

Fig. 2. *Dictyocaulus viviparus* of European bison, female genital system, light microscopy. **(A)** Ovejectors in left lateral view, showing relationships for the vulva (vu), vestibules, and combined anterior infundibulum, and sphincter (ainf + asph), and posterior infundibulum and sphincter (pinf + psph). **(B)** Region of posterior infundibulum (pinf) and posterior sphincter (psph), left lateral view. **(C)** female tail, right lateral view, showing anus and phasmids (ph).

Table 4
Dimensional characterization of morphological features of female reproductive system of *Dictyocaulus viviparus* derived from European bison (own data) in comparison with cattle-derived lungworm data following Divina et al. (2000).

FEATURE		HOST	
		Cattle	European bison
Posterior to vulva opening[a]	Range	13–29.5	14.7–41
	Sample size (*n*)	–	*n* = 21
	Mean ± standard deviation (*X* ± *SD*)	–	27.3 ± 8.2
Body width at vulva opening	Range	322–550	456.9–839.6
	Sample size (*n*)	–	*n* = 26
	Mean ± standard deviation (*X* ± *SD*)	–	634.8 ± 93.3
Length of vestibules	Range	1255–1996	1165.1–2397
	Sample size (*n*)	–	*n* = 15
	Mean ± standard deviation (*X* ± *SD*)	–	1928.4 ± 366.4
Length of anterior sphincter and infundibulum	Range	75–114	106 - 175
	Sample size (*n*)	–	*n* = 26
	Mean ± standard deviation (*X* ± *SD*)	–	141.2 ± 22.4
Length of posterior sphincter and infundibulum	Range	84–113	55 - 171
	Sample size (*n*)	–	*n* = 21
	Mean ± standard deviation (*X* ± *SD*)	–	116.5 ± 30.8
Immature eggs in uterus	Range	46–60 × 74 - 91	67 - 98,6 × 38.9–60
	Sample size (*n*)	–	*n* = 22
	Mean ± standard deviation (*X* ± *SD*)	–	86.8 ± 7.8 × 52.1 ± 5.4
Mature embryonated eggs	Range	43–55 × 79 - 86	72.2–99 × 37–48.4
	Sample size (*n*)	–	*n* = 9
	Mean ± standard deviation (*X* ± *SD*)	–	84.9 ± 8 × 44.8 ± 3.8
Posterior to anus (length of tail)	Range	261–483	323.2–688
	Sample size (*n*)	–	*n* = 31
	Mean ± standard deviation (*X* ± *SD*)	–	484.9 ± 65.8
Posterior to phasmids	Range	–	178 - 286
	Sample size (*n*)		*n* = 29
	Mean ± standard deviation (*X* ± *SD*)		234.7 ± 12.7

[a] All dimensions are given in µm except posterior to vulva opening, given in mm.

3.5. Histopathology of the lung tissue

The histopathological sections of European bison lung tissue revealed various lung pathologies associated with *Dictyocaulus* infection, including numerous cross-sections of mature adult or young adult worms in the lumen of the bronchi and bronchioles (Fig. 5A). Moreover, sections of nematode larvae were detected multifocally in pulmonary alveoli (Fig. 5B). Alveolar walls were infiltrated by lymphocytes, plasma cells, macrophages and eosinophils, whereas the bronchi, bronchioles and blood vessels were surrounded by mononuclear cells (Fig. 5A, C). Histopathological examination revealed lesions consisted with interstitial pneumonia with hyperplasia of the lymphoid follicle (Fig. 5D). At the same time hyperplasia of smooth muscle and bronchial epithelial cells was observed, accompanied by the thickening of alveolar septa and the blood vessel walls (Fig. 5C). Other pulmonary lesions included emphysema and congestion.

4. Conclusion

Investigated European bison were infected exclusively with morphologically-different genotype of *D. viviparus* than cattle. No mixed infections of bison and cattle-derived lungworms was noted in investigated European bison. We propose, therefore, that the lungworm of European bison should be classified as *D. viviparus* subsp. *bisontis*.

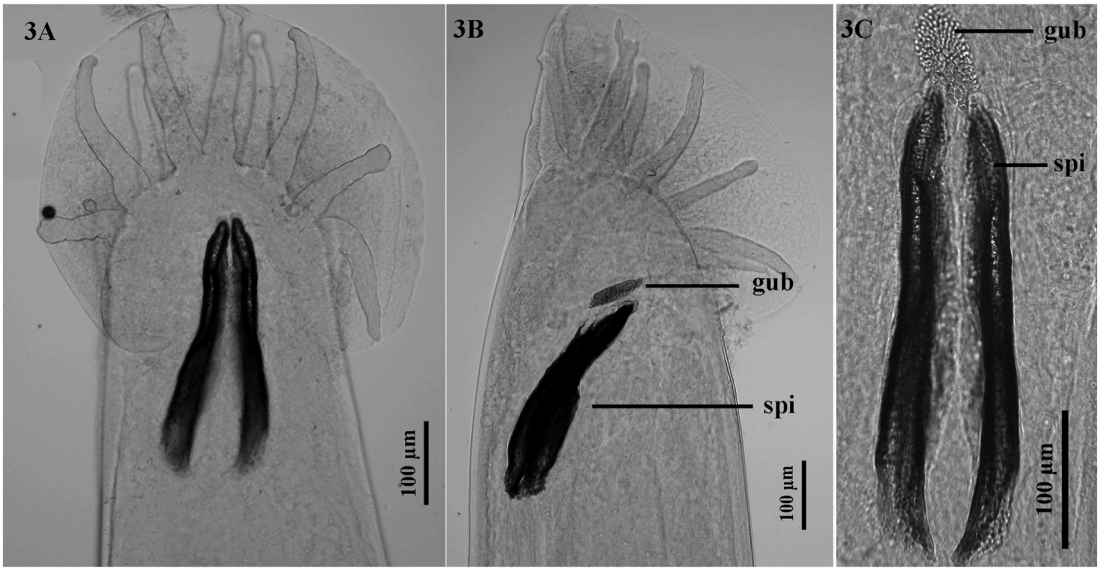

Fig. 3. *Dictyocaulus viviparus* of European bison, male genital system, light microscopy. **(A)** Bursa, dorsal view. **(B)** Bursa, left lateral view, showing gubernaculum (gub), and left spicula (spi). **(C)** Spiculae, dorsal view.

Table 5
Dimensional characterization of morphological features of male reproductive system of *Dictyocaulus viviparus* derived from European bison in comparison with cattle-derived lungworm data following Divina et al. (2000).

FEATURE[a]		HOST		
		Cattle	European bison	Cattle vs. European bison
Length of copulatory bursa	Range	–	205.7–415	–
	Sample size (*n*)		25	
	Mean ± standard deviation ($X \pm SD$)		310.5 ± 59.1	
Width of gubernaculum	Range	–	26.7–44	–
	Sample size (*n*)		19	
	Mean ± standard deviation ($X \pm SD$)		33.4 ± 4.6	
Length of spicules	Range	100.1–286.9	271.5–385	$p < 0.001$
	Sample size (*n*)	64	57	
	Mean ± standard deviation ($X \pm SD$)	209.7 ± 15.4	310.8 ± 18.8	

[a] All dimensions are given in µm.

4.1. Taxonomic summary

The specific epithet: D. viviparus subsp. *bisontis*.

Type and only known host: European bison, *Bison bonasus* (Linnaeus, 1758) (Artiodactyla: Bovidae)

Site of infection: Trachea, bronchi, bronchioli

Type locality: Białowieża Primeval Forest, podlaskie voivodship, Poland (52°29′N - 52°57′N, 23°31′ E – 24°21′E), Borecka Forest, warmińsko-mazurskie voivodship, Poland (54°08′01, 9850″N, 22°05′39, 1214″E)

Specimens deposited: Holotype (male) and allotype (female): MNHW 1353 (Museum of Natural History, Wroclaw University, Poland)

Sequences deposited: GenBank: KC771250.1, KF007338.1 – KF007341.1, KM359411.1 - KM359435.1, KT581635.1 - KT581637.1, MT157226.1, MN503300.1, MN503301.1, MN656991.1, MN656992.1.

Etymology: The specific epithet is derived from host genus name.

Table 6
Pairwise comparison of sequence alignment (1719 bp) consisted of *cox*1 (654 bp), *cyt*B (714 bp) and *nad*5 (351 bp) *mt* DNA nucleotide sequence and inferred amino acid sequence (alignment: 573; *cox*1: 218; *cyt*B: 238; *nad*5: 117) variability among *Dictyocaulus eckerti* and isolates of *D. viviparus* of cattle and European bison. Above diagonal = Number of variable sites in the 1719 base pair (bp). Below diagonal = Number of variable sites in the 573 amino acid. Total number of variable sites between 2 species/isolates is given prior to parenthesis, whereas number of variable sites regarding particular marker genes (*cox*1, *cyt*B and *nad*5, respectively) are given in parenthesis.

Species and host[a]	1	2	3	4	5	6
1. *D. eckerti*, red deer		258 (76; 115; 68)	259 (76; 115; 67)	258 (76; 115; 67)	256 (74; 117; 65)	258 (76; 118; 64)
2. *D. viviparus*, European bison	51 (5; 29; 17)		1 (0; 0; 1)	0	45 (22; 15; 8)	46 (24; 15; 7)
3. *D. viviparus*, European bison	51 (5; 29; 17)	0		1 (0; 0; 1)	44 (22; 15; 7)	45 (24; 15; 6)
4. *D. viviparus*, European bison	51 (5; 29; 17)	0	0		45 (22; 15; 8)	46 (24; 15; 7)
5. *D. viviparus*, cattle	52 (6; 28; 18)	6 (1; 4; 1)	6 (1; 4; 1)	6 (1; 4; 1)		5 (2; 2; 1)
6. *D. viviparus*, cattle	52 (6; 28; 18)	6 (1; 4; 1)	6 (1; 4; 1)	6 (1; 4; 1)	0	

[a] GenBank: 1 = NC_019809.1; 2 = KM359417.1 + KM503300.1 + KM359431.1; 3 = MN656991.1 + MN503301.1 + MT157226.1; 4 = MN656991.1 + MN503301.1 + MN656992.1; 5 = JX519460.1; 6 = AP017683.1.

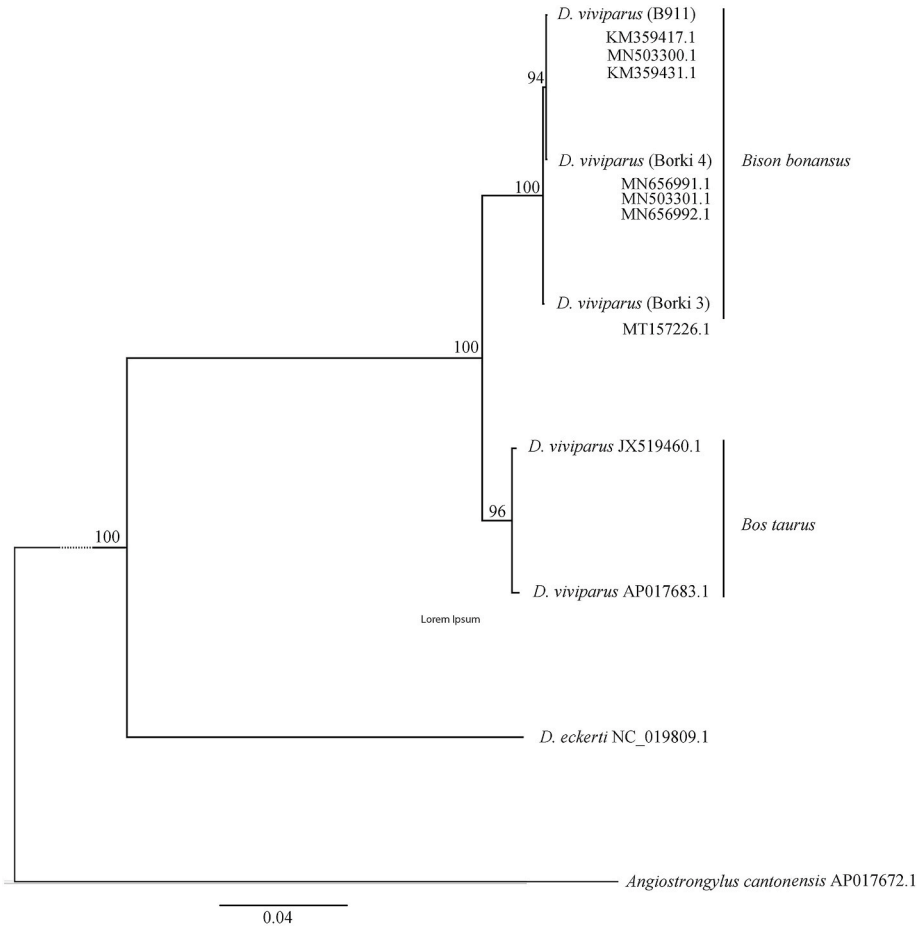

Fig. 4. Phylogenetic tree of *Dictyocaulus* spp. based on *cox*1, *cyt*B and *nad*5 partial sequences, constructed with the use of Bayesian inference (BI) analysis using MrBayes version 3.2. The GTR + G (*cox*1, *cyt*B) and GTR + I (*nad*5) model was chosen based on jModelTest version 2.1.4 using Akaike Information Criterion. The analysis was run for 2,000,000 generations, with 500,000 generations discarded as 'burn-in'. Hosts (for *Dictyocaulus viviparus*) and GenBank accession numbers of origin are shown. Nodal support is indicated as Bayesian posterior probabilities. Sequence from *Angiostrongylus cantoniensis* (AP017672.1) was used as an outgroup.

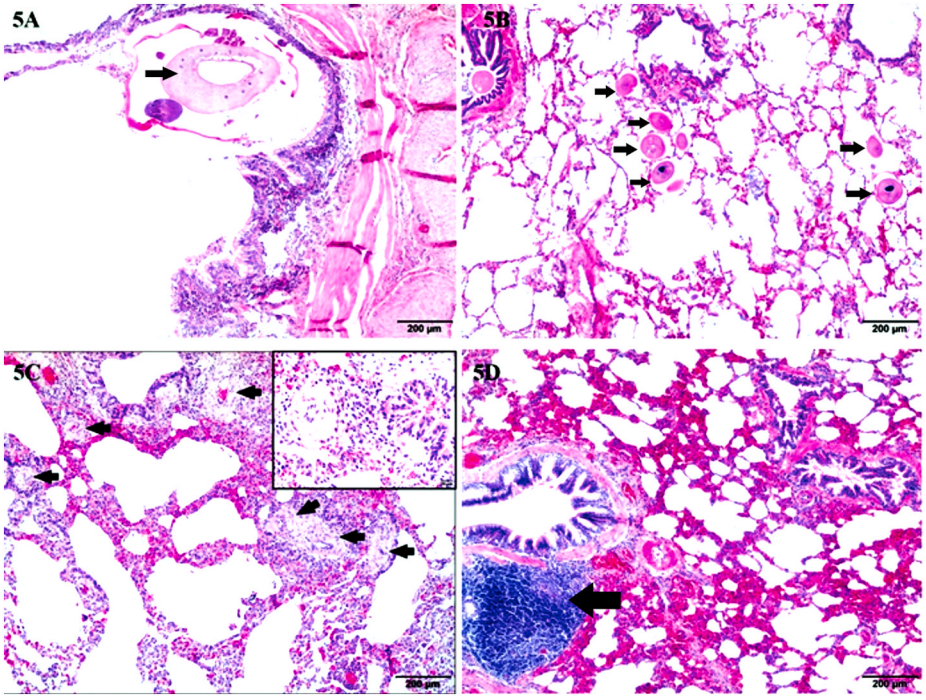

Fig. 5. Histopathological lesions in the lungs of European bison infected with *Dictyocaulus viviparus*. **(A)** The cross-section of a nematode in the lumen of bronchiole (arrow); mononuclear cells infiltration in the mucous membrane of the bronchiole. H-E stain (× 40 magnification). **(B)** Multiple cross-sections of nematodes scattered within alveoli and bronchiole (arrows). H-E stain (× 40 magnification). **(C)** Interstitial pneumonia, diffuse, lymphoplasmacytic, eosinophilic with the hyperplasia of the vascular wall (arrows). H-E stain (× 40 magnification). Inset: Perivascular, peribronchiolar lymphocytic and eosinophilic infiltration; on the left side: the tunica media of vessels is thickened (hyperplastic). H-E stain (× 200 magnification). **(D)** Prominent peribronchiolar hyperplasia of the lymphoid follicle (arrow); pulmonary tissue showed interstitial pneumonia pattern. H-E stain (× 200 magnification).

5. Discussion

This study provides a detailed morphological description of European bison-derived *Dictyocaulus* lungworms, and assess the phylogenetic position of lungworms among closely-related hosts: European bison and domestic cattle. According to Divina et al. (2000), the most important morphological diagnostic characteristics for *Dictyocaulus* spp. are BCW thickness and length, while spicular length does not differ between cattle, roe deer and moose-derived lungworms. In the present study, the specimens from the European bison were found to have longer BCW than those isolated from cattle: this different was almost three-fold in males and more than four-fold in females; in addition, the BCW was narrower in the males of European bison than in those diagnosed in cattle. In addition, like the majority of cattle-derived lungworms, the BCW of European bison lungworm was classified as thin (Divina et al., 2000). Furthermore, the spicules of *Dictyocaulus* were significantly longer in European bison than those of cattle (Divina et al., 2000). Divina et al. (2000) also report that the thickness and length of BCW were positively correlated with the total body length of the lungworm. Our morphological findings suggest that European bison host a distinct *Dictyocaulus* species; however, it is well known that morphological differences (morphotypes) can be induced by the host species (Nadler, 2002). Nevertheless, although our present results suggest that *Dictyocaulus* lungworms of European bison and cattle can be morphologically distinguished on the basis of the BCW length, the total body length, and spicule lengths in males, their phylogenetic position suggests that cattle lungworm can infect both hosts. This suggestion is supported by previous findings involving rDNA datasets, showing that the SSU sequence of European bison lungworms (GenBank: KC771250) was homologous with those from cattle (GenBank: AY168856) (Pyziel, 2014); additionally, both isolates diverged only slightly in the ITS2 region (GenBank: KM359413; KM359411; U37718; AF105257) (Pyziel et al., 2017). Previous studies have found *mt* DNA sequences to be more variable within a nematode species than rDNA (Gasser, 2006; Gasser et al., 2012), and hence *mt* marker genes are more suitable for studying population genetics and systematics in this regard (Blouin, 2002; Gasser et al., 2012). Current and previous comparisons of the *mt* DNA from lungworms from cattle and red deer (Gasser et al., 2012; Pyziel et al., 2017, 2018b) found *cox*1 to capture the least within-genus sequence variation and *nad*5 the greatest. Similarly, *cox*1 was found to demonstrate the least within-species variation of mtDNA genes among populations of *D. viviparus* isolated from cattle (Höglund et al., 2006; Gasser et al., 2012). These findings contrast with those obtained from cattle and European bison-derived lungworms in the present study, which found *nad*5 and *cyt*B to be more conserved than *cox*1. In addition, the worms from both hosts demonstrated comparable levels of sequence diversity for the same gene regions to those noted previously between various *D. viviparus* isolated from cattle; this diversity did not reach 4.0% (Hu et al., 2002; Höglund et al., 2006; Gasser et al., 2012). In contrast, *mt* sequence variation has been found to range from 10 to 20% between closely-related species of nematodes and from 1 to 3% between individuals of inbreeding population (Blouin, 1998).

Our findings also indicate that lungworm infection can cause interstitial pneumonia, bronchitis and bronchiolitis. Similar histopathological lesions have been observed in the lung tissue of cattle and buffaloes (Schnider et al., 1991; Mahmood et al., 2014) exhibiting parasitic pneumonia with extensive mononuclear infiltration in the lung parenchyma, multifocal lymphocytic and eosinophilic bronchiolitis. Additionally, the histopathological picture of European bison dictyocaulosis revealed the presence of similar lesions to those noted previously in red deer dictyocaulosis (Pyziel et al., 2018a).

To summarise, it appears that *D. viviparus* infecting cattle and European bison are characterized by both morphological differences and slightly diverging genotypes. We therefore propose that the lungworm of European bison should be classified as *D. viviparus* subsp. *bisontis*. An additional recommendation is that *mt cyt*B marker gene should be used to distinguish between the genotypes of the lungworms infecting cattle and bison.

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References

Blouin, M.S., 1998. Mitochondrial DNA diversity in nematodes. J. Helminthol. 72, 285–289.
Blouin, M.S., 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Int. J. Parasitol. 32, 527–531.
Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. JModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772.
Demiaszkiewicz, A.W., Pyziel, A.M., Lachowicz, J., Kuligowska, I., 2009. Dictyocaulosis of European bison in Białowieża forest. European Bison Conserv. News. 2, 112–118.
Divina, B.P., Wilhelmsson, E., Mattsson, J.G., Waller, P., Höglund, J., 2000. Identification of *Dictyocaulus* spp. in ruminants by morphological and molecular analyses. Parasitology 121, 193–201.
Eisenback, J.D., 1985. Techniques for preparing nematodes for scanning electron microscopy. In: Barker, K.R., Carter, C.C., Sasser, J.N. (Eds.), An Advanced Treatise on *Meloidogyne*. North Carolina State University, Raleigh, North Carolina, pp. 79–105.
Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.
Gasser, R.B., 2006. Molecular tools – advances, opportunities and prospects. Vet. Parasitol. 136, 69–89.
Gasser, R.B., Jabbar, A., Mohandas, N., Höglund, J., Hall, R.S., Littlewood, D.T.J., Jex, A. R., 2012. Assessment of the genetic relationship between *Dictyocaulus* species from *Bos taurus* and *Cervus elaphus* using complete mitochondrial genetic datasets. Parasites & Vectors Parasite Vector 5, 241.
Guindon, S., Gascuel, O.A., 2003. Simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.
Hu, M., Höglund, J., Chilton, N.B., Zhu, X., Gasser, R.B., 2002. Mutation scanning analysis of mitochondrial cytochrome c oxidase subunit 1 reveals limited gene flow among bovine lungworm subpopulations in Sweden. Electrophoresis 23, 3357–3363.
Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Höglund, J., Morrison, D.A., Mattsson, J.G., Engström, A., 2006. Population genetics of the bovine/cattle lungworm (*Dictyocaulus viviparus*) based on mtDNA and AFLP marker techniques. Parasitology 133, 89–99.
IUCN, 2020. Red list of threatened species. https://www.iucnredlist.org/. (Accessed 21 August 2020).
Keller, L.F., Waller, D.M., 2002. Inbreeding effects in wild populations. Trends Ecol. Evol. 17, 230–241.
Klich, D., Łopucki, R., Stachniuk, A., Sporek, M., Fornal, E., Wojciechowski, M., Olech, W., 2020. Pesticides and conservation of large ungulates and conservation of large ungulates: health risk to European bison from plant protection products as a result of crop depredation. Polos One 15, e0228243.
Mahmood, F., Khan, A., Hussain, R., Anjum, M.S., 2014. Prevalence and pathology of *Dictyocaulus viviparus* infection in cattle and buffaloes. J. Anim. Plant Sci. 24, 743–748.
Nadler, S., 2002. Species delimitation and nematode biodiversity: phylogenies rule. Nematology 4, 615–625.
Pyziel, A.M., 2014. Molecular analysis of lungworms from European bison (*Bison bonasus*) on the basis of small subunit ribosomal RNA gene (SSU). Acta Parasitol. 59, 122–125.
Pyziel, A.M., Laskowski, Z., Demiaszkiewicz, A.W., Höglund, J., 2017. Interrelationships of *Dictyocaulus* spp. in wild ruminants with morphological description of *Dictyocaulus cervi* sp. (Nematoda: trichostrongyloidea) from red deer, *Cervus elaphus*. J. Parasitol. 103, 506–518.
Pyziel, A.M., 2018. Applicability of selected ribosomal and mitochondrial genetic markers in identification of European bison lungworm: a state of the art review. European Bison Conserv. News. 11, 31–38.
Pyziel, A.M., Dolka, I., Werszko, J., Laskowski, Z., Steiner-Bogdaszewska, Ż., Wiśniewski, J., Demiaszkiewicz, A.W., 2018a. Pathological lesions in the lungs of red deer *Cervus elaphus* (L.) induced by a newly-described *Dictyocaulus cervi* (Nematoda: trichostrongyloidea). Vet. Parasitol. 261, 22–26.
Pyziel, A.M., Laskowski, Z., Höglund, J., 2018b. An assessment of the use of cox1 and cox3 mitochondrial genetic markers for the identification of *Dictyocaulus* spp. (Nematoda: trichostrongyloidea) in wild ruminants. Parasitol. Res. 117, 2341–2345.
Radwan, J., Biedrzycka, A., Babik, W., 2010. Does reduced MHC diversity decrease viability of vertebrate populations? Biol. Conserv. 143, 537–544.
Schnider, T., Kauo, F.J., Drommer, W., 1991. Morphological investigations on the pathology of *Dictyocaulus viviparus* infections in cattle. Parasitol. Res. 77, 260–265.