Genetic diversity and population genetics of large lungworms (*Dictyocaulus*, Nematoda) in wild deer in Hungary

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Abstract

Dictyocaulus nematode worms live as parasites in the lower airways of ungulates, and can cause significant disease in both wild and farmed hosts. This study represents the first population genetic analysis of large lungworms in wildlife. Specifically, we quantify genetic variation in *Dictyocaulus* lungworms from wild deer (red deer, fallow deer, roe deer) in Hungary, based on mitochondrial *cytochrome c oxidase subunit 1* (*cox1*) sequence data, using population genetic and phylogenetic analyses. The studied *Dictyocaulus* taxa display considerable genetic diversity. At least one cryptic species and a new parasite-host relationship are revealed by our molecular study. Population genetic analyses for *D. eckerti* revealed high gene flow among weakly structured spatial populations that utilize the three host deer species considered here. Our results suggest that *D. eckerti* is a widespread generalist parasite in ungulates, with a diverse genetic backround and high evolutionary potential. In contrast, evidence of cryptic genetic structure at regional geographic scales was observed for *D. capreolus,* which infects just one host species, suggesting it is a specialist within the studied area. *D. capreolus* displayed lower genetic diversity overall, with only moderate gene flow compared to the closely related *D. eckerti*. We suggest that the differing vagility and dispersal behavior of hosts are important contributing factors to the population structure of lungworms, and possibly other nematode parasites with single-host life cycles. Our findings are of relevance for the management of lungworms in deer farms and wild deer populations.

Key words: dictyocaulosis, helminth, lungworm, deer, population genetics

Introduction

Dictyocaulus lungworms live as parasites in the lower airways of ruminants. Worm burden (worm abundance per individual host) varies from mild to heavy, and can result in severe host pathology, a condition referred to as 'dictyocaulosis'. For example, *Dictyocaulus viviparus* (Bloch, 1782) causes severe and frequently fatal bronchitis and pneumonia in cattle (termed 'husk'), which is of serious veterinary and agricultural importance due to animal welfare issues, reduced production yields, and costs associated with treatment (David 1997; Ploeger 2002; Kutzer 1988, Wooley 1997). *D. eckerti* Skrjabin, 1931 is the major parasite of importance in farmed deer, and heavy infestations in young hosts can lead to anaemia and death, leading to substantial negative consequences for the farming industry (Mason 1994; Sugár 1997). *Dictyocaulus* species are also believed to result in parasitic bronchitis in a wide variety of wild ruminants (Urquhart et al. 1996).

Dictyocaulus species are classified into the monogeneric Dictyocaulidae family and the Trichostrongyloidea superfamily (but see Höglund et al. 2003; Chilton et al. 2006), and have a direct lifecycle (Kassai 1999). The genus *Dictyocaulus* contains seven species: *D. africanus* Gibbons & Khalil, 1988, *D. arnfieldi* (Cobbold, 1884), *D. cameli* Boev, 1951, *D. capreolus* Gibbons & Höglund, 2002, *D. eckerti*, *D. filaria* (Rudolphi, 1809) and *D. viviparus* (Durette-Desset 1988; Gibbons and Khalil 1988; Gibbons and Höglund 2002). In order to confidently separate among *Dictyocaulus* species molecular methods are necessary, due to difficulties associated with morphological identification (Divina et al. 2000; Höglund et al. 2003). Commonly applied molecular techniques for this purpose are amplification of a specific gene fragment, followed by restriction enzyme digestion or single-nucleotide polymorphism (SNP) analysis, as well as PCR assays of *18S*, *28S* and *ITS* rDNA sequences (Schnieder et al. 1996; Epe et al. 1997; Höglund et al. 1999; Johnson et al. 2004; Höglund et al. 2008; Carreno et al. 2009).

Conventional chemotherapeutic treatments to reduce *Dictyocaulus* infections are costly and must be repeated frequently. Consequently, there is a current research focus on developing vaccines to control *Dictyocaulus* lungworms (McKeand 2000; Strube et al. 2015). Thus, knowledge of genetic variation and population genetic structure in *Dictyocaulus* lungworms is important if we are to develop effective measures

of control. Evidence from studies of diverse parasitic nematodes suggest several patterns of population structure can occur in such species (Blouin et al. 1995, 1999; Gilabert and Wasmuth 2013). Generally, population structure in parasitic nematodes, in terms of genetic diversity and divergence, is influenced by levels of gene flow and so an important factor is often host mobility (Blouin et al. 1995, 1999; Hawdon et al. 2001; Braisher et al. 2004).

Currently, population genetic studies of *Dictyocaulus* nematodes have been restricted to the cattle lungworm (*D. viviparus*) among Swedish farms (Hu et al. 2002; Höglund et al. 2004, 2006, 2008). Cattle lungworms display low levels of gene flow and high population genetic structure compared to other worms in the highly diverse trichostrongylid family, but similar levels to the less diverse, highly structured nematode parasite populations known from plants and insects (Hugall et al. 1994; Blouin et al. 1999; Höglund et al. 2004). The majority of trichostrongilid parasite populations appear to have high genetic diversity and little genetic structuring, suggestive of panmictic populations (Blouin et al. 1995, 1998; Archie and Ezenwa 2011).

Mitochondrial DNA sequences are considered particularly useful for studying interspecific and intraspecific variation because of their high evolutionary rates, predominantly maternal inheritance, and limited recombination (Blouin 1998, 2002; Zhu et al. 2000). Variation in the nematode mitochondrial genome appears to be somewhat higher than in many other animal groups (Blouin 1998). Therefore, mitochondrial markers are a suitable and commonly applied choice to conduct estimates of population genetic structure, recent phylogeny, and gene flow among populations. Furthermore, mitochondrial protein coding genes have higher variation than ribosomal genes for *Dictyocaulus* specifically (Höglund et al. 2006). The mitochondrial *cytochrome c oxidase 1* locus *(cox1*) in particular is frequently used in population genetic studies because it exhibits a relatively high mutation rate, conserved primers are available, and the large amount of data available for other species provides a comparison of genetic variation and population structure. Indeed, the mitochondrial *cox1* gene has been employed in a variety of studies on parasite nematodes (Hawdon et al. 2001; Blouin 2002; Hu et al. 2002, Miranda et al. 2008).

In the present study, we analyze genetic diversity at *cox1* for *Dictyocaulus* lungworms parasitizing wild deer species in Hungary. It is important to extend research in this field, so that general biological

insights regarding the evolution and ecology of *Dictyocaulus* lungworms can be made (Höglund et al. 2003). Our study is the first attempt to analyse the population genetic structure of large lungworms living in wild hosts, and our specific objectives are to: (i) examine broad-scale evolutionary patterns among *Dictyocaulus* species in wild deer, (ii) assess host relationships among the observed species, (iii) identify genetic diversity, differentiation, geneflow and demographic history for recovered *Dictyocaulus* species, and, (iv) determine if patterns are similar to those observed for *D. viviparus* in farmed cattle, which represent the only other *Dictyocaulus* species for which population genetic data are available.

Materials and methods

Sampling of parasites

Adult lungworms were collected from the trachea and bronchi of the following deer species harvested during hunting: fallow deer (*Dama dama*), red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*). Samples were taken from 23 sites in Hungary and one locality in neighbouring Romania (Fig. 1). Collecting sites were separated by distances ranging from 20 to 415 km in Hungary. The locality in Romania was situated in the Eastern Carpathians at Kászon, at a distance from the most eastern locality in Hungary (Mikóháza) of 460 km. Worms were collected during the period 2004-2015. After collection, individual worms were washed with physiological saline to avoid contamination and preserved in absolute alcohol at -20°C. Specimens were randomly selected for subsequent genetic analyses, and a portion of approximately 1 cm was excised from the midbody of each individual for DNA extraction. Consequently, the anterior head and posterior end remained intact for morphological examination. Lungworms were identified to genus level using taxonomic keys (Divina et al. 2000; Gibbons and Khalil 1988; Gibbons and Höglund 2002). To identify dictyocaulids to the species level, DNA sequencing of the internal transcribed spacer 2 (*ITS2*) of the nuclear ribisomal DNA was conducted (following Johnson et al. 2004) for selected samples from each clade (sample id.: D18, D24, D82) and compared to sequences of currently known lungworm species using a nucleotide BLAST search in Genbank (https://blast.ncbi.nlm.nih.gov).

DNA sequences

Total genomic DNA was extracted from each worm specimen using a spin-column based extraction method (DNeasy Tissue Kit, Qiagen, Germany), following the manufacturer's protocol. Each DNA sample was eluted using 200µl of EA buffer, as supplied in the kit, and subsequently stored at -20°C. The *cytochrome c oxidase subunit 1* (*cox1*) gene fragment was amplified using the universal barcoding primers LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). Polymerase chain recation (PCR) conditions were as follows: each 25µl reaction mixture contained 0.5µl of each primer (10µM), 2µl of dNTPs (2mM), 2.5µl of x10 PCR buffer, 0.8-2µl of $MgCl_2$ (1.5mM), 1 unit of Taq polymerase (Fermentas), and varying concentrations of DNA and dH_2O depending on the quality of the DNA extraction. Samples lacking genomic DNA were included in each PCR amplification as negative controls, and no products were detected in these negative controls. Amplification was preceded by 1 cycle of initial denaturation at 94°C for 120s, followed by 6 cycles of 94°C for 30s, 50°C for 90s, 72°C for 60s, and then 36 cycles of 94°C for 30s, 55°C for 90s, 72°C for 60s, with a terminal extension of 72°C for 5 minutes. The yield and quantity of DNA was analysed using ethidium-bromide staining and agarose gel-electrophoresis. PCR products were cleaned using shrimp alkaline phosphatase and *Eschericia coli* exonuclease I (Fermentas) and sequenced directly on an ABI Prism 3730 Genetic Analyser machine using ABI BigDye Terminator Sequencing chemistry. Purified PCR-products were sequenced using the same primers as for the PCR reaction, in both directions to minimise PCR artefacts, ambiguities and base-calling errors. Chromatogram output was checked by eye using Bioedit v.7 (Hall 1999). In a small proportion of cases direct sequencing of *cox1* PCR products revealed multiple fragments, suggesting contamination by host (deer) DNA. In such cases, these results were eliminated from the study. Since *cox1* is a protein coding gene, only specimens for which a single open reading frame (ORF) was identified were included in the analyses. In total, our analyses include 103 new sequences, each derived from a single worm specimen, as well as 9 sequences retrieved from GenBank (accession numbers for *D. viviparus*: JX519460, KM359418, KM359416, KM359417; *D. eckerti* (cf. red

deer): JX519459; *Aelurostrongylus abstrusus*: JX519458; *Metastrongylus pudendotectus*: GQ888714; *Metastrongylus salmi*: GQ888715; *Protostrongylus rufescens*: KF481953). The last four species are related to Dictyocaulidae within the Strongylida order, and their sequences were included as outgroups in the phylogenetic analysis. All sequences generated in this study were deposited in GenBank under accession numbers KT372244-KT372346 (*cox1*) and KT438069-KT438071 (*ITS2*) (Table 1).

Evolutionary relationships

Sequences of *cox1* were aligned using ClustalX version 2.0 (Thompson et al. 1997). To infer the most likely model of sequence evolution for the *cox1* dataset, we used the Akaike and Bayesian Information Criterions (AIC, BIC) as implemented in MODELTEST (Posada and Crandall 1998) and MEGA6 (Tamura et al. 2013). The best fitting model of sequence evolution was the Tamura-Nei model with gamma distributed rate variation and a proportion of invariable sites (TN93+G+I) according to both AIC and BIC. Mitochondrial sequences evolve relatively rapidly (in comparison to many nuclear genes), and this can affect the signal to noise ratio for phylogenetic datasets, which in severe cases can lead to the inference of erroneous relationships among taxa. To investigate this possibility we implemented a test of mutational saturation in the DAMBE5 (Xia 2013) across each codon position for our dataset. To examine the evolutionary relationships among lungworm samples we reconstructed a maximum likelihood (ML) phylogenetic tree using MEGA. Bootstrap clade support was inferred using 1,000 bootstrap replicates.

Population genetic analysis

To infer the population structure of lungworms and examine the processes that have shaped present distributions, several analyses of amplified *cox1* sequences were performed. Genetic diversity values, including polymorphic sites (*S*), *GC* nucleotide content, haplotype number (*H*), haplotype diversity (*Hd*), average number of nucleotid differences within groups (*K*), and nucleotide diversity (π), were calculated

within species and populations using DnaSP version 5 (Librado and Rozas 2009). All estimates were calculated using DnaSP, including those described below.

We measured genetic variation at four levels (individual host, host species, locality, and region) relative to the entire population (for group specification see Table 2), as well as making between species comparisons. Population structure and gene flow were evaluated by analysis of molecular variance (AMOVA). Genetic differentiation between populations of each lungworm species was estimated using F_{ST} (Hudson, Slatkin, Maddison 1992). Nei's G_{ST} was calculated to estimate population differentiation based on differences in allele frequencies (Nei 1973). Estimates of population differentation were based on nucleotide diversity using N_{ST} (Lynch and Crease 1990). Additionally, we also calculated *Nm*, which is the mean per generation estimate of the absolute number of migrants exchanged among populations as inferred from F_{ST}. These analyses test whether the *a priori* populations defined by collecting locality, region and host represent distinct genetic groups.

The population history of *Dictyocaulus* species was also estimated. Tajima's *D* (Tajima 1989) and Fu's F_s, which is based on the haplotype frequency distribution (Fu 1997), were used to identify genetic signals of deviation from neutrality and population decline or expansion. Tajima's *D* is based on the difference between estimates of the number of segregating sites and the average number of pairwise differences. These values were estimated via 10,000 computer simulations based on observed pair-wise differences. Positive values of both parameteres indicate population decline, while negative values suggest population expansion. Fu's F_s test is more sensitive to demographic changes (Ramos and Rozas 2002). Mismatch distribution analyses (examining the distribution of pairwise differences) are frequently used to estimate population history. Such analyses compare the frequency distribution of pairwise differences between haplotypes with that expected under a model of population expansion (Slatkin and Hudson 1991). The multimodal mismatch distribution predicts that the population has a stable size over its history. Sudden demographic expansion leads to a unimodal distribution of pairwise differences. The smoothness of the mismatch distribution was quantified by the raggedness statistic *r* (ranked pairwise differences in the population), as described by Harpending et al. (1993). The time (*t*) to the most recent common ancestor (tMRCA) for our samples was also estimated. This estimates the number of generations since the population

expanded, and was calculated from the peak distribution (τ) using the equation: $t = \tau / 2\mu$ (Li 1977). The parameter μ is the mutation rate per gene per generation, and is obtained by multiplying the mutation rate per site per generation by the number of nucleotides in the studied fragment (657bp in this case). The mutation rate of substitutions per site per generation was estimated using values for the mtDNA of *Caenorhabditis elegans*: $1.57\times10^{-7} \pm 3.1\times10^{-8}$ (Denver et al 2000). Optimally *D. viviparus* requires 3-4 weeks to develop from an egg to a mature adult (Kassai 1999; Johnson et al. 2004), however, environmental and host related factors can delay its lifecycle by an additional 3-4 weeks. The reproductive season for dictyocaulid worms occurs during April to October in Hungary, leading to an estimate of 4 generations per year. The number of generations since population expansion (t) divided by generations per year gives an estimate of time in terms of number of years.

Results

Sequence analyses and evolutionary relationships

A total of 103 *cox1 Dictyocaulus* sequences were amplified. Each sequence originates from a single lungworm specimen. In total, lungworms were collected from 47 individual deer (Table 1). Collection localities are grouped according to region (Fig. 1). All *cox1* sequences were the same length (657 bp), and could be aligned unambiguously. The resultant *cox1* alignment corresponds to positions 69-725 bp of the complete mitochondrial genome sequence of *D. eckerti* cf. red der (GenBank accession number: JX519459 (Gasser et al. 2012)). Based on the invertebrate mitochondrial genetic code, all amplified *cox1* sequences possessed a single ORF without the existence of stop codons. Nucleotide composition was heavily biased towards A and T bases, as is usual for nematode mtDNA (G+C content: 0.299 to 0.337, Table 3) (Blouin et al. 1998). Tests of mutational saturation for the analysed *cox1* fragment, as well as each codon position individually, were negative ($P<0.0001$).

Amplified *Dictyocaulus cox1* sequences grouped into four main clades according to maximum likelihood phylogenetic analysis, revealing that lungworms collected from wild deer belong to three distinct

clades (Fig. 2). Sequence differences between clades were high (Table 4) compared to within group variability (Table 3), suggesting the clades represent separate species: between clade sequence differences exceeded 10%, which is an empirical limit applied to species differentiation for nematodes (Blouin 2002). Additionally, *ITS2* sequences of selected samples from two clades (D24 - KT438070, D82 - KT438071) showed high similarity to *D. eckerti* (96% and 100% nucleotide identity) (GenBank acc. no. U37716, Epe et al. 1997) and *D. capreolus* (GenBank acc no. AF105255, Höglund et al. 1999), identifying the clades as *D. eckerti* and *D. Capreolus* respectively. An *ITS2* sequence for the D18 sample (KT438069) from the additional clade did not show close similarity to any currently known *Dictyocaulus* species, therefore we consider it an unknown, probably undescribed species, and refer to it here as *D*. sp. S-HU (reflecting the collecting region: South Hungary). Additionally, the lungworm sequence collected from a red deer in New Zealand (JX519459) is divergent with respect to the Hungarian *D. eckerti* samples, and the 0.094 mean pairwise sequence difference between the New Zealand sample and Hungarian sequences within the *D. eckerti* clade suggest these sequences may belong to different species.

The structure of our *cox1* tree indicates high genetic differentiation between the 3 *Dictyocaulus* species from wild deer, but little or no differentiation within each species according to locality or geographical region (Fig. 2). *D. eckerti* was the most prevalent lungworm species, and is represented by 79 sequences collected across 20 sampling localities from 5 geographical regions in Hungary (Fig. 1), and 1 locality in the Eastern Carpathians in Romania. Sequences of *D. eckerti* were recovered from all three deer species examined, however, no host structuring was apparent (Fig. 2). Additionally, specimens of *D. eckerti* were predominantly recovered from red deer: 32 red deer produced 68 worms, with only 6 worms recovered from 3 fallow deer, and 5 worms from 1 roe deer. The 17 sequences obtained for *D. capreolus* were sampled from 5 localities and 3 geographical regions. Of the 17 *D. capreolus* worms included here 16 originated from 10 roe deer, while only 1 worm was collected from red deer. The 7 sequences of the *D*. sp. S-HU isolates were collected exclusively from red deer within 3 localities and 2 regions.

Genetic diversity

Interspecific pairwise sequence distances between sequences from separate *Dictyocaulus* clades (12.6-13.8%, Table 4) are one magnitude higher than intraspecific variation (0.8-1.8%) (Table 3), indicating substantial isolation among the 3 species examined. Levels of genetic diversity for *D. eckerti*. *D. capreolus* and *D*. sp. S-HU were determined using the statistics listed in Table 3 (*Hd*, *K*, π). Mean nucleotide differences and nucleotide diversity for *D. eckerti* were ~2 times higher than those for *D. capreolus* and *D*. sp. S-HU (mean nucleotide diversity (π) for *D. eckerti* was 0.018). The genetic diversity of *D. eckerti* within populations was consistent (range: 0.0081-0.0239), and much higher than for *D. capreolus* (overall nucleotide diversity for *D. capreolus*: 0.0086, and that within populations ranged between 0.0027-0.0152). *Hd* values are close to 1 for all examined species, showing a diverse haplotype distribution. The number of haplotypes for *D. eckerti*, *D. capreolus* and *D*. sp. S-HU were 51, 13 and 6 respectively, and there were many polymorphic sites (2.9-14%). Most haplotypes were represented by a single specimen (55 singletons, 79% of lungworms from all 3 species). The most common haplotype of *D. eckerti* (HP5) comprises samples distributed through 4 regions (NW-HU, S-HU, SW-HU and RO).

Genetic structure and gene flow across spatial distribution

Population genetic analyses were conducted for the *Dictyocaulus* species separately at several study levels. Because *D*. sp. S-HU is represented by relatively few samples, we focus our analyses on the datasets of *D. eckerti* and *D. capreolus*. The lungworm species examined showed different population genetic structures. The genetic structure of *D. eckerti* was low, with population estimates of F_{ST}, G_{ST}, N_{ST} consistently within the range 0.034-0.050 at all levels (Table 5). Consequently, gene flow estimator (*Nm*) values were high, indicating high gene flow among *a priori* defined populations. Estimated pairwise F_{ST} between populations of *D. eckerti* defined by collecting region ranged from 0.0037-0.0598 (Table 6). The highest genetic differences were found between N-HU and NW-HU regional populations. Interestingly the geographically distant samples from the Eastern Carpathians did not correspond to the most isolated population, and are incorporated within Hungarian populations (Fig. 2). *D. capreolus* showed moderate genetic structure at the regional scale. The *D. capreolus* dataset did not indicate substantial genetic structure

at either the infrapopulation (host individual) or locality levels. Based on roughly equal pairwise F_{ST} calculations, the 3 *D. capreolus* populations are equally isolated from each other (Table 6). The overall population structure estimator values ranged between 0.133-0.153 (Table 5), and indications of moderate gene flow (*Nm* = 3.27) suggest that *D. capreolus* has intermediate genetic structure. The samples of *D*. sp. S-HU grouped into 2 populations, revealing limited genetic differences, but this result should be regarded cautiously due to small sample size.

Gene flow across host species

Genetic structure was tested for *D. eckerti* samples collected from three host species (fallow deer, red deer, roe deer). Maximum likelihood phylogenetic analyses revealed that samples of *D. eckerti* lungworms grouped into subclusters, which were not correlated with host species (Fig. 2). Haplotypes from different host species were randomly distributed across the *D. eckerti* clusters. In addition there was no evidence for genetic structuring within host species based on F_{ST}, G_{ST} or N_{ST} (Table 5). The low pairwise F_{ST} values between host defined populations (Table 6) and the high rate of overall gene flow (*Nm*=17.34) between host species suggest that *D. eckerti* uses multiple hosts and has well connected populations in Hungary and with the Carpathian population.

Population history

Tajima's *D* neutrality tests showed negative values for all three *Dictyocaulus* species (weakly supported, Table 3). Similarly, Fu's F_s test estimated negative values in all species overall, although none were significant. Neutrality tests for *D. eckerti* indicated strong departures from a mutation-drift equilibrium (Table 3). Deviations from equilibrium can stem from the effects of selection or demographic processes (population size change). The highest deviations from a mutation-drift equilibrium were recorded with Fu's F_s test, which is one the most sensitive tests for detecting demographic changes. Therefore, we assumed a demographic process was the most likely explanation for these results and proceeded to estimate the

magnitude of historical population size change. Negative values for Tajima's *D* and Fu's F_s might suggest a population-wide demographic change or a recent range expansion for *D. capreolus* also. As expected under population expansion, the mismatch distributions for both species had an unimodal shape (Fig. 3a-b). The low raggedness value was also a sign of an expanded population. Also, *r* indices were low for both species (r=0.0044 for *D. eckerti, r*=0.0147 for *D. capreolus*).

Mismatch analyses were carried out for each species to date potential population expansion events. A tMRCA analysis was performed for all the samples in the case of *D. eckerti*, since there was no or only weak genetic evidence for differentiated populations. The peak of the unimodal distribution (τ) was 9.529, corresponding to a population expansion approximately 46,000 generations ago, which equals ~11,500 (9,600-14,400 CI) years ago, placing the event at the end of the last ice age, assuming 4 generations per year. These calculations assume that the *D. eckerti* population is at equilibrium. Based on the unimodal mismatch distribution, a similar analysis was performed for all samples of *D. capreolus* collectively, as well as separately for two populations. For all samples collectively, the estimate of τ was 3.669, suggesting an expansion estimate ~4,500 years ago (3,700-5,500 CI). Because of evidence of moderate genetic structure for *D. capreolus* and the observation that eastern (E) and southwestern (SW) populations differ in genetic variability by about 5 times (Table 2), separate mismatch analyses were also carried out. The eastern population showed negative Tajima's *D* and F_s values: -0.368 and -1.579 respectively (P> 0.10), and low raggedness (0.0363) indicating an expanded population. The unimodal mismatch distribution for the eastern population (Fig. 3c) also indicates an expanded population. According to tMRCA analyses, the onset of the eastern population expansion was approximately 7,000 (5,900–8,800 CI) years ago (τ=5.827). However, the SW population of *D. capreolus* exhibited a low negative Tajima's *D* (-0.4410, P> 0.10) and low positive Fu's F_s (0.469, P> 0.10) with moderate raggedness (0.23), suggesting a roughly constant population size. The mismatch distribution of the SW population of *D. capreolus* (Fig. 3d), shows a multimodal distribution under a constant model, indicating occasional bottlenecks in population history.

Discussion

In addition to their evolutionary and ecological relevance, information regarding the genetic variability of *Dictyocaulus* lungworms is of direct applied interest given their status as important parasites of farmed and wild animals. Among the macroparasites of deer (Cervidae), lungworms are believed to be pathogenic in farmed or fenced circumstances within temperate regions (Mason 1994, Sugár 1997). Prior to this study, population genetic analysis of dictyocaulid lungworms was restricted to the cattle lungworm, *D. viviparus* in Sweden (Hu et al. 2002; Höglund et al. 2004, 2006, 2008). Studies on *D. viviparus* genetic diversity and gene flow among cattle farms have revealed a signature of strong population genetic structure, possibly influenced by human activities. Our survey aimed to reveal the population genetic structure of *Dictyocaulus* lungworms in natural wildlife, focussing on host deer species, at small and medium geographic scales.

Phylogenetic analyses reveal that *Dictyocaulus* sequences group into 3 strongly supported clades (100% bootstrap support). Given patterns of sequence divergence within (<2%) and between clades (>13%), our results strongly suggest that these 3 clades correspond to separate lungworm species in Hungary. While support for our clades of interest is strong, values among major clades are poor, limiting our ability to elucidate evolutionary relationships among them. A previous phylogenetic analysis of European *Dictyocaulus* species using rDNA *ITS2* recovered a different pattern to that observed in our results, with *D. capreolus* more basal, although support among clades was similarly low (Höglund et al. 2003). We identify an undescribed species in our analysis, referred to here as *D.* sp. S-HU. Interestingly, Höglund et al. (2003) also noted an undescribed species in their phylogenetic study of European *Dictyocaulus*. However, the undescribed species was recovered from fallow deer, whereas *D.* sp. S-HU was collected from red deer here. Thus, it is clear that there is currently at least one undescribed species of *Dictyocaulus* present in European deer. In the future, efforts should be made to compare these lineages, to examine if they represent the same or different cryptic species, with formal descriptions following. Additionally, further sampling of New Zealand lungworms (and additional European sampling), would be of interest to determine the origin and identity of these worms.

Regarding host relationships, *D. eckerti* is a frequent parasite in wild and semi-domesticated hosts, and is recorded from several host species: fallow deer, hog deer (*Axis porcinus*), Indian muntjac (*Muntiacus*

muntjak), moose (*Alces alces*), musk ox (*Ovibos moschatus*), red deer, reindeer (*Rangifer tarandus*), sika deer (*Cervus nippon*), wapiti (*Cervus canadensis*) (Epe et al. 1997; Gibbons and Khalil 1988; Höglund et al. 2003). However, it is unclear if this is indeed the correct host range, due to the problem of cryptic species, since only very limited consideration using molecular markers has been undertaken. Höglund et al. (2003) found that *D. eckerti* from red deer, moose, reindeer, and musk ox group together in their molecular phylogenetic study, suggesting that it is a truly generalist species (at least among these hosts). We find that red deer is the primary host for *D. eckerti* within the region that we sampled. Although *D. eckerti* samples were recovered from all three deer species considered here, the vast majority of worms originated from red deer. However, to some extent this may reflect sampling bias, since 70% of the deer sampled in this study were red deer. All of the six lungworms collected from fallow deer were identified as *D. eckerti*. The prevalence and abundance of dictyocaulid worms in fallow deer are very low in comparison to values in red deer (unpublished results). The distribution of fallow deer, which is patchy, may provide only a secondary host for the parasite, but more sampling is required to confirm this, and to ascertain the focal host of *D. eckerti* and if this varies across its large range.

In contrast to the findings for *D. eckerti*, only 1/17 *D. capreolus* worms originated from red deer, with the rest sampled from roe deer. Thus, our data suggest that *D. capreolus* is a roe deer specialist in Hungary, although in Sweden it was also recovered from moose suggesting a complex pattern of host associations (Höglund et al. 2003). To our knowledge, the occurence of *D. capreolus* in red deer (id. number: D22, Table 1), is a new host-parasite record. In addition, we believe this is the first time that *D. eckerti* has been recorded from roe deer confirmed by molecular analysis. As mentioned above, *D*. sp. S-HU isolates were collected exclusively from red deer. Thus, despite previous suggestions that *Dictyocaulus* species have a broad host spectrum (Eckert et al. 1992; Kassai 1999; Sprehn 1932), it is now clear from studies using molecular identification methods (including this one), that lungworm species generally infect more limited sets of hosts (Divina et al. 2002; Höglund et al. 2003).

Additional detailed genetic data host-parasite data are required to clarify the extent to which all lungworm species use focal hosts across their range. Additionally, we demonstrate that *D. eckerti* and *D*. S-HU. share similar ecological habitats, and the same host species (red deer). We could not identify any

ecological factors underlying genetic differentiation between *D. eckerti* and *D.* S-HU and hence an interesting question is what factors exist to promote reproductive isolation between them. We did not investigate the closely related cattle lungworm, *D. viviparus* here, but studies report that it is widespread in Hungary (Kassai and Holló 1962). As wild deer and cattle use often the same grazing sites, there could in theory be a high likelihood of cross-infection between deer and cattle lungworms. However, we did not observe any *D. viviparus* lungworms in deer. Earlier reports that deer species host *D. viviparus* in Hungary (Kutzer et al. 1987; Sugár 1990, 1994), may originate from erroneous identification based on morphology alone (Divina et al. 2000).

Levels of nucleotide diversity for *D. eckerti* samples are on a par with mtDNA diversity reported in various parasites of vertebrate hosts (~2% nucleotide diversity for mtDNA) (Blouin et al. 1995, 1999; Blouin 2002; Brashier et al. 2004). *D. capreolus* samples showed lower genetic diversity than that for *D. eckerti* samples. Studies of *D. viviparus* in Sweden have indicated that mitochondrial sequences show moderate genetic diversity (Höglund et al. 2006; Hu et al. 2002). Our study indicates high nucleotide variation for wild lungworm species, with haplotype diversity approaching 1. It is striking that 70 haplotypes, belonging to 3 species, were identified from 103 lungworm specimens in Hungary. In comparison, for cattle lungworms in Sweden, 12 haplotypes from 252 cattle lungworm specimens were found (Hu et al. 2002). The *D. eckerti* populations included here are variable, but there were no clear differences between populations according to haplotype distributions. Our analyses detected higher levels of nucleotide variation in the *cox1* gene of lungworms from wild host species than was found in Sweden for cattle lungworms (Höglund et al. 2006). This comparison is not altogether straigthforward since the gene regions utilised in these studies are from two neighbouring fragments, as in this study we examined the 5' end of *cox1*, while in the cattle lungworm study the 3' end of *cox1* was analysed. However, the mutation rate is only somewhat higher at the 5'-end of *cox1*, than at the 3' end in dictyocaulid lungworms (Gasser et al. 2012). Therefore, there appears to be considerably higher nucleotide diversity in *Dictyocaulus* lungworms from wild deer hosts than there is in those from farmed cattle hosts.

The estimated distributions of lungworm species examined in this study are larger than the sampling area. While *D. capreolus* are recorded only from Europe (Spain: Carreno et al. 2009; Sweden: Divina et al.

2002; France: Durette-Desset et al. 1988) and Asia Minor (Turkey: Umur et al. 2012), *D. eckerti* is widely distributed in temperate regions worldwide, such as North America (Höglund et al. 2003), Europe (Epe et al. 1997), Siberia (Skrjabin et al. 1954) and New Zealand (Mason 1994; Gasser et al. 2012). Spatial structuring is evident where all populations of a species are not completely panmictic. The *Dictyocaulus* species considered here show three distinct population genetic classes across the examined range. First, *D. eckerti* has high host vagility, and shows low population differentiation, and consequently high *Nm* values. The high *Nm* values indicate that populations of *D. eckerti* show strong genetic connectivity. Second, *D. capreolus* in host populations with moderate vagility show moderate population structure, close to the critical F_{ST}=0.2 value (Allendorf 1983). Genetic structure in *D. capreolus* appears distance-dependent, which may be a consequence of limited dispersal behaviour of its roe deer hosts. Third, F_{ST} of *D. viviparus* in hosts with very low vagility (i.e. cattle in farms), shows high population genetic structure far above the critical F_{ST} value. It is likely that *D. viviparus* has very low gene flow, as the cattle hosts of *D. viviparus* are isolated by farms. Wild deer are not suitable hosts for *D.viviparus* (Höglund et al. 1999; Gasser et al. 2012). Thus, gene flow in *D. viviparus* populations is highly limited. Our results regarding the genetic structure of *D. capreolus* are similar to findings from a population genetic analysis of a different parasitic nematode of wild deer. Specifically, the white-tailed deer (*Odocoileus virginianus*) nematode parasite *Mazamastrongylus odocoilei* in North America, shows high genetic diversity and moderate genetic structure (N_{ST}=0.12 and 0.31) (Blouin et al. 1995). However, it should be noted that *M. odocoilei* was studied using mitochondrial DNA sequences of the *ND4* region, which is more variable than the *cox1* locus, and also that the sites examined in America were situated at larger distances than those in our study.

Several studies have reported that the most important factor to impact on parasite population structure is the vagility of hosts (Levin et al. 2013; McCoy et al. 2003). This may be especially true for trichostrongylid parasites, for which the infective larvae lack means of long distance dispersal (Blouin et al. 1995). There are considerable differences in the dispersal patterns of the examined hosts. Fallow deer and red deer, which host *D. eckerti*, can migrate large distances, while roe deer migrate less and are considered to be a territorial species (Kropil et al. 2015; Cagnacci et al. 2011). Roe deer usually disperse individually (bucks) or in small goups (doe with fawn/s) during spring to autumn when lungworm infection is most

likely. However, roe deer have two ecotypes in Hungary: forest-based roe deer live in groups of 4-8 animals (SW-HU roe population), while field-based roe deer live in larger groups of dozens or even hundreds of individuals (E-HU roe population) during autumn to spring. The field-based bucks leave mixed sex groups in March, but females stay with the group until the second half of May, and fawns stay together for some additional weeks. We assume that cross infection is more probable among group members (red deer and field-based roe deer) than it is among dispersed forest-based roe deer individuals. This hypothesis corresponds to observed levels of infection by *Dictyocaulus* in red and roe deer. The prevalence of infection was 8.3%, 13.0% and 46.6% for forest-based roe deer, field-based roe deer, and red deer respectively (Sugár 1994, 1997). Prevalence is highest in the youngest age group of roe (33.3%), and red deer (75%) (Sugár 1997). Therefore, dispersal behavior of hosts may be the best explanation for the different population genetic structures observed among *Dictyocaulus* species.

The differing levels of gene flow observed in lungworms have consequences for population dynamics and evolutionary potential (Barrett et al. 2008). Parasites such as *D. eckerti* with high gene flow between host species probably switch hosts often and may not experience such extreme population bottlenecks compared to worms restricted to a single host species. *D. capreolus* is reported to utilise an additional host species to roe deer in Sweden, the moose (Gibbons and Höglund 2002), which has a different dispersal behaviour. Consequently, one expectation is that populations of *D. capreolus* in Sweden may show lower genetic structure than those examined here in Hungary, particularly as the moose is a long distance disperser (Sweanor and Sandegren 1989) and it would be interesting to test this prediction.

Our results suggest that *D. eckerti* has not experienced a severe recent population bottleneck, and that there was a population expansion ~11,500 years ago (although these results should be interpreted with caution, e.g. see Morrison and Höglund 2005). Our 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, since climate warming began approximately 15,000 years ago (Denton et al. 2010). Further, archeological and genetic data indicate that red deer and other wild ungulate hosts in Europe experienced population expansions approximately 10,000 years ago (Sommer et al. 2008). Red deer have three genetically differentiated

70 populations in Europe: eastern, western and mediterranean (Skog et al. 2009). Our sampling was performed
71 on the eastern population of red deer, which arose from the Balkan glacial refugium. Sampling of *D. eckerti*
72 across a larger spatial scale, including western and mediterreanean populations may indicate greater genetic
73 structure, following the main host's genetic structure. In future studies, it would be interesting to examine if
74 lungworm genetic structure reflects that of its red deer hosts at larger scales across Europe, and to what
75 extent worms arising from different refugial populations have spread across distinct European host
76 populations.

77 The high gene flow observed for *D. eckerti* in this study likely reflects a parasite population that
78 extends over a larger spatial scale than our study area. When a population expands it is expected to gain rare
79 alleles, which we observe here for *D. eckerti*. The predicted large distribution, high genetic diversity, and
80 high gene flow for *D. eckerti* have important evolutionary consequences and offer the potential for new
81 mutations to spread rapidly. The majority of red deer in Hungary are infected by lungworms during their
82 first year (Sugár 1990, 1997), while only low prevalence in roe deer was recorded (Sugár 1997), with the
83 mean intensity of lungworm infection per individual higher in red deer than roe deer (Sugár, *unpublished*
84 *data*). Taking into account lungworm distribution, host range, host vagility, prevalency and intensity, *D.*
85 *capreolus* is likely to have a much smaller overall population size than *D. eckerti*. The high population size
86 of *D. eckerti* could maintain high genetic diversity, and an ability to respond quickly to forces of selection,
87 and the impact of genetic drift should be negligible compared to that of natural selection. These predictions
88 have considerable implications for lungworm management, particularly since high gene flow enhances the
89 efficient evolution of resistance to treatment methods.
90

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94

95 **Ethical Standards**

96 The study was performed in compliance with current national laws and regulations.

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Conflict of Interests

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 Map of collecting sites of *Dictyocaulus* in Hungary. Host species are indicated using different symbols (triangle: fallow deer, square: red deer, circle: roe deer) as are lungworm species (filled symbol: *D. eckerti*, empty symbol: *D. capreolus*, leaky symbol: *D.* sp. S-HU).

Fig. 2 Maximum likelihood phylogenetic tree, constructed using the mitochondrial *cox1* gene for 103 *Dictyocaulus* lungworms originating from Hungary and 5 lungworms from GenBank indicated by their accession numbers (one dictyocaulid worm of red deer in New Zealand and 4 sequences of *D. viviparus*). Lungworms were collected from hunted deer (fallow, red and roe deer), indicated by triangle, square and circle symbols respectively. Geographical collecting regions are indicated for each sample.

Fig. 3 Observed and simulated (expected) mismatch frequency distributions under a model of population expansion for (a) *D. eckerti* overall, (b) *D. capreolus* overall, (c) the eastern population of *D. capreolus,* and under model of constant population size for (d) the western population of *D. capreolus*.