Identification of *Dictyocaulus* spp. in ruminants by morphological and molecular analyses

B. P. DIVINA[1,2], E. WILHELMSSON[1], J. G. MATTSSON[1], P. WALLER[1] *and* J. HÖGLUND[1]*

[1] *Department of Parasitology (SWEPAR), National Veterinary Institute and Swedish University of Agricultural Sciences, 751 89 Uppsala, Sweden*
[2] *College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, 4031 Philippines*

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SUMMARY

Lungworms of the genus *Dictyocaulus* from cattle, roe deer, and moose in Sweden were subjected to morphological and molecular analyses. The objectives of the study were to investigate whether mixed or monospecific *Dictyocaulus* infections occur in Swedish cattle and whether wild cervids may act as reservoirs. The morphological characters examined were thickness and shape of the buccal capsule wall (BCW) and total spicular length (TSL). Morphometry was also done on the total body length, and BCW thickness and length. In the molecular identification, we used a PCR-linked hybridization assay to probe worm DNA with species-specific oligonucleotide probes to the second internal transcribed spacer (ITS2). The results showed that the BCW shape was the most reliable morphological character for identification. Significant differences were observed in this character, but an overlap occurred between lungworms from each of the host species. With the hybridization assay, all lungworms from cattle were identified as *D. viviparus*, whereas those from roe deer represented a novel *Dictyocaulus* species demonstrating that each host had a monospecific lungworm infection. In moose, 61 (78·2 %) worms belonged to the new species and 17 (21·8 %) were *D. eckerti*. This study shows the usefulness of hybridization assay as an epidemiological tool for the specific identification of lungworms of cattle and wild cervids.

Key words: *Dictyocaulus*, morphology, hybridization, ITS2, ruminants, Sweden.

INTRODUCTION

According to the latest taxonomic revision (Gibbons & Khalil, 1988), the genus *Dictyocaulus* contains 6 species. Cattle are the primary hosts of *D. viviparus* while cervids play this role for *D. eckerti*. Although it is possible to distinguish the bovine lungworm from that of cervids by morphological characters, such as shape of the oral opening, thickness of the buccal capsule wall (buccal ring), and longitudinal ridges, these differences are small. Other authors emphasized that the shape (Jansen & Borgsteede, 1990; Bienioschek, Rehbein & Ribbeck, 1996) and thickness (Jansen & Borgsteede, 1990) of the buccal capsule wall are features that allow the differentiation of *D. viviparus* from *D. eckerti*. In addition to these two characters, Durette-Desset, Hugonnet & Chabaud (1988) included the height of the buccal ring in their redescriptions of *D. viviparus* and *D. noerneri*. The latter species, from roe deer, was considered a *species inquirenda* by Jansen & Borgsteede (1990).

There are reports that cattle under experimental conditions are susceptible to a range of *Dictyocaulus* spp., namely *D. eckerti* (Bienioschek *et al*. 1996), the ovine *D. filaria* (Enigk & Hildebrandt, 1965), and red deer-derived *Dictyocaulus* (Corrigall *et al*. 1988). The possibility of cross-transmission of *Dictyocaulus* spp. between cattle and wild cervids is of practical importance, particularly in Sweden. Current estimates on the number of roe deer (*Capreolus capreolus*) and moose (*Alces alces*) are in the order of 1 million and 250000, respectively (Swedish Hunters' Association, personal communication). These cervids co-graze in cattle-raising areas of the central and southern parts of Sweden (Cederlund & Markgren, 1987). The increase in the cervid populations during the past few decades has coincided with increasing frequency of reports of clinical lungworm disease in cattle. Consequently, it is important to elucidate whether roe deer and moose may serve as reservoirs of infection for this parasite, as this has been suggested previously (Nilsson, 1971).

Given the limited morphological differences, especially between lungworms in cattle and cervids, and the apparent existence of variation in host-specificity among lungworms as shown by results of cross-transmission experiments (Enigk & Hildebrandt, 1965; Corrigall *et al*. 1988; Bienioschek *et al*. 1996) and reports of natural infections (Enigk & Hildebrandt, 1965; Nilsson, 1971; Corrigall *et al*. 1982; Rehbein & Haupt, 1984; Mason, 1985; Pybus,

* Corresponding author: Department of Parasitology (SWEPAR), National Veterinary Institute and Swedish University of Agricultural Sciences, 751 89 Uppsala, Sweden. Tel: +46 (0)18 674156. Fax: +46 (0)18 67 309162. E-mail: johan.hoglund@sva.se

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1990), various molecular approaches have been used for the characterization of species within the genus. Marked genetic differences even between the closely similar *D. viviparus* and *D. eckerti* were reported in Germany (Epe *et al.* 1995, 1996; Schnieder, Epe & Samson-Himmelstjerna, 1996; Samson-Himmeltsjerna *et al.* 1997; Epe, Samson-Himmeltsjerna & Schnieder, 1997). More recently, we showed that *Dictyocaulus* isolates from roe deer and moose in Sweden differed genetically from *D. viviparus* in Swedish cattle, as well as from *D. eckerti* in fallow deer (*Cervus dama*) and *D. filaria* in sheep of German origin. This was based on substantial differences in the nucleotide sequences of the second internal transcribed spacer (ITS2). The results suggested that wild cervids in Sweden are parasitized by a novel species of *Dictyocaulus*. However, these observations were only based on 3 worms from each host (Höglund *et al.* 1999).

The aims of the present study were to determine whether mixed or monospecific *Dictyocaulus* infections occur in Swedish cattle, and whether wild cervids act as reservoir hosts. We used morphological examination in combination with a genetic approach to identify individual worms.

MATERIALS AND METHODS

Collection of lungworms

Lungs from cattle from various abattoirs in Sweden and of cervids sent by local hunters were examined for the presence of worms upon arrival at the laboratory. The bronchi were dissected with scissors, and each worm was carefully recovered using flat-tipped forceps and placed in a Petri dish with 40 mM KCl solution.

The Petri dish containing the worms was placed under a stereomicroscope and all intact worms were collected. Each worm was straightened over a clean paraffin sheet, the total body length (TBL) measured, and then the anterior and posterior ends were cut off with a scalpel. The anterior and posterior ends of each worm were fixed together in a microwell with 10% formalin solution. The remainder of the worm was placed in a labelled microcentrifuge tube and stored at −75 °C. Thus, it was possible to subsequently relate the morphological and molecular findings. The entire microwell plate was covered with adhesive plastic film to prevent evaporation, and stored at room temperature.

Morphological examination

The formalin-preserved anterior and posterior ends of each worm were mounted on microscope slides with lactophenol as a mounting and clearing medium, and covered with a cover-slip. The slides were identified with randomly chosen numbers in order to examine the worms without knowledge of their origin. Because *D. viviparus* and *D. eckerti* can be distinguished more by the shape and thickness of their buccal capsule wall (BCW) (Jansen & Borgsteede, 1990; Bienioschek *et al.* 1996), morphological identification was based mainly on those features. Using the illustrations of Jansen & Borgsteede (1990) as a guide, the BCW was categorized as 'thin' (as in *D. viviparus*) or 'thick' (as in *D. eckerti*), with a third category of 'intermediate' for worms whose BCW were intermediate between the 'thin' and 'thick' categories. Based on the BCW shape, those with triangular shape were identified as *D. viviparus* while those with kidney- or bean-shaped BCW were considered to be *D. eckerti* (Jansen & Borgsteede, 1990; Bienioschek *et al.* 1996).

The specimens were photographed using an Olympus DP 10® (Olympus Optical Co.) digital camera attached to an Olympus BH-2 compound microscope and a computer. The images obtained were in tagged image file (TIF) format. Image analysis software (Olympus DP-Soft® ver. 3.0 for Windows®, Soft Imaging Systems) was then used to obtain measurements of the BCW thickness (widest portion of wall), BCW length and total spicular length (TSL). The software converts a pixel count covered by a line drawn on the image into its micrometer (μm) equivalent based on previous calibration.

Extraction and purification of total DNA

Total DNA was extracted and purified from a thawed portion of each worm by using QIAmp Tissue Kit (QIAGEN). Each DNA sample was eluted into 400 μl of buffer (Buffer AE) and stored at −20 °C.

PCR amplification of the lungworm ITS2

The second internal transcribed spacer (ITS2) of each DNA sample was amplified by polymerase chain reaction (PCR) (Saiki *et al.* 1985). The reaction mixture and thermocycler settings were identical to those described previously (Höglund *et al.* 1999). Aliquots (5 μl) of PCR products were analysed by 1·5% agarose gel electrophoresis, the products stained with ethidium bromide and detected using a UV light. The ITS2 samples, contained in their original PCR tubes, were placed in small self-sealing plastic bags and stored at −20 °C until further use.

Molecular probing of amplified ITS2

Samples were thawed at room temperature, denatured at 95 °C for 10 min and then chilled immediately. One μl of each undiluted and diluted (1:5) ITS2 sample was blotted on to a nylon membrane (Roche). Three membrane blots were

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made for samples from each worm. The DNA was subjected to ultraviolet cross-linking (UV Stratalinker™ 1800), and the membranes were probed separately with digoxigenin-end-labelled oligonucleotide probes (OP): OP108 (5′-GAA GAC GAT ATA AGG CAG-3′), OP109 (5′-TAG CAG TAC ACA TAC ATA-3′), and OP110 (5′-TAA GAA CGG CGG TAA TAT-3′) for *D. viviparus*, *D. eckerti*, and *Dictyocaulus* sp., respectively. The probes were based on species-specific ITS2 sequences of large lungworms in Sweden and Germany (Höglund *et al.* 1999). The specificity of probes was tested using previously sequenced samples.

Pre-hybridization was performed at 40 °C in a DIG Easy Hyb Solution (Roche). After 30 min, the pre-hybridization solution was replaced with a fresh solution that included one of the probes (10 pmol/ml solution) and the hybridization was continued for 90 min at 40 °C. The membranes were washed separately under agitation at 40 °C twice each with 2× SSC (5 min per wash) and 0·5× SSC (15 min per wash), both solutions containing 0·1% SDS. Using a fresh container, each membrane was incubated with 1× blocking solution on a shaking platform for 30 min at room temperature. The blocking solution was decanted and the membranes were incubated for 30 min with a fresh blocking solution containing anti-digoxigenin-alkaline phosphatase (Roche) at a 1:10000 dilution. After conjugated incubation, each membrane was washed twice (15 min per wash) with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7·5) containing 0·3% Tween 20 (Merck). Hybridization signals were detected by chemiluminescence using CSPD® (Roche) as substrate. After exposure to Hyperfilm ECL® (Amersham Pharmacia Biotech) for 20 min, the films were developed according to the manufacturer's protocol.

Cloning of ITS2

One of the *D. eckerti* probe-positive samples was randomly selected and the longest ITS2 fragment band (about 580 bp) was extracted from the gel using a Geneclean® kit (Bio 101) and cloned into the pGEM®-T vector (Promega). One recombinant plasmid was purified from cultured cells with Wizard® Plus Midipreps DNA Purification System (Promega) and sequenced using an ABI Prism (model 377) automatic sequencer. The DNA sequence analysis was conducted using the Basic Local Alignment Tool (BLAST).

Statistical analysis

Differences in the various thickness categories and shapes of the BCW among the hosts were determined using the χ^2-test. Pearson correlation analysis was also done between worm TBL, and the BCW thickness and length. The effects of host, sex of worm, and TBL on the two BCW variables were determined by using the General Linear Model (GLM) procedure in the Statistical Analysis System (SAS Institute, Inc., 1989). Least square means were compared by using the probability of difference option in SAS. All tests were considered significant at the $P < 0{\cdot}05$ level.

RESULTS

Morphological examination

Buccal capsule wall (BCW) thickness category. A total of 273 lungworms from 20 cattle, 8 roe deer, and 10 moose were examined microscopically to determine the thickness of BCW (Fig. 1). Out of these worms, 122 (44·6%), 89 (32·6%), and 62 (22·7%) had thin, intermediate, and thick BCW, respectively (Table 1). On a host basis, 104 out of 146 (71·2%) worms from cattle were in the thin BCW category, 66·0% of 50 worms from roe deer had thick BCW, and in moose, the majority (55·8%) of worm BCW was considered as intermediate. Significant differences in the proportions of the 3 BCW categories within and among the hosts were observed ($P < 0{\cdot}001$).

Shape of BCW. Based on the shape of the BCW, 91·4% of examined worms from cattle had triangular (see Fig. 1: thin category) BCW as described for *D. viviparus*. Only 1·4% of the worms was observed to have a kidney- or bean-shaped (see Fig. 1: thick category) BCW while the rest of the worms (7·1%) had indistinct BCW shape. In roe deer, 90·7% of the worms had the *D. eckerti*-like appearance, but also 9·3% could not be determined. No worm with *D. viviparus*-like BCW was found in roe deer. With regard to the worms from moose, a lower percentage (86·6%) of specimens was regarded as having *D. eckerti*-like BCW, with 7·5% and 6·0% of worms categorized as being *D. viviparus*-like and non-distinct BCW shape, respectively. Significant differences in the frequency of worms were obtained based on shapes in each host ($P < 0{\cdot}001$). In addition, each shape classification among the hosts was significantly different, except between roe deer and moose where they had statistically similar percentages (90·7% *vs.* 86·6%, respectively) of worms with kidney-shaped BCW.

Gross and digital measurement. The number of worms measured from each host species as shown in Table 2 is less than that presented in Table 1, because bad orientation hindered examination of some specimens and some were not included due to incomplete data. As with other parasitic nematode parasites, male lungworms were significantly ($P < 0{\cdot}001$) shorter than females regardless of host. Female worms from roe deer were significantly longer ($P < 0{\cdot}001$) than their counterparts in the

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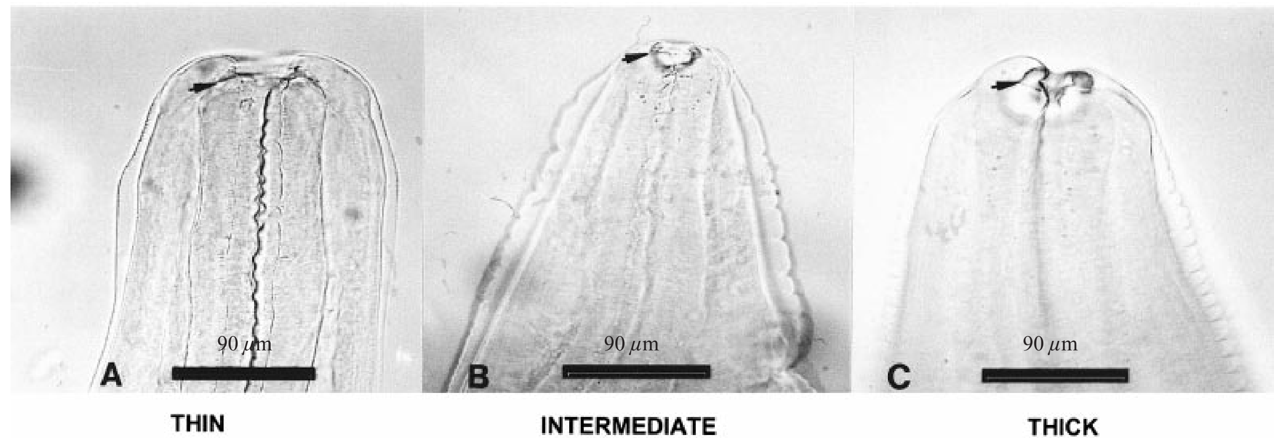

Fig. 1. Digital photomicrographs of anterior ends of *Dictyocaulus* spp. with thin, intermediate, and thick buccal capsule walls (arrows) from cattle (A), moose (B) and roe deer (C), respectively.

Table 1. Distribution of *Dictyocaulus* spp. from Swedish cattle, roe deer, and moose according to thickness of the buccal capsule wall (BCW)

Host	No. of animals	Total no. of worms examined	BCW thickness category* (%)		
			Thin	Intermediate	Thick
Cattle	20	146	104 (71·2)	32 (21·9)	10 (6·8)
Roe deer	8	50	3 (6·0)	14 (28·0)	33 (66·0)
Moose	10	77	15 (19·5)	43 (55·8)	19 (24·7)
Total	38	273	122 (44·6)	89 (32·6)	62 (22·7)

* As judged from the illustrations of Jansen & Borgsteede (1990), with 'Thin' category corresponding to the figure for *D. viviparus* and 'Thick' for *D. eckerti*. 'Intermediate' is an arbitrary category that represents BCW thickness in between them.

Table 2. Least square means of total body length (TBL) and buccal capsule wall (BCW) measurements of *Dictyocaulus* spp. from Swedish cattle, roe deer, and moose according to the sex of the worms

Character/statistics	Host and sex of worm					
	Cattle		Roe deer		Moose	
	Female	Male	Female	Male	Female	Male
TBL(cm)						
Range	1·4–7·6	1·4–5·5	3·3–8·1	2·8–6·2	2·5–8·2	1·8–6·3
Mean ± S.E.M.	4·8 ± 0·1	3·8 ± 0·2	6·0 ± 0·3	4·5 ± 0·3	4·2 ± 0·2	3·7 ± 0·3
n	89	37	27	21	41	26
Pooled*	$4·3^{a} \pm 0·1$		$5·3^{b} \pm 0·2$		$3·9^{a} \pm 0·2$	
n	126		48		67	
BCW thickness (μm)						
Range	2·0–16·6	3·9–15·8	8·1–14·9	8·0–12·6	4·9–11·6	5·3–11·9
Mean ± S.E.M.	8·8 ± 0·2	8·1 ± 0·4	11·6 ± 0·6	10·2 ± 0·5	8·4 ± 0·4	8·2 ± 0·5
n	77	35	23	19	33	18
Pooled*	$8·5^{a} \pm 0·2$		$10·5^{b} \pm 0·3$		$9·0^{a} \pm 0·3$	
n	112		42		51	
BCW length (μm)						
Range	6·8–30·9	9·9–20·9	14·5–23·4	13·2–23·8	9·1–19·8	8·9–18·8
Mean ± S.E.M.	16·1 ± 0·4	16·2 ± 0·6	18·8 ± 0·9	17·2 ± 0·7	15·7 ± 0·6	15·1 ± 0·8
n	77	35	23	19	33	18
Pooled*	$16·1^{a} \pm 0·3$		$17·8^{b} \pm 0·6$		$15·4^{a} \pm 0·5$	
n	112		42		51	

* Mean ± S.E.M. (standard error of the mean). In a row, means with different letters did not differ significantly ($P < 0·05$) based on the GLM.
n, Number of worms.

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Table 3. Simplified formulae for predicting the BCW thickness and length of *Dictyocaulus* spp. based on the SAS General Linear Model procedure

Host	Sex of worm	BCW thickness*	BCW length*
Cattle	Female	$y = 4{\cdot}1 + 0{\cdot}7(t)$	$y = 9{\cdot}6 + 0{\cdot}5(t)$
	Male	$y = 4{\cdot}4 + 0{\cdot}7(t)$	$y = 9{\cdot}2 + 0{\cdot}5(t)$
Roe deer	Female	$y = 7{\cdot}0 + 0{\cdot}7(t)$	$y = 15{\cdot}8 + 0{\cdot}5(t)$
	Male	$y = 7{\cdot}3 + 0{\cdot}7(t)$	$y = 15{\cdot}4 + 0{\cdot}5(t)$
Moose	Female	$y = 6{\cdot}1 + 0{\cdot}7(t)$	$y = 10{\cdot}92 + 0{\cdot}5(t)$
	Male	$y = 6{\cdot}4 + 0{\cdot}7(t)$	$y = 10{\cdot}5 + 0{\cdot}5(t)$

* t, Total body length of worm in cm.

Table 4. Total spicular lengths of *Dictyocaulus* spp. from Swedish cattle, roe deer, and moose measured with the use of an image analysis software, Olympus DP-Soft®

			Spicule	
Host	No. of worms	No. of spicules measured*	Mean (range) length (μm)	C.I. (μm)†
Cattle	36	64	209·7[a] (100·1–286·9)	±15·4
Roe deer	24	33	225·1[a] (127·1–293·7)	±20·6
Moose	20	33	214·2[a] (125·0–301·0)	±20·2

* In some specimens, only 1 spicule was possible to measure.
† 95% confidence interval.
In a column, values with the same letter did not differ significantly ($P > 0{\cdot}05$).

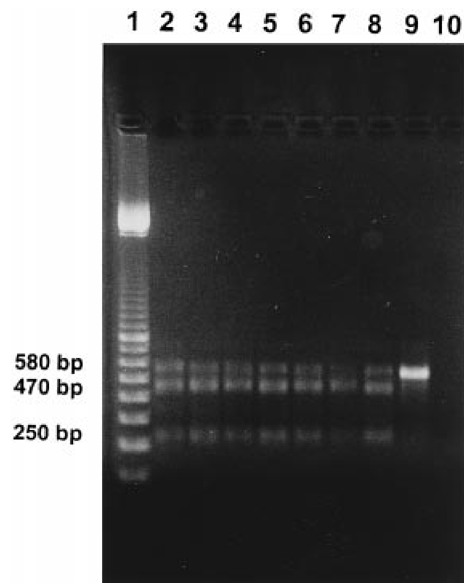

Fig. 2. Picture of ethidium bromide-stained 1·5% agarose gel with PCR products following amplication of ITS2 from lungworms of moose and cattle. Lane 1, 100 bp ladder; lanes 2–8, moose; lane 9, positive control (cattle); lane 10, negative control.

other hosts. However, the male worms from this host were longer ($P < 0{\cdot}05$) than their counterpart in moose only. Sex-wise comparison between worms from cattle and moose showed that only the female worms from the former were significantly longer ($P < 0{\cdot}05$) than female worms from the latter. Disregarding the sex of the worms, those from roe deer were significantly much longer ($P < 0{\cdot}001$) than worms from cattle and moose while no significant differences ($P > 0{\cdot}05$) in the TBL of worms from the last 2 hosts (Table 2).

It appeared that the BCW thickness and length of lungworms from the 3 animals exhibited wide variation (Table 2). Significant mean differences ($P < 0{\cdot}01$) were found in the 2 variables among the 3 hosts except between cattle and moose ($P > 0{\cdot}05$). Worms from roe deer had the largest mean BCW thickness and length compared to worms from the other hosts, which is in agreement with the result in Table 1. Note that worms from moose had lower mean BCW measurements than those worms from cattle although these were not significant. Using pooled data from the 3 hosts, there were no sexual differences ($P > 0{\cdot}05$) in the BCW measurements. These two BCW variables had significant ($P < 0{\cdot}001$) positive linear relationships to the worm TBL except between TBL and BCW length of worms from roe deer.

Three GLM models were tried with the last model excluding the non-significant interactions of the independent components. This last model showed that host and TBL had a significant effect on the BCW thickness while host, worm sex, worm

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Table 5. Result of molecular probing of PCR-amplified *Dictyocaulus* spp. ITS2 region with dig-labelled oligonucleotide probes using the filter hybridization assay

Host	No. of probed PCR-positive samples	Probe and no. of positive hybridization*		
		OP 108 (D viv)	OP 109 (D eck)	OP 110 (D sp)
Cattle	148	148	0	0
Roe deer	49	0	0	49
Moose	78	0	17	61
Total	275	148	17	110

* D viv, *Dictyocaulus viviparus*; D eck, *D. eckerti*; D sp, *Dictyocaulus* n. sp.

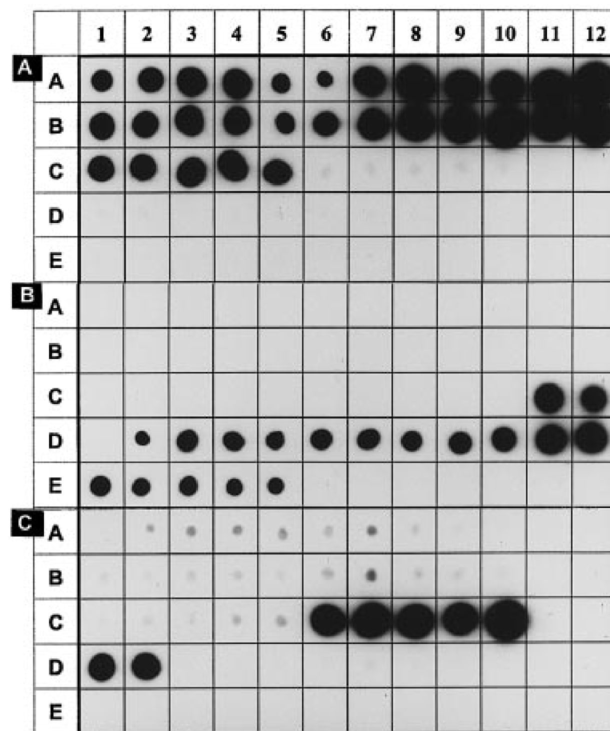

Fig. 3. Representative photograph of exposed X-ray film with superimposed grids showing amplified lungworm ITS2 samples probed with species-specific digoxigenin-labelled oligonucleotides: (A) *Dictyocaulus viviparus* probe (OP 108); (B) *D. eckerti* probe (OP 109); (C) *Dictyocaulus* sp. probe (OP 110). Grids A1–C5, ITS2 blots from cattle samples; C6–C9, samples from roe deer; C10–E5, samples from moose.

TBL, and host–TBL interaction had significant effect on the BCW length. These indicate that there were more factors that could explain the BCW length variations than in BCW thickness, although, only about 37·4% and 38·5% of the variations was explained by the model for each variable, respectively. The derived formulae for predicting the BCW thickness and length are shown in Table 3. The general formula for BCW thickness and length based on the GLM analysis is:

$$y_i = a + bx_i + cz_i + dt_i + bd + e_i,$$

where, y = BCW thickness or length in μm; a = y-intercept; b = estimate for host (x); c = estimate for sex (z); d = estimate for TBL (t); bd = estimate for host–TBL interaction; e = error term which is assumed as 0.

There was no significant difference ($P > 0·05$) between the length of the right and left spicules of the male worms from each host nor were there differences in between worms from the different hosts ($P > 0·05$) (Table 4).

Molecular analysis

DNA was extracted from 150, 50, and 78 worms from cattle, roe deer, and moose, respectively. These came from 20 cattle, 11 roe deer, and 9 moose. All but 3 worms, 1 each from cattle and roe deer, resulted in a successful ITS2 PCR amplification. For the majority of samples, a single band (about 580 bp) was visible on agarose gels after the ITS2 PCR. However, from 1 moose, 17 of 19 samples resulted in multiple bands after amplification (Fig. 2).

Table 5 shows the result after the hybridization of amplified ITS2 with species-specific probes. Figure 3 is a picture of an exposed X-ray film from the dot blot screening. All probed PCR-positive samples from cattle lungworms resulted in positive hybridization with the *D. viviparus* (OP 108) probe only. One PCR-positive sample was analysed by direct sequencing. The ITS2 samples from roe deer lungworms only hybridized with the OP 110 probe (*Dictyocaulus* sp.). On the other hand, 61 (78·2%), and 17 (21·8%) samples from moose hybridized positively with the OP 110 probe only, and OP 109 probe only, respectively. Furthermore, 1 sample from moose, which was positive to the *Dictyocaulus* sp. probe, was derived from the same animal where the samples that were positive to the *D. eckerti* probe were obtained. The 17 samples displaying multiple bands on agarose gels were all shown to be *D. eckerti* in the hybridization analysis.

To confirm the results with the *D. eckerti* probe, 1 of the positive samples was analysed by sequencing

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after cloning. The analysed ITS2 was found to be 96·5% similar to the ITS2 of German fallow deer *D. eckerti*, and the observed differences were all due to differences in the number of microsatellite repeats.

DISCUSSION

There are limitations to the use of morphological characters alone for helminth identification. For example, host-induced morphological variability is now a well-established problem in some characters of parasitic helminths (Michel, Lancaster & Hong, 1971; Waller & Thomas, 1978; Ulmer & Rhode, 1981). With DNA-based techniques, this problem can be overcome (for a review, see McKeand, 1998). However, to prevent a bias towards one method, comparative morphology and molecular methods are required. This would allow a meaningful description and natural classification of species (Monis, 1999).

In this study on identification of the lungworms of the genus *Dictyocaulus* in cattle, roe deer, and moose, both morphological and molecular approaches were applied on all worms recovered. In the blind test that we used, we found classification of the BCW alone could lead to a relatively high degree of correct identification. However, identification to the genus level only and misidentification may occur as shown by small percentages (6·8%) of worms in cattle belonging to the thick BCW category and small proportions of worms (6·0% in roe deer and 19·5% in moose) from the wild cervids under the thin BCW category. The visual examination of the lungworm BCW thickness was consistent with the morphometric results on this character. For instance, most of the worms from cattle were categorized as having thin BCW and had significantly lower mean BCW thickness. Hence, they were identified as *D. viviparus*, whereas those from roe deer belonged mainly to the thick category and had significantly thicker BCW, and they were accordingly identified as *D. eckerti* (Jansen & Borgsteede, 1990). The worms from moose, mostly with intermediate BCW, represented a diagnostic dilemma because the mean BCW thickness was not different from those of worms from cattle. Identification to the species level according to this feature was not made. Consequently, presentation of the morphological and morphometric data according to host was considered the correct approach.

With respect to the BCW length, the mean obtained for *D. viviparus* in this study was much lower than that reported of 22–25 μm. In contrast, the mean BCW length of worms from moose was close to the 12–14 μm range reported for *D. noerneri* by Durette-Desset *et al*. (1988). However, the worms from roe deer in this study had longer mean BCW length than *D. noerneri* which is proposed to be a synonym of *D. eckerti* by the same authors. This suggested that they were infected by a different species. The result of the hybridization supports this interpretation. Morphologically, the worms from roe deer were also significantly longer in their TBL compared with worms collected from the other hosts.

Results from the BCW shape indicated that this morphological character is to be preferred over the BCW thickness in identifying lungworms. This was shown by much higher percentages of worms identified as *D. viviparus* in cattle and *D. eckerti* in roe deer and moose. However, problems with identification were also encountered because the BCW shape of some worms was not distinct. In many instances, this was related to difficulty in orientating the specimens for correct examination. Thus, as with the BCW thickness, misidentifications can occur.

Morphometric analysis indicated a positive correlation of the BCW thickness and length with the hosts and TBL of the worms. This could be a helpful finding in terms of identifying the parasites, but there is also a question as to what extent the characters of the BCW are host induced. A transmission experiment on calves, fallow deer fawns and lambs by Bienioschek *et al*. (1996) involving *D. viviparus*, *D. eckerti* and *D. filaria* showed that the difference in BCW shape of the first two species was maintained after heterologous infections. This indicates that there is no host-induced variability in this character. However, any differences in the thickness and length were not mentioned. In the present study, the BCW thickness and length were significantly positively correlated with the worm TBL. In addition, the host species also had appeared to have a significant effect on these variables. Nevertheless, the BCW measurements obtained could contribute to the characterization of these parasites.

It should be noted that the worm host–TBL interaction also affected the BCW length. This may be because there were significant differences in mean TBL amongst worms from each host. On the TSL, all means obtained were within the range reported by Bacinský (1973) and Gibbons & Khalil (1988) for *D. viviparus* and *D. eckerti*, although the ranges obtained in this study were wider. This finding confirmed that the TSL is not a valid character in distinguishing among *Dictyocaulus* spp. from cattle, roe deer and moose.

Molecular analysis revealed that lungworms of cattle reacted only with the *D. viviparus* ITS2 probe, indicating that the 20 Swedish cattle studied had a monospecific infection. The absence of another *Dictyocaulus* sp. in this host provides evidence that lungworm-infected wild cervids may not be a reservoir of infection for cattle. Similarly, the roe deer in this study were infected with a single species as shown by the hybridization results. Neither the *D. eckerti* nor the *D. viviparus* probes hybridized with

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the worm ITS2 samples from this host. This supports a previous proposal (Höglund *et al*. 1999) that the roe deer in Sweden serves as a host for a novel species of *Dictyocaulus*. Nevertheless, *D. viviparus* has been reported in roe deer in Central Europe (Enigk & Hildebrandt, 1965) and Sweden (Nilsson, 1971). Given the close morphological similarity between *Dictyocaulus* spp. in cattle and wild cervids, it is likely that misidentifications in previous taxonomic studies may have occurred.

In moose, 78·2% of lungworm-derived ITS2 samples hybridized exclusively with the *Dictyocaulus* sp. (OP 110) probe. However, some also hybridized with the *D. eckerti* probe (OP 109). This result was reproducible as confirmed by sequencing one sample of the *D. eckerti*-positive samples. This shows that *D. eckerti* as defined by Epe *et al*. (1997) also occurs in Swedish moose. The presence of OP 110-positive and OP 109-positive samples indicates that mixed species infection between *D. eckerti* and *Dictyocaulus* n. sp. occurs. Specimens of the latter species are presently being examined in preparation for a formal taxonomic description.

The occurrence of more than one ITS2 band in the PCR products from DNA of lungworms from moose is most likely related to different sequence types in an individual worm. It has been observed that for some nematode species, the ITS2 varies within individuals (McKeand, 1998) which can be manifested in mobility differences in the agarose gels. Such differences have been linked to the presence of microsatellites that vary in number between individual ITS2 copies (Conole *et al*. 1999). The only differences between the *D. eckerti* ITS2 sequence from moose in this study and the *D. eckerti* sequence from fallow deer are the number of repeats of microsatellites. However, a more detailed analysis is necessary to address the question of intra-individual variability of *D. eckerti* ITS2.

In conclusion, the BCW thickness and length were found to be important morphological diagnostic characters for *Dictyocaulus* spp. However, they are of limited use for identifying the parasites to the species level because their dimensions appeared to be affected by host species and worm total body length. Because the total length of the worms is related to their age, identification would be difficult when only immature specimens are present. This study also confirmed that the spicular length is not a diagnostic morphological feature for these parasites in cattle and wild cervids, which is in agreement with Gibbons & Khalil (1988). On the other hand, with PCR-linked hybridization assay, *D. viviparus*, *D. eckerti*, and *Dictyocaulus* n. sp. were readily distinguished from each other. With this assay, it was possible to determine a monospecific infection with *D. viviparus* in cattle, the existence of *D. eckerti* in Swedish moose, and the presence of an hitherto morphologically undescribed *Dictyocaulus* sp. which is markedly distinct genetically from the above species. Equally important, we have not been able to detect the presence of *D. viviparus* in Swedish roe deer. As there are no previous reports on the presence of *D. eckerti* in Sweden, this comprises a new locality record. Moreover, this study also showed that when identification relied solely on morphology, most of the lungworms in wild cervids, especially those from roe deer, would have been misidentified as *D. eckerti*. In contrast, taking advantage of specifically designed oligonucleotide probes, all the lungworms from roe deer and most worms from moose were classified as the new species of *Dictyocaulus* (see also Höglund *et al*. 1999). Therefore, the PCR-linked hybridization assay provides a useful complementary tool.

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