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CHARACTERISTICS OF INCIDENCE AND HOST SPECIFICITY OF *DICTYOCAULUS* LUNGWORMS IN DEER INVESTIGATED BY DNA EXAMINATIONS

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BACKGROUND OF RESEARCH , OBJECTIVES

Lungworm (*Dictyocaulus* spp.) infection in ruminants and horses is a well known problem for livestock farmers and wildlife managers throughout the world. The reason for sporadic outbreaks of dictyocaulosis in domestic animals is not completely understood, but it has been suggested that wild animals may serve as reservoirs of the parasites. Lungworms are considered to be the most important parasites in deer (Cervidae). There is evidence that several *Dictyocaulus* species in wild ruminants in Europe (*D. capreolus, D. eckerti, D. filaria, D. viviparus,*). *D. viviparus* is widespread in cattle in the temperate zone, while *D. filaria* is common in sheep and goats. Mild dictyocaulosis infection causes growth decline, while more severe cases may cause mortality.

In spite of the worldwide occurrence of *Dictyocaulus* worms and their economic impact, there is limited knowledge regarding the identification and distribution of existing species. It is often difficult to morphologically distinguish closely related *Dictyocaulus* species, but molecular markers provide a powerful alternative means to define them. Considering the literature based on traditional morphological identifications of *Dictyocaulus* species, they have a broad and overlapping host spectrum. But if we look only at literature dealing with lungworms identified or separated by molecular (DNA) methods, we find *Dictyocaulus* species with a rather narrow host range.

Prior to this study, population genetic analyses of lungworms were restricted to the cattle lungworm, *D. viviparus* in Sweden. Studies on *D. viviparus* genetic diversity and gene flow among cattle farms have revealed a strong population genetic structure, likely influenced by human activities. However, genetic study on wildlife populations of lungworms

have not been conducted yet. While *D. capreolus* are recorded only from Europe and Asia Minor, *D. eckerti* is widely distributed in temperate regions worldwide, such as North America, Europe, Siberia and New Zealand.

Resistance to current anthelmintics is now widespread and commonplace, motivating the search for other control methods. With increased knowledge about lungworms it is possible to find weak points in the lifecycle and hence to improve control methods. To separate and know the biology of lungworm species requires more knowledge, which can contribute to understanding, prevention and management of dictyocaulosis.

The aims of the present work were the following:

1. Identification of lungworm species living in red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and European roe deer (*Capreolus capreolus*) based on DNA analyses, and establishment of presumed biological species based on genetic distances of isolated lungworms;
2. Analysis of phylogenetic relationships within the *Dictyocaulus* genus based on newly generated sequences and earlier published data;
3. Testing and description of PCR based methods which are fast, inexpensive and practical to identify genetically separated lungworm species;
4. Description of the host-parasite relationships of lungworms in deer at the species level, comparison to the international data, and thus, establishment of the host specificity of *Dictyocaulus* species;
5. Investigation of the population genetics of lungworms in deer, including: description of the genetic diversity of populations divided

by individual hosts, host species, collecting locality and region, analysis of the separation of lungworm populations, and estimation of gene flow and historical demography.

2. MATERIALS AND METHODS

2.1. Adult large lungworms were collected from the trachea and bronchi of the following deer species harvested during hunting: fallow deer, red deer and roe deer. Samples were taken from 23 sites in Hungary and one locality in neighbouring Romania. Total genomic DNA was extracted from each of 312 worm specimens. Five gene fragments were PCR amplified and sequenced: ribosomal small subunit (18S rDNA), ribosomal large subunit (28S rDNA), ribosomal internal transcribed spacer 2 (ITS2), major sperm protein 1 (MSP1) and mitochondrial cytochrome c oxidase subunit 1 (cox1).

2.2. I conducted multigene phylogenetic analyses for large lungworms of Hungarian deer species, based on 5 genetic loci mentioned above. Patterns in polymorphisms between the 4 nuclear and 1 mitochondrial loci were different. Therefore analyses were applicable to provide recent and distant evolutionary insights. I aligned the DNA sequences using ClustalX version 2.0. I choose the best fitting models of sequence evolution using Modeltest for each locus, and applied this to examine the evolutionary relationships among lungworm samples. I constructed Maximum Likelihood (ML), Maximum Parsimony (MP), Neighbor Joining (NJ) and Bayesian inference (BI) phylogenetic trees using MEGA6 and MrBayes for each examined locus. Bootstrap clade support was inferred using 1,000 bootstrap replicates for ML, MP, and NJ analyses.

2.3. My work is considered to be of practical use to find a method by which the morphologically difficult or impossible to separate deer parasites of the *Dictyocaulus* can be distinguished. To this aim, the following DNA testing methods were used: melting point analysis of PCR

fragments, length polymorphism of PCR fragments, restriction fragment length polymorphism of PCR fragments (PCR-RFLP), random amplification of polymorphic DNA (RAPD).

2.4. I evaluated host-parasite relationships based on the host species of lungworms identified by the molecular markers of cox1 and RAPD (own results), as well as additional data I collected from scientific publications.

2.5. To infer the population structure of lungworms and examine the processes that have shaped present distributions, I utilised cox1 DNA sequences. I calculated genetic diversity values using DnaSP software. I measured variation within and between species and populations at four levels (individual host, host species, locality and regional collecting area) relative to the entire population. I evaluated the population structure and gene flow using analyses of nucleotid variability. I estimated the population history of *Dictyocaulus* populations based on Tajima's *D* and Fu's F_s tests, mismatch distribution analyses and the time to the most recent common ancestor (tMRCA).

3. RESULTS

3.1. 106 *Dictyocaulus* specimens were classified to species based on DNA sequences. Phylogenetic analyses revealed that *Dictyocaulus* sequences of Hungarian lungworm samples grouped into 3 strongly supported clades (99% bootstrap support). The average DNA sequence divergences within clades were less than 2%, and those between clades were over 13%. I identify an undescribed cryptic species in these analyses, which I refer to in this work as *D.* sp. n. The new species is supported by analyses of all 5 gene sequences examined. Specimens of *D.* sp. n. were collected from red deer only in South-West Hungary.

3.2. Three evolutionary lineages differ significantly within the *Dictyocaulus* genus: (1) *D. filaria*, (2) *D. arnfieldi*, and (3) lungworms living in deer (Cervidae) and ungulates with cloven hooves (Bovinae). Five *Dictyocaulus* species can be separated based on cox1 phylogenetic analyses for group (3): *D. capreolus*, *D. eckerti*, *D. viviparus*, *D.* sp. n. and '*D.* sp. red deer-New-Zealand'. This last lungworm species, which has a genotype identified from red deer in New Zealand, likely represents an additional new cryptic species within the genus *Dictyocaulus*, with a close relationship to European *D. eckerti* species. *D. capreolus* is positioned more basally to the other species within the clade. The closest evolutionary relatives of *Dictyocaulus* lungworms are species of the Metastrongyloidea superfamily.

3.3. I tested 12 RAPD primers to identify the 3 *Dictyocaulus* species in deer in Hungary. Based on separation by cox1 sequences, 3 lungworm species were distinguishable based on patterns of the OPB-01 primer for all 106 samples. I carried out RAPD analysis for a further 125 samples of lungworms, which were unknown at species level. I carried out species

identifications for these lungworm specimens via the RAPD method by assessing similarity to patterns of control samples from previously sequenced data for cox1. The results gave consistently three patterns, which agreed with previous patterns for lungworms having known sequence data.

3.4. Regarding host relationships, I show that red deer is the primary host for *D. eckerti* within the region sampled. Although *D. eckerti* samples (n=184) were recovered from all three deer species considered here, the vast majority of worms originated from red deer (n=158). All of the 13 lungworms collected from fallow deer were identified as *D. eckerti*. The patchy distribution of fallow deer may mean this species acts only as a secondary host for the parasite. In contast, only one *D. capreolus* worm originated from red deer, with the additional 34 worms sampled from roe deer. The identification of *D. capreolus* from red deer represents a new host-parasite record. In addition, this is the first time that *D. eckerti* has been recorded from roe deer confirmed by a molecular study (n=13). *D.* sp. n. specimens were collected exclusively from red deer.

3.5. This study represents the first population genetic analysis of large lungworms in wild animals. My work indicates high nucleotide variation for wild lungworm species of deer, with haplotype diversity approaching 1. It is striking that in total, 70 haplotypes, belonging to 3 species, could be identified from 106 lungworm specimens collected in Hungary. *D. capreolus* samples showed lower genetic diversity (π=0.0086) than *D. eckerti* samples (π=0.0184). Population genetic analyses for *D. eckerti* revealed high gene flow among weakly structured spatial populations and the three sympatric host deer species considered here. These results suggest that *D. eckerti* is a widespread generalist parasite in ungulates with a diverse genetic background. The *D. eckerti* populations included

here are genetically variable (π=0.0099-0.0239), but there were no clear differences between populations according to haplotype distributions. The *Dictyocaulus* species considered show two distinct population genetic classes: (1) *D. eckerti* has high host vagility, and shows low population differentiation (F_{ST}≤0.044), and consequently high migration *Nm* (number of effective immigrants) values. The high *Nm* value indicates that populations of *D. eckerti* have strong genetic connectivity. (2) *D. capreolus* in host populations with moderate vagility shows moderate population structure (F_{ST}=0.15). The cryptic genetic structure in *D. capreolus* appears to be distance dependent, which may be a consequence of the limited dispersal behaviour of its roe deer hosts. It is clear that there are population genetic differences between *D. eckerti* and *D. capreolus* in Hungary. The observed population structure of *D. capreolus* may indicate lower dispersal capacities (*Nm*=3) than in *D. eckerti* (*Nm*=11-17).

4. CONCLUSIONS

In addition to the general scientific relevance of the evolutionary and ecological results of this dissertation, this work has applied consequences for animal health, since large lungworms are important parasites of domestic animals and big game species, and are considered a issue substantial in wildlife management.

Phylogenetic analyses revealed that sequences of *Dictyocaulus* living in Hungarian deer grouped into 3 clades. The variability of DNA sequences within and between clades differed by one order of magnitude, consequently the 3 clades identified correspond to separate lungworm species, that live in sympatry in Hungary. Based on these results, sequences of the 5' end of the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene are useful to identify *Dictyocaulus* species, reveal new species, estimate phylogenetic relationships, and analyse population genetics.

Based on my results, RAPD analysis using the OPB-01 primer appears to be suitable to separate and identify *Dictyocaulus* species living in deer, at least for lungworms in Hungary. Further validation of this method is required using a larger number of samples. The cox1 DNA sequence analysis can be recommended for reliable identification of *Dictyocaulus* species.

The host preferences of lungworms holds great importance because of their animal health relevance. The host preference of *D. capreolus* is determined by the host species primarily, that is, *D. capreolus* is a roe deer specialist, at least in Hungary. The host preference and specificity of *D. eckerti* and *D.* sp. n. are affected presumably by other factors also. I demonstrated the sympatric occurrences of *D. eckerti* and *D.* sp. n. in the

same host species (red deer) in a few localities also (Gemenc, Hőgyész, Gálosfa). I did not find any ecological factors underlying the genetic differentiation between them. Further research is required to elucidate the factors leading to the genetic isolation of these two lungworm species. Contrary to previous suggestions that *Dictyocaulus* species have a broad host spectrum, it is clear from recent studies and my own that lungworm species are capable of infecting only one or a few host species, except *D. eckerti*, which is a generalist species.

As wild deer and cattle often use the same grazing sites, theoretically there could be a high possibility of cross infection between deer and cattle lungworms. However, I did not find any *D. viviparus* lungworms in deer. Based on my results deer are not reservoirs for the cattle parasite *D. viviparus*. However, clarification is required to determine whether lungworms of cervids (*D. capreolus*, *D. eckerti*, *D.* sp. n.) are parasites in cattle. The identification of *Dictyocaulus* species by molecular markers used in my work, such as RAPD patterns or cox1 sequences, can be used to answer this question. Additional host-parasite studies are required to know with greater confidence the full host spectrum of lungworms, identified by molecular markers, across their whole distributional area, to understand which ruminant is the primary host for a given parasite in a given geographical region. This knowledge would be beneficial to animal health as it would provide useful information on the reservoir host species of the parasitic worms.

My survey aimed to reveal the population genetic structure of *Dictyocaulus* lungworms in wildlife, focussing on deer host species. The cryptic genetic structure in *D. capreolus* appears distance-dependent, which may be a consequence of limited dispersal behaviour of its roe deer hosts. There are significant population genetic differences between *D.*

eckerti and *D. capreolus* in Hungary. The observed population structure of *D. capreolus* may indicate lower dispersal capacities than in *D. eckerti*, leading to reduced gene flow between populations of *D. capreolus*, which may explain the pattern of stronger differentiation. There are considerable differences in the dispersal patterns of the hosts examined. Fallow deer and red deer, which host *D. eckerti*, may migrate across great distances, while roe deer migrate to a lesser extent, and are considered to be a territorial species. Roe deer usually disperse individually (bucks) or in small groups (does with fawns) from spring to autumn when lungworm infection is most likely. However, roe deer have two ecotypes in Hungary: field-, and forest-based. Forest-based roe deer live in groups of 4-8 animals, while field-based roe deer live in larger groups of dozens or even hundreds of individuals from autumn to spring. It is assumed that cross-infection is more probable among group members (red deer and field-based roe deer) than among dispersed forest-based roe deer individuals. Therefore I suggest that the differing vagility and dispersal behaviour of host species are important contributing factors to the population structure of lungworms.

Regarding differing levels of gene flow in lungworms, I have observed consequences for parasite population dynamics and evolutionary potential. These results suggest that *D. eckerti* has probably not experienced a severe population decrease recently. Furthermore, results suggest that the species experienced a population expansion, indicated at ~11,500 years ago. The estimate for a relatively recent *D. eckerti* population expansion is likely to be driven by the population expansion of its hosts. The population expansion time estimate is concordant with host migration and population expansion after the last ice age. Red deer in

Europe experienced population expansions approximately 10,000 years ago.

The majority of red deer in Hungary become infected by lungworms during their first summer (75% prevalence in calves), while in roe deer fawns 31-40% prevalence was recorded. The currently known host species of *D. eckerti* (red deer, fallow deer, roe deer, reindeer, moose and musk ox) have large geographic distributions that overlap substantially. The large distribution, high genetic diversity, and high gene flow of *D. eckerti* have important evolutionary consequences, and together with its host generality offer the potential for new beneficial mutations to spread rapidly.

Livestock are protected against a variety of worm infestations by use of broad-spectrum anthelmintic drugs. Thus, increasing resistance against anthelmintic drugs poses an increasingly serious challenge for livestock producers. Consequently, scientific developments are required to develop new approaches to protecting livestock from worm infestation, in order to safeguard agricultural production and food security.

The findings in this thesis have considerable implications for lungworm management, particularly since high gene flow enhances the efficient spread of anthelmintic resistance, which is a serious problem for the management of several parasitic worms. Evidence of anthelmintic resistance against abamectin and trichlorfon in cattle lungworm in Southern-America has already been published. These anthelmintics are often also used in Hungary, so the spread of resistant worms can be assumed or anticipated here also. Resistance in lungworms living in cervids has not yet been reported, but this thesis shows there is considerable potential risk, especially in the case of *D. eckerti*.

5. NEW SCIENTIFIC RESULTS

1. I applied molecular techniques to *Dictyocaulus* lungworms living is Hungary for the first time, for species identifications based on international literature, and showed the occurrence of *Dictyocaulus capreolus* and a new species, *Dictyocaulus* sp. n. in Hungary.

2. *Dictoycaulus*. sp. n., is considered to be a new species. For this species, I determined DNA sequences for 5 genes, the following of which represent new data: cytochrome c oxidase subunit 1 (cox1), 28S ribosomal RNA (28S rDNA) and major sperm protein 1 (MSP1).

3. I determined the synonymy of *D. noerneri* Raillet & Henry, 1907 and *D. capreolus* Gibbons & Höglund, 2002 based on my own analysis of DNA sequences. As the description of *D. noerneri* is incomplete, the name of *D. capreolus* must be used for that species.

4. I carried out the first population genetic study of *Dictyocaulus* worms from wild ruminants. Based on DNA sequence analyses, cox1 gene showed less genetic diversity for *D. capreolus* and *D.* sp. n., than for *D. eckerti* species.

5. I showed that wild populations of *D. eckerti* lungworms are weakly structured, presumably due to high gene flow. This is probably due to the social behaviour, casual migration, or population expansion of the host, red deer.

6. It can be assumed on the basis of limited sampling, that populations of *D. capreolus* in Hungary are moderately fragmented, showing moderate gene flow. The population in the Great Plain has more than four times greater genetic diversity compared to the population in Southern Transdanubia. The inferred reason is the social behaviour of roe deer from autumn to spring.

7. I identified the following new host-parasite relationships: *Cervus elaphus – Dictyocaulus capreolus*, *Cervus elaphus – Dictyocaulus* sp. n. I also demonstrated the parasitic relationship *Capreolus capreolus – Dictyocaulus eckerti* for the first time using molecular markers.

8. I described the phylogenetic relatedness between *Dictyocaulus* species based on the DNA sequences of 5 genes. I separated three main evolutionary lineages within the genus: (1) *D. filaria*, (2) *D. arnfieldi*, and, (3) a group of lungworms living in deer (Cervidae) and ungulates with cloven hooves (Bovinae), which containing the species *D. capreolus*, *D. eckerti*, *D. viviparus*, *D.* sp. n. and '*D.* sp. red deer-New-Zealand'. I showed that *D. capreolus* is positioned most basally within the clade (3).

6. PUBLICATIONS AND PRESENTATIONS ON THE SUBJECT OF THE DISSERTATION

Publication in foreign language

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Publications in Hungarian

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Ács, Z., Sugár, L. (2010). Hazai szarvasfélék tüdőférgeinek genetikai változatossága. Magyar Biológiai Társaság, Állattani Szakosztály, 986. előadóülés, 06. 10. 2010.

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Ács, Z., Sugár, L. (2015). A szarvasfélék nagy tüdőférgeinek (*Dictyocaulus* spp.) genetikai változatossága. Magyar Parazitológusok Társasága 50 éves Jubileumi Emlékülése, 03. 06. 2015.