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Contributions to Knowledge of the Dictyocaulosis of the Red Deer

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Simple Summary: The genus *Dictyocaulus* consists of eighteen species of worms, but only four of these infect red deer. Infection causes damage to the respiratory tract, ranging from emphysema or edema to microscopic inflammatory and hemorrhagic lesions. Larval eggs are expelled to the outside by coughing and release of L1 into the environment. Worms from positive lungs collected in Extremadura (Spain) were assessed by morphological identification, but anatomopathological lesions and molecular barcode identification were also performed. The presence of three genetic groups was supported by significant subdivision using the ϕ_{ST} measure but *D. cervi* and *D. viviparus* showed its respective matrilineal ancestry while *D. eckerti* and *D. cervi* showed matrilineal sharing. Consequently, the need to evaluate introgression between these two species was highlighted. *D. viviparus* was discarded despite being reported in the same Spanish location by morphological methods and *D. cervi* and *D. eckerti* were found for the first time in the geographical area explored.

Abstract: Dictyocaulosis is a parasitic disease affecting ungulate species, including red deer (*Cervus elaphus*). *Dictyocaulus* genus consist of nine species but only four are reported to infect red deer. The disease is characterized by respiratory tract infection, particularly in lungs, bronchi and bronchioles, causing inflammatory and hemorrhagic microscopic lesions but also emphysema and edema. Biological cycle consists of a female that oviposits larvae eggs in the bronchi and trachea to be expelled to the exterior by coughing and releasing the L1 in the environment. In this study 106 adult red deer were collected from seven locations in Extremadura (Spain). Eight positive lungs were primarily assessed by morphological identification, leading to a mean intensity of 13.25 adult worms/infected lung, but globally descending to an average of 1.78 adults/sampled lung. The presence of adult worms in the upper and middle respiratory tract was confirmed by anatomopathological analysis. Molecular identification was made by sequencing the COI gene. As a result, we have detected the presence of three genetic groups supported by significant subdivision using ϕ_{ST} measure but *D. cervi* and *D. viviparus* showing its respective matrilineal ancestry while *D. eckerti* and *D. cervi* showing matrilineal sharing. Consequently, introgression between these two species was suggested. *D. viviparus* has been identified in the same Spanish region on the basis of morphological characters, but *D. cervi* and *D. eckerti* were reported for the first time in the explored geographic area.

Keywords: COI-barcoding; Dictyocalulus; Morfolofical and Molecular identification; Red Deer

1. Introduction

Throughout the history of mankind, attention has been paid to the study of zoonoses and how wildlife has been involved in health alerts, which includes the transmissible diseases to livestock, as is the case of tuberculosis, protozoan or helminths as dictyocaulus [1,2]. Helminths parasitic diseases are among those of high importance for wild ruminants' dynamics but four genera, *Ascaris*, *Dictyocaulus*, *Strongyloides*, and *Trichuris* are of special attention for health and husbandry in ruminant

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[3]. Among them Dictyocaulus species (Nematoda: Trichostrongyloidea) are distributed worldwide and affect both even-toed ungulates – *D. viviparus*, *D. filaria*, *D. eckerti*, *D. murmanensis*, *D. africanus*, *D. capreolus*, *D. cervi*, *D. cameli* - and odd-toed ungulates – *D. arnfieldi*, *D. pandionis* within Artiodactyla mammals.

The genus *Dictyocaulus* (Nematoda: Dictyocaulidae) was described by Karl Moritz (1851) for the first time based on morphological traits. Advances in optical microscopy and scanning electron microscopy from the end of the 19th to the 20th century, further the development of histological techniques, allowed more accurate morphological identification of these nematodes [4] improving species taxonomic classification of distinct species, as in the case of *Dictyocaulus capreolus* [5]. Despite this, most species of the genus Dictyocaulus are still identified by the parasitized host, even in the 21st century [3]. In Spain, *Dictyocaulus viviparus* and *Dictyocaulus filaria* have been reported in domestic ruminants [6,7]. However, some reports acknowledged major difficulties for species identification occurring in wild ruminants, due to the high level of morphological similarity exhibited among several species [8]. To this respect, prior to the advent of molecular identification, all infections of lung worms from red deer were identified as *D. viviparus* which parasitized to cattle. However, molecular characterization of *Dictyocaulus* spp. has revealed the existence of several distinct clades or species [2,3,7,9–11] and have also been of value in estimating phylogenetic relationships among trichostrongyloid and metastrongyloid nematodes [6,12]. Although the genus Dictyocaulus belongs in the monotypic family Dictyocaulidae with 18 nominal species, only five species have been confirmed valid based on molecular genetic data: *D. viviparus*, *D. filaria*, *D. eckerti*, *D. capreolus,* and *D. cervi* [3 and references therein].

This parasite is found in the small and large airways of the host, potentially causing parasitic bronchitis (dictyocaulosis), sometimes a fatal disease, especially in cattle, sheep and farmed red deer [13]. Under this scenario, a major breakthrough for the development of control strategies to prevent dictyocaulosis was a topic of concern because different species of cervids carrying these parasites behaves as true vectors to livestock [1,14,15]. In this regard, relevant researchs have provided control strategies based on vaccine preparations with effective immunological response against this parasite [16,17]. However, it was noticing as the nematodes became more resistant to the former vaccines reason why repeated improvement has been performed, until the “Bovilis Huskvac” vaccine was available offering a 95-98% level of protection [18]. Unfortunately, this vaccine against pulmonary verme is not of wide use despite its usefulness. Practical concerns, such as a short shelf life and the availability of anthelmintics with persistent efficacy against dictyocaulus, have apparently made vaccination a less attractive control option [18]. Currently, it has been described that resistance to antiparasitic drugs, such as fenbendazole and albendazole [19] and macrocyclic Lactones [20] in cattle reactivated the interest in these parasites. All lungworms were reported as *Dictyocaulus viviparus* in feral deer species [21,22] and a few studies on native Iberian red deer in Extremadura (Spain) suggested a low level of infection [23]. However, recent research advocates for investigating the full host range, epidemiology, the potential impacts and cross-transmission events with livestock of *Dictyocaulus* spp. with special attention on cervids world-wide [13,24] because levels of infection with lungworms in free-ranging deer remains largely unknown [7,13,24,25]. In addition, a global opinion suggests that objective methods of species-specific identification should be recommended [3], as cross-transmission has not yet been described [26]. For all these reasons, this report was aimed to contributed to knowledge of *Dictyocaulus* species by aspect such as the morphology, prevalence and associated lesions, as well as to carry out a genetic analysis using DNA molecular techniques in order to accurate specie-specific identification of parasite involved in dictyocaulosis in Extremadura at southwestern of Spain in free ranging red deer.

2. Materials and Methods

A total of 36 deer lungs from 7 fences in the province of Cáceres (Figure 1). Table 1 showed the fences/estates where sampling was done after carried out hunting activities. Carcass inspection was performed by dedicated official veterinarian (Junta de Extremadura, Spain) following the regulations

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(EC) No. 853/2004 of the European Parliament and the execution reglament (EU) 2015/1375. Accordingly, 5-6 lungs with their respective tracheas were collected in each site. Each lung was stored individually in an airtight bag and transported at 4ºC for preservation.

Table 1. Location and date of material collection.

Name	Location	Date
Cuadrillas Bajas	Cedillo	21/09/23
San Fermín	Torrejón el Rubio	03/12/23
Sierra Palomares	Alía	27/01/24
Jabalina	Salorino	02/02/24
Valdelayegua	Aliseda	09/02/24
Cerro Verde	Carbajo	11/02/24
El Águila	Serradilla	17/02/24

Figure 1. Maps of collected áreas in north Extremadura.

2.1. Necropsies and Microscopic Examination

The lungs were opened in a parasitology dedicated laboratory in the Clinical Veterinary Hospital (University of Extremadura, Spain) using appropriated preventive measures. After, careful examinations by compression were performed to check the presence of any vermecia. Macroscopic vermeus were introduced in a 150 mL propylene bottle containing 96% Ethanol and stored at 4ºC until processing. Finally, microscopic assessment confirmed *Dictyocaulus* spp. under a NIKON H550S (Japan) light microscope. Morphological traits were assayed following keys [4,27], focusing on the shape of the anterior end (mouth and esophagus) and on the spicules of males. In any case, the lungs were additionally subjected to complementary exams by scraping the trachea and bronchi to find eggs and/or larval stages. All data was recorded in an Excel sheet. Statistical agreement between macroscopic and microscopic was performed using Cohen's Kappa statistic in SPSS 15.0. (under UNEX License).

2.2. Anatomopathologival Study

The macroscopic and microscopic lesions caused by the worms were studied. Specially, affected areas of positive lungs were embedded in formalin. Subsequently, slices were processed by conventional hematoxylin eosin histopathological methods.

 doi:10.20944/preprints202504.1246.v1

2.3. Molecular Procedures

2.3.1. DNA Extraction

A total of 15 worms were processed, 5 for each fence from the positive lung. Samples from Sierra Palomares (SP), Jabalina (JB) and Cerro Verde (CV) fences were processed for molecular analysis. Cuadrillas Bajas fence was rejected due to sampling conservation error. Excluding genitalia, half cm from the medial portion of adult or larvae parasites were stuffed for generic material (DNA) extraction under sterility condition in dedicated room, avoiding cross-contamination. DNA template was purified by a salting-out protocol [28] modified using Zymo-Spin II C columns and eluted in 400 µL molecular biology grade water.

2.3.2. End-Point PCR and Primers

End-point PCR was made with primers described [29] which targeting a portion of the COI mitochondrial gene as follows: COX I_F (5'-TTTTTTTTGGGCATCCTGAGGTTTAT-3') and COX I_R (5'-TAAAGAAAGAAAGAACATAATGAAAAAATG-3'). The PCR master-mix followed published protocols [30]. Briefly, 10 µL of each 10x NH4 buffer, 2mM dNTPs, 10 µM COX I_R and F primers and 3 µL MgCl2 (50mM) and completing up to 90 µL with molecular biology grade water. Individual assays were performed with 18 µL of 2 µL of template DNA. Thermocycler conditions were as follows: Pre-Denaturation at 95ºC for 5 minutes; 35 cycles at 94ºC 60 seconds, 50ºC 60 seconds, 72ºC 60 seconds and final extension of 72ºC 7 minutes. PCR products were visualized by 1.6% (w/v) agarose electrophoresis SYBR safe stains. Molecular weight 100 bp ladder profile 100 bp ladder was used to monitor amplicon size under 312 nm ultraviolet light transilluminator.

2.3.3. Sequencing, Alignment and Comparison Through Phylogenetic Networks

5 µL PCR products were intimately purified with Ex-Spure (NimaGen, The Netherlands) following manufacturer's recommendations and later diluted to 10 µL with ultrapure water. PCR product templates (10 µL) were sequenced using the Big Dye® ver 3.1 cycle sequencing Kit (Thermo Fisher Scientific) and residual eliminated with Performa®DTR cartridges (Edge Bio). Profile of sequencing products obtained by capillary electrophoresis (Applied Biosystems™ 3130 DNA Analyzer; USA) and analyzed with "ABI Sequencing Analysis" ver. 5.2 software (Applied Biosystems Company, USA).

Genbank database NCBI (USA) was filtered to find species belonging to the genus *Dictyocaulus*. The search was limited to 250 sequences but having the same or higher coverage with respect to the COI segment sequenced in this study. These sequences were selected based on a similarity cutoff level of ≥ 90% aimed to gather all sequences of the genus *Dictyocaulus* [31]. The last species downloaded was *Arthrosoma* spp. after filtering under these criteria. Thirty sequences among those downloaded belonged to the genus *Dictyocaulus* spp. with 100% coverage (320 bp) with respect to the 13 sequences from this study. As a result, a total of 115 sequences were selected but 102 from GenBank (NCBI, USA). These sequences were collapsed into haplotypes using DnaSP ver 6.1 [32]. These haplotypes were further used to create a standard Median-Joining (MJ) Network [33] of the genetic relationships with the program PopArt v. 1.7. PopArt v. 1.7 (Population Analysis with Reticulate Trees) [34] but their positions are visualized on a physical map using GPS data. Divergence among phylogenetic cluster was measured using the fixation index statistic (Φ_{ST}) by grouping sequenced data as follows: (1) *D. cervi*, (2) *D. Eckerti/D.cervi* and (3) *D. vivparus*, in PopArt v. 1.7.

3. Results

3.1. Prevalence of Dictyocaulosis in Deer at Sampling Location

Table 2 recorded the number of positive animals and the number of adults in each of the 36 lungs processed.

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Table 2. Number of deers, location, macroscopic, microscopic results and adults found.

Lungs	Location	Macroscopic	Microscopic	Adult number
Deer 1	Carrillas	+	+	4
Deer 2	Carrillas	-	+	
Deer 3	Carrillas	-	-	
Deer 4	Carrillas	-	-	
Deer 5	Carrillas	-	-	
Deer 6	San Fermín	-	-	
Deer 7	San Fermín	-	-	
Deer 8	San Fermín	-	-	
Deer 9	San Fermín	-	+	
Deer 10	San Fermín	-	-	
Deer 11	San Fermín	-	-	
Deer 12	Sierrra Paloma	-	-	
Deer 13	Sierrra Paloma	-	-	
Deer 14	Sierrra Paloma	-	-	
Deer 15	Sierrra Paloma	-	+	
Deer 16	Sierrra Paloma	+	+	8
Deer 17	Jabalina	+	+	17
Deer 18	Jabalina	+	+	22
Deer 19	Jabalina	-	-	
Deer 20	Jabalina	-	-	
Deer 21	Jabalina	-	-	
Deer 22	Valdelayegua	-	-	
Deer 23	Valdelayegua	-	-	
Deer 24	Valdelayegua	-	-	
Deer 25	Valdelayegua	-	-	
Deer 26	Valdelayegua	-	-	
Deer 27	Cerro Verde	-	-	
Deer 28	Cerro Verde	-	-	
Deer 29	Cerro Verde	-	-	
Deer 30	Cerro Verde	-	-	
Deer 31	Cerro Verde	+	+	13
Deer 32	El Águila	-	-	
Deer 33	El Águila	-	-	
Deer 34	El Águila	-	-	
Deer 35	El Águila	-	-	
Deer 36	El Águila	-	-	
TOTAL		5	8	64

The frequency of infected lung was 13.89% after the macroscopic analysis but an averaged adult count of 12.80 (SD 7.12). However, higher frequency was found after microscopic analysis with 22.22% of positive lungs. The results highlight the relevance of the microscopic analysis over the macroscopic inspection because larvae are much more common in lungs than macroscopically observable adults. Although at first glance Cohen's Kappa test suggested substantial agreement (κ=0.722) between the macroscopic and microscopic analysis with significant p-value ($p<0.001$), there was not perfect agreement indicating some discrepancies. Furthermore, the mean value for adults suggested a large variability in the count of adult parasites per infected red deer. Globally, the mean abundance of worms in all the lungs was calculated, giving an average of 1.78 (SD = 5.09) adults/lung.

 doi:10.20944/preprints202504.1246.v1

3.2. Morphological Identification and Anatomopathological Finding in Dictyocaulosis

After worm collection, preservation and preparation for microscopic observation, the adults were studied in detail for their correct identification. It was found that the buccal capsule was small with an oval shape and surrounded by simple lips (Figure 2A) as expected. Also, differences between males and females were examined. The male has an expansion of the cuticle (copulatory pouch) with a series of supporting ribs, as well as a series of rays for allowing its expansion during copulation. The posterior part in the female was conical in shape, showing a small and rounded protrusion called the vaginal diverticulum (Figure 2B,C). Finally, the presence of eggs in the uterus of the female is noteworthy with a uterus full of larvae eggs (L1 stage) (Figure 2C).

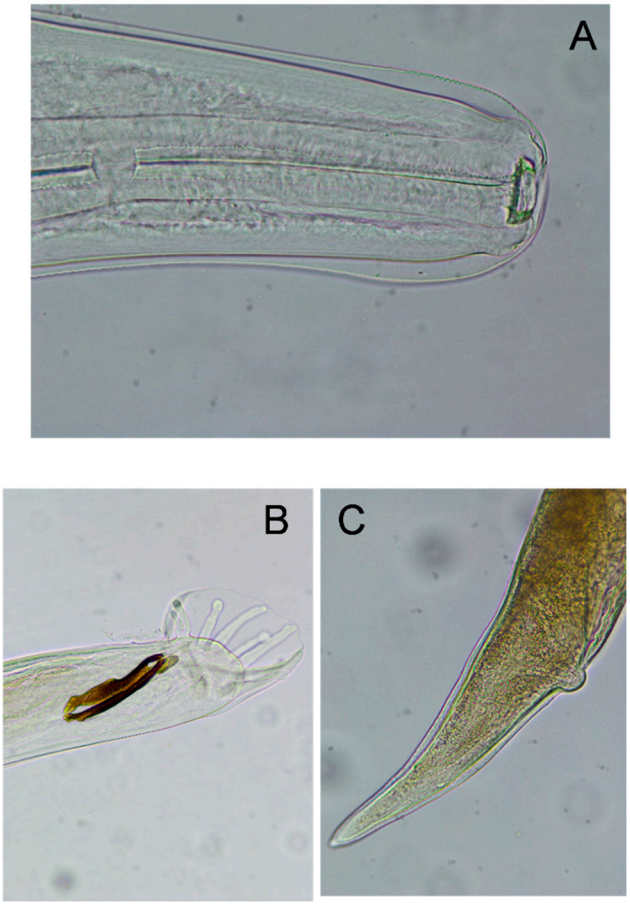

Figure 2. (A) Anterior part of *Dictyocaulus* spp.: Buccal capsule; (B) and (C) posterior part of male and female, respectively.

Anatomopathological study showed that dictyocaulus produces bronchopneumonia in red deer due to the presence of adult or larvae worms in the upper and middle respiratory tract. Figure 3A showed bronchioles inflammation and destruction of the bronchiolar epithelium (bronchiolitis) as well as intraluminal exudate of eosinophil, lymphocytes and plasmatic cells. Intraluminal parasitic structures (Figure 3B) compatible with the nematode surrounded by massive intraluminal hemorrhage and slight peri-bronchial lymphoid reaction were also identified. Figure 3C highlighted a clear parasitic membrane with three structural components: fibrous capsule (C), acellular laminated membrane (L), and germinative membrane (G). Figure 3D showed a non-specific interstitial pneumonia with a diffuse mononuclear cell infiltration that expands and widens the interalveolar septa, with little collagenization and a mild hyperplasia of the alveolar epithelium. No foci of fibroblastic proliferation and panalization phenomena.

 doi:10.20944/preprints202504.1246.v1

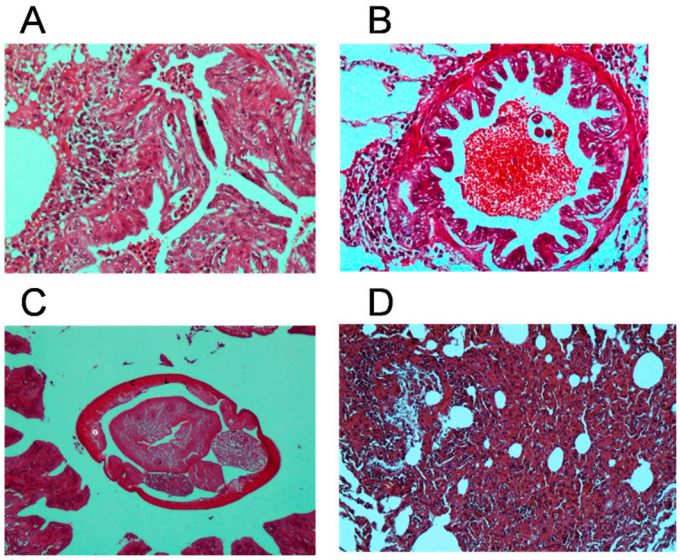

Figure 3. (A) Bronchioitis with a destructuring of the bronchiolar epithelium; (B). Cross section of adult *Dictyocaulus* spp. in the bronchial lumen, surrounded by hemorrhages. (C). Cross section of adult *Dictyocaulus* sp.. int he bronchial lumen and structural components: fibrous capsule (C9, acellular laminated membrane (L) and germinative membrane 8G). (D) Non-specific interstitial pneumonia and mild hyperplasia of the alveolar epithelium.

3.3. Barcoding Finding Through Sequencing COI Gene

Sequences from this study resulted in thirteen successful COI profiles using Sanger sequencing. These sequences collapsed in seven different haplotypes (Hap_31 to Hap_37, Figure 4). These haplotypes were compared with 102 additional sequences which collect molecular information from twenty-four nematode worm genus or species (table S1). Firstly, globally, the 115 sequences yielded up to 84 different haplotypes (Figure S1). The obtained overall sequence similarity varies between 96.97% and 99.66% respect to the sequences from Genebank (NCBI, USA) except one sequence from Cerro Verde (CV) that showed the highest similarity value (100%) and unclear species assignation that might better belong to *D. eckerti* (see Hap_32 vs. Hap_73, Figure 4). All haplotypes drawn very conspicuous different clusters which mostly assigned haplotypes to taxonomic species level as reported by the GenBank entries (Figure S1). Particularly important was the fact that the haplotype network very clearly separated three species from the *Dictyocaulus* genus, *D. viviparus*, *D. eckerti* and *D. cervi*. For this reason, it was decided to analyze them separately for a better visualization of the results (Figure 4). Secondly, it was constructed another network containing exclusive haplotypes from *Dictyocaulus* genus. In this redraw picture the *Dictyocalus* genus showed three well distinguishable matrilineal groups. Of these, two *groups* contained haplotypes exclusively assigned to *D. viviparus* and *D. cervi* species. However, *D. viviparus* was never found in this study. Four haplotypes (Hap_33, 34, 36 and 37) belonged to a group containing solely *D. cervi* species. A third genetic groups collect very similar haplotypes assigned to *D. cervi* or *D. eckerti* (Hap_ 31 and 32, plus Hap_35) species. Because these lasts were assigned to different species despite sharing a most probable common ancestor respect to *D. viviparus* or *D. cervi*, assignment confusion or admixture throughout hybridization could be further investigated. Furthermore, Hap_32 was the only showing a 100% identity with other sequences from Genbank but assigned to *D. cervi* (Acc N. PP922991) within the admixed group (Figure 4).

 doi:10.20944/preprints202504.1246.v1

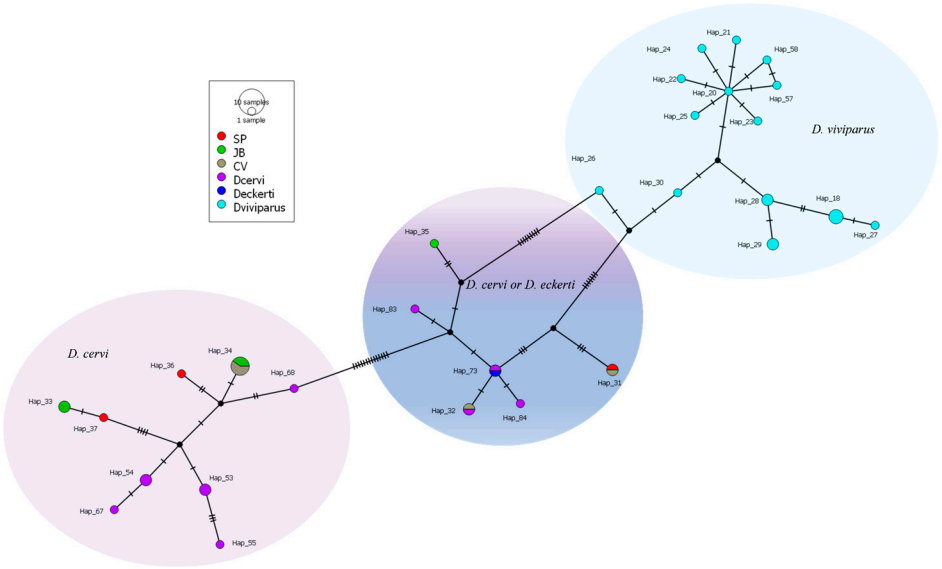

Figure 4. Graphical philogenetic relationship among the three *Dictyocaulus* spp. genetic groups under a similarity threshold greater then 90% respect to Genebank sequences (NCBI, USA). Central group consist of conflicting species assignations.

According with phylogenetic analysis (color assignment in the network, Figure 4) should be noted that Hap_31 was found in SP (Sierra Palomares, n=1) and CV (n=1), Hap_32 in CV (n=1), Hap_34 in both CV (n=3) and JB (La Jabalina, n=2), Hap_36/Hap_37 in SP (n=1 each), Hap_33/hap _35 in JB (n=2 and n=1, respectively)(Figure 4). In addition to phylogenetic evidence of molecular divisions, also, AMOVA analysis demonstrated genetic subdivision among the three dictyocaulus groups with 89.82% genetic variance explained among groups, two with conspicuous species status, and 10.18% variance within groups, supporting by a significant ϕ_{ST} index (p-value < 0.001). This result conforms with matrilineal ancestor isolation among haplotype groups with perfect match to species assignation. Conversely, *D. cervi* and *D. eckerti* shared matrilineal ancestor in one of the genetically differentiated groups, reason why this gene cluster (central cluster, Figure 4) does not match the species assignment (see table S1: haplotypes and species) with suggestive introgression. Furthermore, haplotypes of the admixed group and *D. cervi* groups were present in all sampling sites. Also, it can be stated that both *D. cervi* and *D. eckerti* coexist in the same host, since Hap_31, _32 (mixed group) and_34 (*D. cervi* group) were simultaneously identified in the only lung obtained at CV. As well the same scenario happened for haplotypes 31, 36 and 37, identified in one lung obtained from SP.

4. Discussion

4.1. Prevalence of Dictyocaulosis in Deer in Extremadura

If we focus on the adult count after macroscopic analysis, our results agreed with those obtained by other authors [13,35] who found an averaged intensity per positive lung of 11.7 adults and 6.33, respectively. However, extremely large discordances have been detected with others [4,36–38] because they reported disparate prevalence as less than one adult among 27 positive lungs or all positive deer presenting adults. All of these different results may be due to multifactorial reasons, ranging from the environmental and physiological status of the animals to the study design, but those particularly related to the age of the sampled animals and the state of their immune system have been suggested. Also, differences in sample size may be claimed. Several authors [13,23,35,39–42] suggested taking as large a possible number of samples, especially in the locations sampled. From an epidemiological point of view, a prevalence of 22.22% has been obtained, very similar to those obtained previously [40] in roe deer in the north of Spain and in deer in the Italian Alps [13]. However,

 doi:10.20944/preprints202504.1246.v1

prevalence rates of 50% and 54% in Norway have been found [40,43] respectively. Also, in Poland [4,38] and in Extremadura [23] reported prevalence three times higher in the order of 44%/68% and 62.5% in red deer, respectively. All these data suggest that a high variation in prevalence over time and geography would be expected.

Furthermore, it would have been interesting to perform coprological analysis to determine how many larvae, if any, they expel in their faeces [23]. Despite this application of feces, given that genetically different worms can be found from the same lung (even with small samples), caution is advised when using conventional sequencing analysis based on genetic material obtained from feces. Therefore, adults or larvae should be individualized when genetic analysis is among the objectives. This is especially relevant when hybridization is suspected. Furthermore, our results are significant because individualized specimens contribute 44.44% (4/9), 33.33% (2/6) of haplotypes to *D. cervi* and to admixed *D. eckerti*/*D. cervi* group, respectively, but 0% (0/14) to *D. viviparus* group; additionally, to those currently present in the NCBI databases.

4.2. Findings After Anatomopathological Study

In agreement to some authors [4,27] morphological traits observed in worms may only assess its belonging to the genus *Dictyocaulus* spp. However, these traits may distinguish *Dictyocaulus* from other genuses of pulmonary nematodes. Specifically, the adults collected in our study presented a simple buccal capsule, with a long esophagus, the presence of a copulatory pouch with ribs and rays that allow its expansion during copulation. In males, the dark-colored spicules with a boot or sock shape that are directed by the gubernaculum, and in females, the presence of the uterus filled with easily discernible larval eggs. However, identification at species level has been suggested to be extremely complicated by light microscopy since we have to rely on subjective measurements with no great difference among species. These reasons led to the need for more standardized diagnostic methods to identify dictyocaulus species level, such as molecular DNA methods. Furthermore, they open up new and unexpected scenarios out of the scope of traditional anatomopathological methods.

In the analysis of lung lesions caused by dictyocaulus worms, our findings show similarities and important differences compared to those reported in previous studies. Specifically, our observations present notable discrepancies with other authors [38] because no significant presence of macrophages was confirmed in the exudates of this study. In addition, they reported massive hyperplasia in follicular lymphoid follicles, bronchial vessels, and bronchiolar epithelium, leading to strong and widespread inflammatory and proliferative response in the affected lungs [38]. In contrast, our study showed only mild hyperplasia in the alveolar epithelium, which could indicate a less intense inflammatory response to different stages of the alveolar epithelium with reduced inflammatory response. Another study [44] largely corroborated the lesions described by us, reinforcing the consistency of many pathological patterns associated with different scenarios of dictyocaulosis. Also, compared with other study [45] findings were congruent, except for some specific details. Described the presence of slight fibroblastic proliferation, necrotic changes and proliferation of type II pneumocytes which were not a prominent observation in our study. These differences may be due to environmental and/or genetic variations between different red deer populations. Furthermore, we cannot discard differences in the histological and sampling techniques used. All these data may suggest multifactorial and differential infection stages, underscore possible differential responses among *Dictyocaulus* species and/or species mixtures.

4.3. Molecular of Dictyocaulus spp. Assessment

Mitochondrial COI gene outcomes were consistent with some authors [13,29] because relatively large molecular variability allowed for effective barcoding to discern all three *Dictyocaulus* species: *D. viviparus, D. eckerti and D. cervi*. Of the 18 nominal species within the genus only four are reported to infect European deer species: *D. eckerti*, *D. capreolus*, *D. cervi* and *D. skrjabini* [46]. Except *D. viviparus* from cattle, the remaining species in the genus were found in sheep and goats (*D. filaria*), donkeys and horses (*D. arnfeldi*), camels (*D. cameli*), African artiodactylids (*D. africanus*) and, also, *D. skrjabini*

 doi:10.20944/preprints202504.1246.v1

were not present in the data set due to either their lower similarity threshold, different sequence coverage or absence of data.

As a result, the downloaded molecular data only collected information from *D. viviparus* reinforcing a closer genetic relationship between these three species (*D. viviparus*, *D. eckerti* and *D. cervi*) within the genus *Dictyocaulus*. There are also subspecies, or possible new species, of *D. viviparus* within both European [47] and North American bisons [48] which were also in our data set (Hap_58 and Hap_57, Figure 4 and table S1) but it has not been possible to corroborate even different lineage level for this subspecies with the available data. All these facts gives support to a high genetic variability and longlife evolutionary history of this genus. The global phylogenetic analysis (115 sequences) allowed for the identification of three major groups in the genus *Dictyocaulus* supported by a great genetic variability between (89,9% variance) and less within (10,12% variance) groups, which agreed with last studies [4,13,38,48]. However, from the three genetic groups (*D. eckerti*, *D. cervi* and *D. viviparus*), only the two formers were found by us in red deer. Interestingly, this wide genetic variability has been associated with a possible adaptation to environmental changes of the nematode, even the development of resistance to anthelmintics. This resistance has been reported in a few studies from [19,20,49]. The first study carried out in the same region of Extremadura identified infection *D. viviparus* [23] but based on challenged morphological characters. However, a better barcoding-based model identified *D. cervi* and, probably, *D. eckerti* at this location (this study). A MJN analysis showed estrict genetic relationships within lineages of *D. cervi* and *D. viviparus* sequences but allocating *D. eckerti* with several *D. cervi* haplotypes (specially Hap_32) suggesting a mixed origin for this group (*D. Eckerti*/*D. cervi* groups, Figure 4). These findings were also reported [46]. Φ_{ST} statistic supported a significantly high divergence among the three groups from this study with strongly different mitochondrial COI alleles within the *Dyctiocaulus* genus was detected.

Accordingly, it was formerly confirmed the presence of *D. eckerti* and *D. cervi* in Extremadura and Spain. In addition, our results suggest a better and precise identification of *Dictyocaulus* species and discarding *D. viviparus* as causing dictyocaulosis in the examined red deer populations but discarding *D. viviparus*. According to some authors [4,13,38], there is firm evidence, even morphological, as *D. cervi* recovered from red deer was well distinguished from *D. eckerti*, assessing the importance of the use of COI sequences for its diagnosis. Despite, our results suggested more research must be performed to unravel the presence of two species in one of the three genetic groups, especially when hybridization might be a reasoned event since in this study both species have been found in the same red deer lung. The hybridization in nematode species has been demonstrated [50]. This state of hybridization in which they share genetic material could be facilitated by similarities in climatic conditions, between hosts and their life cycles. So, our findings further strengthen the idea that the genetic complexity and diversity among *Dictyocaulus* lungworms infecting wildlife ruminants is larger than previously believed and requires further investigation.

5. Conclusions

The prevalence of *Dictyocaulus* spp. was low, which leads to suggest as the immune system of red deer balanced these infestations and concordance between the macroscopic and microscopic diagnosis was also confirmed. The molecular analysis was valuable to assess taxonomic level among groups because of the high divergence of *Dictyocalus* species. So, the COI gene differentiated *Dictyocaulus* species in Spain for the first time, even when worms were sampled in the same infected lung. Furthermore, some sequences attributed to different species within the same genetic group were revealed, suggesting the need of new studies unraveling two competing questions aimed to differentiate between natural species mixtures or hybridization in these worms.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Figure S1: Graphical representation of the distribution of the different 81 haplotypes used for MJN analysis. Color were selected according to assigned species in genebank report, sampling sites when form those study and others

 doi:10.20944/preprints202504.1246.v1

from non Dictyocaulus genus.; Table S1: Species of worms from Genbank (NCBI, USA) with a higher 90% threshold similarity.

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References

1. Huaman, J.L., Helbig, K.J., Carvalho, T.G., Doyle, M., Hampton, J., Forsyth, D.M., Pople, A.R., Pacioni, C. A Review Of Viral And Parasitic Infections In Wild Deer In Australia With Relevance To Livestock And Human Health. Wildl. Res., 50 (2023), Pp. 593-602, 10.1071/Wr22118;
2. S. Shamsi, K. Brown, N. Francis, D.P. Barton, D.J. Jenkins. First Findings Of *Sarcocystis* Species In Game Deer And Feral Pigs In Australia.Int. J. Food Microbiol. (2024), 10.1016/J.Ijfoodmicro.2024.110780
3. Vainutis, K.S., Voronova, A.N., Andreev, M.E. et al. Morphological and molecular description of Dictyocaulus xanthopygus sp. nov. (Nematoda: Trichostrongyloidea) from the Manchurian wapiti Cervus elaphus xanthopygus. Syst Parasitol 100, 557–570 (2023). https://doi.org/10.1007/s11230-023-10105-4).
4. Pyziel, A. M., Laskowski, Z., Demiaszkiewicz, A. W., Y Höglund, J. (2017). Interrelationships of Dictyocaulus Spp. In Wild Ruminants With Morphological Description Of Dictyocaulus Cervi N. Sp. (Nematoda: Trichostrongyloidea) From Red Deer, Cervus Elaphus. Journal Of Parasitology, 103(5), 506-518. Https://Doi.Org/10.1645/16-75
5. Gibbons, L. M., Y Höglund, J. (2002). Dictyocaulus Capreolus N. Sp. (Nematoda: Trichostrongyloidea) From Roe Deer, Capreolus Capreolus And Moose, Alces Alces In Sweden. Journal Of Helminthology, 76(2), 119-124. Https://Doi.Org/10.1079/Joh2001108
6. R. A. Carreno And S. A. Nadler . 2003. Phylogenetic Analysis Of The Metastrongyloidea (Nematoda: Strongylida) Inferred From Ribosomal Rna Gene Sequences. Journal Of Parasitology 89:965–973.
7. Carreno, R. A., Diez-Baños, N., Hidalgo-Argüello, M. del R., & Nadler, S. A. (2009). Characterization of Dictyocaulus Species (Nematoda: Trichostrongyloidea) from Three Species of Wild Ruminants in Northwestern Spain. Journal of Parasitology, 95(4), 966-970. https://doi.org/10.1645/GE-1791.1
8. L. M. Gibbons And L. F. Khalil . 1988. A Revision Of The Genus Dictyocaulus Railliet And Henry, 1907 (Nematoda: Trichostrongyloidea) With The Description Of D. Africanus N. Sp. From African Artiodactylids. Revue De Zoologie Africaine 102:151–175.
9. J. Höglund, D. A. Morrison, B. P. Divina, E. Wilhelmsson, And J. G. Mattsson . 2003. Phylogeny Of Dictyocaulus (Lungworms) From Eight Species Of Ruminants Based On Analyses Of Ribosomal Rna Data. Parasitology 127:179–187.
10. R.B. Gasser, A. Jabbar, N. Mohandas, J. Höglund, R.S. Hall, D.T.J. Littlewood, A.R. Jex. Assessment Of The Genetic Relationship Between *Dictyocaulus* Species From *Bos Taurus* And *Cervus Elaphus* Using Complete Mitochondrial Genomic Datasets. Parasites Vectors, 5 (2012), P. 241, 10.1186/1756-3305-5-241
11. Pyziel, A. M., Laskowski, Z., Klich, D., Demiaszkiewicz, A. W., Kaczor, S., Merta, D., Kobielski, J., Nowakowska, J., Anusz, K., & Höglund, J. (2023). Distribution of large lungworms (Nematoda: Dictyocaulidae) in free-roaming populations of red deer Cervus elaphus (L.) with the description of Dictyocaulus skrjabini n. sp. Parasitology, 150(10), 956-966. https://doi.org/10.1017/S003118202300080X

 doi:10.20944/preprints202504.1246.v1

12. Chilton, N. B., Huby-Chilton, F., Gasser, R. B., & Beveridge, I. (2006). The evolutionary origins of nematodes within the order Strongylida are related to predilection sites within hosts. Molecular Phylogenetics and Evolution, 40(1), 118-128. https://doi.org/10.1016/j.ympev.2006.01.003
13. Cafiso, M. Castelli, P. Tedesco, G. Poglayen, C. Buccheri-Pederzoli, S. Robetto, R. Orusa, L. Corlatti, C. Bazzocchi, C. Luzzago. Molecular Characterization Of *Dictyocaulus* Nematodes In Wild Red Deer *Cervus Elaphus* In Two Areas Of The Italian Alps. Parasitol. Res., 122 (2023), Pp. 881-887, 10.1007/S00436-022-07773-4
14. D. Jenkins, A. Baker, M. Porter, S. Shamsi, D.P. Barton. Wild Fallow Deer (*Dama Dama*) As Definitive Hosts Of *Fasciola Hepatica* (Liver Fluke) In Alpine New South Wales. Aust. Vet. J., 98 (2020), Pp. 546-549, 10.1111/Avj.13001.,
15. J. Lamb, E. Doyle, J. Barwick, M. Chambers, L. Kakhn. Prevalence And Pathology Of Liver Fluke (*Fasciola Hepatica*) In Fallow Deer (*Dama Dama*). Vet. Parasitol., 293 (2021), Article 109427, 10.1016/J.Vetpar.2021.109427
16. Jarrett, W. F. H., Jennings, F. W., Mcintyre, W. I. M., Mulligan, W., Thomas, B. A. C., Y Urquhart, G. M. (1959). Immunological Studies On Dictyocaulus Viviparus Infection. Immunology, 2(3), 252-261.
17. Jarrett, W. F. H., Y Sharp, N. C. C. (1963). Vaccination Against Parasitic Disease: Reactions In Vaccinated And Immune Hosts In Dictyocaulus Viviparus Infection. The Journal Of Parasitology, 49(2), 177-189. Https://Doi.Org/10.2307/3275980
18. Claerebout, E., Y Geldhof, P. (2020). Helminth Vaccines In Ruminants: From Development To Application. Veterinary Clinics: Food Animal Practice, 36(1), 159-171. Https://Doi.Org/10.1016/J.Cvfa.2019.10.001
19. Molina, V. M., Arbeláez, J. M., Prada, J. A., Blanco, R. D., Y Oviedo, C. A. (2016). Posible Resistencia De Dictyocaulus Viviparus Al Fenbendazol En Un Bovino. Revista De La Facultad De Medicina Veterinaria Y De Zootecnia, 63(1), 54-63. Https://Doi.Org/10.15446/Rfmvz.V63n1.56904
20. Campbell, P., Forbes, A., Mcintyre, J., Bartoschek, T., Devine, K., O'neill, K., Laing, R., Y Ellis, K. (2024). Inefficacy Of Ivermectin And Moxidectin Treatments Against Dictyocaulus Viviparus In Dairy Calves. Veterinary Record, E4265. Https://Doi.Org/10.1002/Vetr.4265
21. R.A. Mckenzie, P.E. Green, A.M. Thornton, Y.S. Chung, A.R. Mackenzie, D.H. Cybinski, T.D. St George. Diseases Of Deer In South Eastern Queensland Aust. Vet. J., 62 (1985), P. 424. G.E. Mylrea, R.C. Mulley, A.W. English Gastrointestinal Helminths In Fallow Deer (Dama Dama) And Their Response To Treatment With Anthelminthics. Aust. Vet. J., 68 (1991), Pp. 74-75
22. G.E. Mylrea, R.C. Mulley, A.W. English. Gastrointestinal Helminths In Fallow Deer (Dama Dama) And Their Response To Treatment With Anthelminthics. Aust. Vet. J., 68 (1991), Pp. 74-75
23. Habela Martínez-Estéllez, M. Á., Moreno Casero, A. M., Peña, J., Montes, G., Gómez Carmona, J. M., Y Hermoso De Mendoza Salcedo, J. (2006). Parásitos Asociados A Tuberculosis En Ciervos (Cervus Elaphus) De Extremadura. Xxxi Jornadas Científicas Y X Internacionales De La Sociedad Española De Ovinotecnia Y Caprinotecnia (Seoc): Zamora, 20-22 De Septiembre De 2006, 2006, Isbn 84-934535-8-7, Págs. 337-339, 337 339. Https://Dialnet.Unirioja.Es/Servlet/Articulo?Codigo=8691504
24. A.M. Pyziel, Z. Laskowski, J. Höglund. Development Of Amultiplex Pcr For Identification Of *Dictyocaulus* Lungworms In Domestic And Wild Ruminants. Parasitol. Res., 114 (2015), Pp. 3923-3926, 10.1007/S00436-015-4657-Y
25. P. Halvarsson, P. Baltrušis, P. Kjellander, J. Höglund.Parasitic Strongyle Nemabiome Communities In Wild Ruminants In Sweden. Parasites Vectors, 15 (2022), P. 341
26. Bangoura, B., Brinegar, B., & Creekmore, T. E. (2020). DICTYOCAULUS CERVI-LIKE LUNGWORM INFECTION IN A ROCKY MOUNTAIN ELK (CERVUS CANADENSIS NELSONI) FROM WYOMING, USA. Journal of Wildlife Diseases, 57(1), 71-81. https://doi.org/10.7589/JWD-D-20-00023
27. Pato Rivero, F. J. (2012). Estudio Epidemiológico De Las Infecciones Que Afectan Al Aparato Respiratorio Y Gastrointestinal De Los Corzos En Galicia [Tesis Doctoral]. Https://Minerva.Usc.Es/Xmlui/Handle/10347/3700
28. Miller, S. A., Dykes, D. D., Y Polesky, H. F. (1988). A Simple Salting Out Procedure For Extracting Dna From Human Nucleated Cells. Nucleic Acids Research, 16(3), 1215.

 doi:10.20944/preprints202504.1246.v1

29. Bowles, J., Blair, D., Y Mcmanus, D. P. (1992). Genetic Variants Within The Genus Echinococcus Identified By Mitochondrial Dna Sequencing. Molecular And Biochemical Parasitology, 54(2), 165-173. Https://Doi.Org/10.1016/0166-6851(92)90109-W
30. Zhang, D. X., Y Hewitt, G. M. (1997). Assessment Of The Universality And Utility Of A Set Of Conserved Mitochondrial Coi Primers In Insects. Insect Molecular Biology, 6(2), 143 150. Https://Doi.Org/10.1111/J.1365-2583.1997.Tb00082.X
31. Wollan, G. T., & Quevedo, E. M. (2024). Molecular Methods Used to Identify a New Species of Dictyocaulus (Family Dictyocaulidae) in White-Tailed Deer.
32. Rozas Liras, J. A., Librado Sanz, P., Sánchez Del Barrio, J. C., Messeguer Peypoch, X., Y Rozas, R. (2010). Dnasp Version 5. Dna Sequence Polymorphism. Programari (Genètica, Microbiologia I Estadística). Https://Diposit.Ub.Edu/Dspace/Handle/2445/53451
33. Bandelt, H. J., Forster, P., Y Röhl, A. (1999). Median-Joining Networks For Inferring Intraspecific Phylogenies. Molecular Biology And Evolution, 16(1), 37-48. Https://Doi.Org/10.1093/Oxfordjournals.Molbev.A026036
34. Leigh, J. W., Y Bryant, D. (2015). Popart: Full-Feature Software For Haplotype Network Construction. Methods In Ecology And Evolution, 6(9), 1110-1116. Https://Doi.Org/10.1111/2041-210x.12410
35. Panadero, R., Carrillo, E. B., López, C., Díez-Baños, N., Díez-Baños, P., Y Morrondo, M. P. (2001). Bronchopulmonary Helminths Of Roe Deer (Capreolus Capreolus) In The 45 Northwest Of Spain. Veterinary Parasitology, 99(3), 221-229. Https://Doi.Org/10.1016/S0304-4017(01)00465-4
36. Borgsteede, F. H. M., Jansen, J., Van Nispen Tot Pannerden, H. P. M., Van Der Burg, W. P. J., Noorman, N., Poutsma, J., Y Kotter, J. F. (1990). Untersuchungen Über Die Helminthen-Fauna Beim Reh (Capreolus Capreolus L.) In Den Niederlanden. Zeitschrift Für Jagdwissenschaft, 36(2), 104-109. Https://Doi.Org/10.1007/Bf02241807
37. Shimalov, V., Y Shimalov, V. (2002). Helminth Fauna Of Cervids In Belorussian Polesie. Parasitology Research, 89(1), 75-76. Https://Doi.Org/10.1007/S00436-002-0700-X
38. Pyziel, A. M., Dolka, I., Werszko, J., Laskowski, Z., Steiner-Bogdaszewska, Ż., Wiśniewski, J., Demiaszkiewicz, A. W., Y Anusz, K. (2018). Pathological Lesions In The Lungs Of Red Deer Cervus Elaphus (L.) Induced By A Newly-Described Dictyocaulus Cervi (Nematoda: Trichostrongyloidea). Veterinary Parasitology, 261, 22-26. Https://Doi.Org/10.1016/J.Vetpar.2018.08.003
39. Hugonnet, L., Y Cabaret, J. (1987). Infection Of Roe-Deer In France By The Lung Nematode, Dictyocaulus Eckerti Skrjabin, 1931 (Trichostrongyloidea): Influence Of Environmental Factors And Host Density. Journal Of Wildlife Diseases, 23(1), 109-112. Https://Doi.Org/10.7589/0090-3558-23.1.109
40. Dacal, V., Vázquez, L., Pato, F. J., Cienfuegos, S., Panadero-Fontán, R., López Sández, C., Y Morrondo, P. (2010). Cambios En La Capacidad Pulmonar En Corzos (Capreolus Capreolus) Del Noroeste De España Infectados Por Nematodos Broncopulmonares. Galemys 22, 22, 233-242. Https://Doi.Org/10.7325/Galemys.2010.Ne.A14
41. Kuzmina, T., Kharchenko, V., Y Malega, A. (2010). Helminth Fauna Of Roe Deer (Capreolus Capreolus) In Ukraine: Biodiversity And Parasite Community. Vestnik Zoologii, 44(1), E-12-E-19. Https://Doi.Org/10.2478/V10058-010-0002-1
42. Handeland, K., Davidson, R. K., Viljugrein, H., Mossing, A., Meisingset, E. L., Heum, M., Strand, O., Y Isaksen, K. (2019). Elaphostrongylus And Dictyocaulus Infections In Norwegian Wild Reindeer And Red Deer Populations In Relation To Summer Pasture Altitude And Climate. International Journal For Parasitology: Parasites And Wildlife, 10, 188-195. Https://Doi.Org/10.1016/J.Ijppaw.2019.09.003
43. Stoican, E.; Olteanu, G. (1959). Beitriige Zum Studium Der Helminthofauna Des Rehes (C. Capreolus) In Rumiinien. Probleme Der Parazitologie, 7: 38-46
44. Llada, I. M., Gianechini, L. S., Lloberas, M. M., Morrell, E. L., Odriozola, E. R., Y Cantón, G. J. (2020). Dictiocaulosis En Vacas De Cría En La Provincia De Buenos Aires, Argentina: Descripción De Dos Brotes. Analecta Veterinaria, 40(1). Https://Revistas.Unlp.Edu.Ar/Analecta/Article/Download/9495/9286?Inline=1#Redalyc_2 51357005_Ref2

 doi:10.20944/preprints202504.1246.v1

45. Mahmood, F., Khan, A., Hussain, R., Y Anjum, M. S. (2011). Prevalence And Pathology Of Dictyocaulus Viviparus Infection In Cattle And Buffaloes. Veterinary Record, 169(19), 494-494. Https://Doi.Org/10.1136/Vr.D4736
46. Keira Brown, David J. Jenkins, Alexander W. Gofton, Ina Smith, Nidhish Francis, Shokoofeh Shamsi, Diane P. Barton, The First Finding Of Dictyocaulus Cervi And Dictyocaulus Skrjabini (Nematoda) In Feral Fallow Deer (Dama Dama) In Australia, International Journal For Parasitology: Parasites And Wildlife, Volume 24, 2024, 100953. Issn 2213-2244, Https://Doi.Org/10.1016/J.Ijppaw.2024.100953.
47. Pyziel, A. M., Laskowski, Z., Dolka, I., Kołodziej-Sobocińska, M., Nowakowska, J., Klich, D., Bielecki, W., Żygowska, M., Moazzami, M., Anusz, K., & Höglund, J. (2020). Large lungworms (Nematoda: Dictyocaulidae) recovered from the European bison may represent a new nematode subspecies. International Journal for Parasitology: Parasites and Wildlife, 13, 213-220. https://doi.org/10.1016/j.ijppaw.2020.10.002
48. H.A. Danks, C. Sobotyk, M.N. Saleh, M. Kulpa, J.L. Luksovsky, L.C. Kones, G.G. Verocai. Opening A Can Of Lungworms: Molecular Characterization Of *Dictyocaulus* (Nematoda: Dictyocaulidae) Infecting North American Bison (*Bison Bison*). Int. J. Parasitol.: Parasites And Wildlife, 18 (2022), Pp. 128-134, 10.1016/J.Ijppaw.2022.04.011
49. Molento, M. B., Depner, R. A., & Mello, M. H. A. (2006). Suppressive treatment of abamectin against Dictyocaulus viviparus and the occurrence of resistance in first-grazing-season calves. Veterinary Parasitology, 141(3), 373-376. https://doi.org/10.1016/j.vetpar.2006.01.061
50. Blanc-Mathieu, R., Perfus-Barbeoch, L., Aury, J.-M., Rocha, M. D., Gouzy, J., Sallet, E., Martin-Jimenez, C., Bailly-Bechet, M., Castagnone-Sereno, P., Flot, J.-F., Kozlowski, D. K., Cazareth, J., Couloux, A., Silva, C. D., Guy, J., Kim-Jo, Y.-J., Rancurel, C., Schiex, T., Abad, P., … Danchin, E. G. J. (2017). Hybridization And Polyploidy Enable Genomic Plasticity Without Sex In The Most Devastating Plant-Parasitic Nematodes. Plos Genetics, 13(6), E1006777. Https://Doi.Org/10.1371/Journal.Pgen.1006777