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Ramon A. Carreno

Ohio Wesleyan University, racarren@owu.edu

Natividad Diez-Baños

University of León

Maria del Rosario Hidalgo-Argüello

University of León

Steven A. Nadler

University of California - Davis, sanadler@ucdavis.edu

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CHARACTERIZATION OF *Dictyocaulus* SPECIES (NEMATODA: TRICHOSTRONGYLOIDEA) FROM THREE SPECIES OF WILD RUMINANTS IN NORTHWESTERN SPAIN

Ramon A. Carreno, Natividad Diez-Baños*, Maria del Rosario Hidalgo-Argüello*, and Steven A. Nadler†

Department of Zoology, Ohio Wesleyan University, Delaware, Ohio 43015. e-mail: racarren@owu.edu

ABSTRACT: Specimens of *Dictyocaulus* spp. were extracted from the respiratory tracts of 3 ruminant hosts including roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and chamois (*Rupicapra rupicapra*) from wild populations in the province of León, northwestern Spain. The near-complete nuclear small-subunit ribosomal RNA gene, and 2 regions of the large-subunit ribosomal RNA gene, were amplified by PCR and sequenced. The SSU rDNA gene sequences indicated a high level of similarity between the isolate from *C. elaphus* and the published sequences for *Dictyocaulus eckerti*. SSU rDNA gene sequences were identical in the isolates from *C. capreolus* and *R. rupicapra*, and both corresponded to published sequences for *D. capreolus*. The LSU rDNA gene sequences differed in isolates from the latter 2 hosts, indicating the possible presence of an undescribed *Dictyocaulus* sp. in *R. rupicapra*. These results showed that the LSU rDNA gene sequences are useful indicators of genetic and species diversity in species of *Dictyocaulus*.

Trichostrongyloid nematodes of *Dictyocaulus* spp. Railliet and Henry, 1907 may be important pathogens in the respiratory tracts of domestic and wild ruminants. The cross-transmission of *Dictyocaulus* spp., from wild to domestic ruminants and vice versa, has rarely been demonstrated. Although little evidence of such cross-transmission between ruminants exists, a thorough understanding of the parasites' geographic distribution and host ranges is important for monitoring *Dictyocaulus* transmission between wild- and domestic-host populations.

In Spain, *Dictyocaulus viviparus* (Bloch, 1782) Railliet and Henry, 1907 and *Dictyocaulus filaria* (Rudolphi, 1809) Railliet and Henry, 1907 have been reported in domestic ruminants (Cordero del Campillo et al., 1994; Astiz et al., 2000). There have also been recent reports of species occurring in wild ruminants, including *Dictyocaulus noeneri* Railliet and Henry, 1907 in roe deer (*Dama dama* Linn.), a nematode redescribed by Durette-Desset et al. (1988), but considered incertae sedis by several authors (and suggested to represent *Dictyocaulus eckerti* Skrjabin, 1931). In addition, a *Dictyocaulus* sp. has been reported in chamois (*Rupicapra rupicapra* Linn.) following recovery and examination of adult specimens. Identification of larvae typical of *Dictyocaulus* spp. has also been the basis of 3 reports (Diez-Baños et al., 1990; Carrillo Gonzalez et al., 1994; Panadero et al., 2001). In these cases, the morphological features of these nematodes have been difficult to use for species identification, given the high level of similarity that exists among several species (Gibbons and Khalil, 1988).

In recent years, advances in molecular characterization of *Dictyocaulus* spp. have resulted in the identification of a distinct species from roe deer, namely *Dictyocaulus capreolus* Gibbons and Höglund, 2002. The use of DNA sequences, coding for small-subunit ribosomal RNA genes and other loci such as internal transcribed spacer (ITS2) sequences, has revealed the existence of several distinct clades or species of *Dictyocaulus* (Epe et al., 1997; Höglund et al., 1999; Hogland et al., 2003). Genes from the ribosomal RNA repeat have also been of value in estimating

phylogenetic relationships among trichostrongyloid and metastrongyloid nematodes (Carreno and Nadler, 2003; Chilton et al., 2006). In the present study, we report the characterization of *Dictyocaulus* spp. found in 3 ruminant hosts in northwestern Spain, including chamois (*R. rupicapra*), red deer (*Cervus elaphus* Linn.), and roe deer (*Capreolus capreolus* Linn.) using sequences from the small- and large-subunit ribosomal RNA genes.

MATERIALS AND METHODS

Specimens of *Dictyocaulus* spp. were collected in the Cantabrian Mountains of the province of León, northwest Spain from chamois, red deer, and roe deer. Hosts were killed (31, 21, and 37 hosts, respectively) by hunters, and the respiratory tracts of each animal were examined for *Dictyocaulus* spp. Adult nematodes were extracted from the respiratory tract, placed in 1.5 ml microcentrifuge tubes, and frozen at -20°C . DNA was extracted from 1 specimen of each of the 3 hosts, using overnight digestion of nematode tissues with proteinase K, followed by DNA extraction using DNAzol (Molecular Research Center, Cincinnati, Ohio) or MasterPure™ (Epicentre Technologies, Madison, Wisconsin) kits. Approximately 1,750 bp of the SSU rDNA was amplified using primers 18S1A forward (5'-GGCGATCGAAAAGATTAAGC-CATGCA) and 136 reverse (5'-TGATCCTTCTGCAGGTTACCTAC). A 950-bp fragment of the 5' end of the LSU rDNA containing the D2 and D3 domains was amplified using primers 391 forward (5'-AGCGGAG-GAAAAGAAACTAA) and 501 reverse (5'-TCGGAAGGAACCAGC-TACTA). The third amplified region consisted of 850–900 bp of the 3' end of the LSU rDNA. This region was amplified using primers 537 forward (5'-GATCCGTAACCTCGGGAAAAGGAT) and 531 reverse (5'-CTTCGCAATGATAGGAAGAGCC). PCR reactions of 25 μl consisted of 0.5 μM of each primer, 200 μM deoxynucleoside triphosphates, and a MgCl_2 concentration of 3 mM. Proofreading polymerase (Finnzymes DyNAzyme EXT, New England Biolabs, Ipswich, Massachusetts) was

TABLE I. *Dictyocaulus* sp. isolates from different hosts sequenced for comparative analysis. GenBank accession numbers for cloned sequences are in bold. Others were derived from direct sequencing of PCR products.

| Host | Sequence | GenBank accession no. |
|----------------------------|------------|-----------------------|
| <i>Capreolus capreolus</i> | 18S | FJ589016 |
| | 28S 5' end | FJ589017 |
| | 28S 3' end | FJ589011 |
| <i>Rupicapra rupicapra</i> | 18S | FJ589019 |
| | 28S 5' end | FJ589018 |
| | 28S 3' end | FJ589012 |
| <i>Cervus elaphus</i> | 18S | FJ589015 |
| | 28S 5' end | FJ589013 |
| | 28S 3' end | FJ589014 |

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*Department of Animal Health, Faculty of Veterinary Medicine, University of León, Spain.

†Department of Nematology, University of California, Davis, California 95616.

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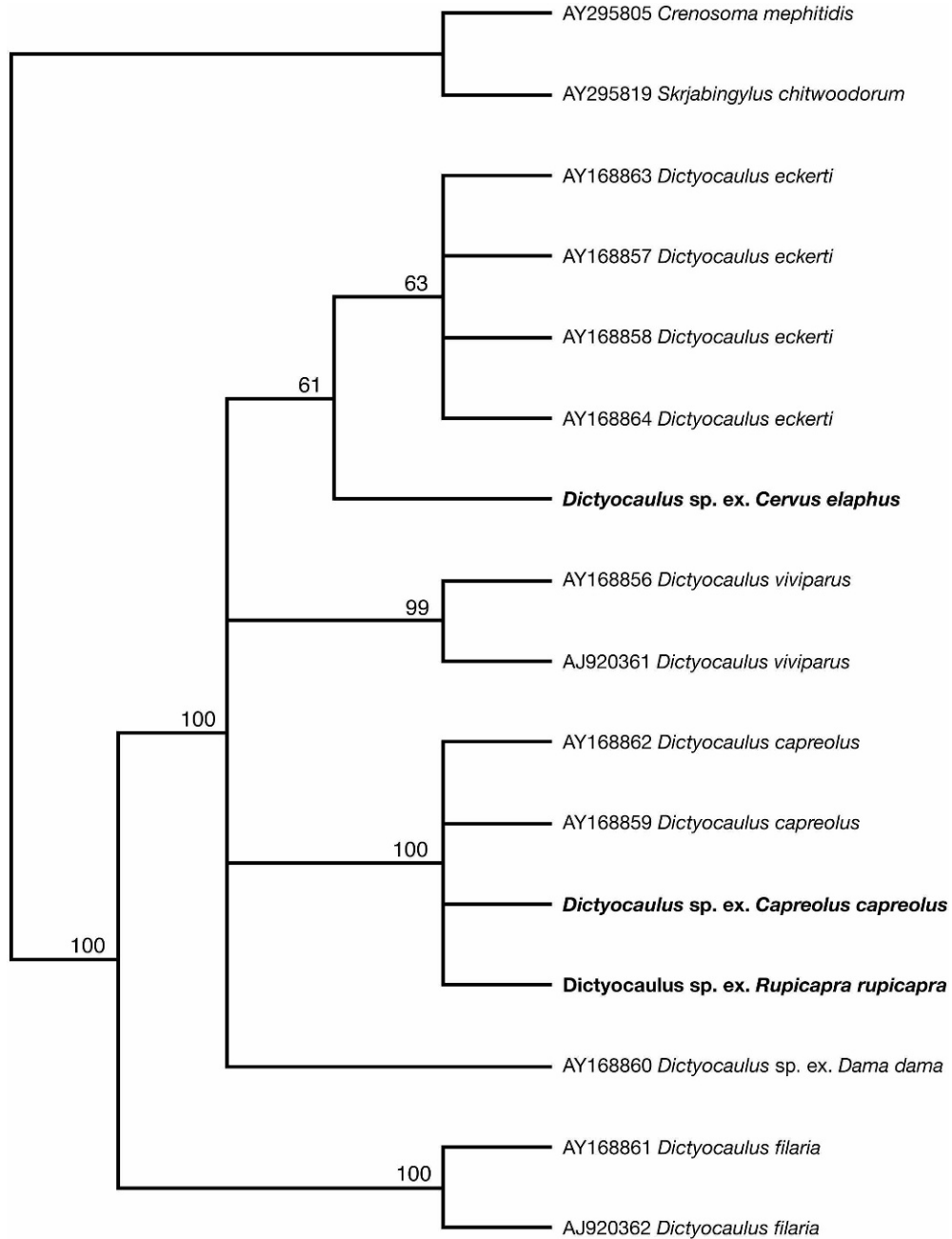


FIGURE 1. Strict consensus of 3 equally parsimonious trees depicting phylogenetic relationships among *Dictyocaulus* spp. inferred from SSU rDNA sequences. Bootstrap values (from 2,000 replicates) for clades exceeding 50% support are mapped onto the tree. Sequences acquired from Spanish ruminants are underlined.

used for amplification. PCR cycling parameters for the SSU rDNA reactions included denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 30 sec, 54 C for 30 sec, and 72 C for 1 min, followed by a post-amplification extension of 72 C for 7 min. The LSU rDNA regions were amplified using an annealing temperature of 54–58 C (5' end) or 54 C (3' end).

Amplifications that yielded only the target product (as assessed by agarose gel electrophoresis) were prepared for direct sequencing using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation, Cleveland, Ohio). Amplifications that yielded one or more non-target products, in addition to the expected amplicon, were cloned prior to sequencing (Table I). For cloning, PCR amplicons were gel-isolated, washed 3× with TE buffer (pH 7.0) by spin filtration (Millipore Ultrafree-MC 30,000 NMWL,

Millipore Corporation, Billerica, Massachusetts), ligated into pGEM-T vector (Promega, Madison, Wisconsin), and cloned into competent JM109 *Escherichia coli*. Plasmid DNA was obtained for sequencing using Qiaprep spin miniprep kits (Qiagen Inc., Valencia, California). Sequencing reactions were performed using dye-terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer (Applied Biosystems Inc., Foster City, California). DNA templates were sequenced for both strands; for cloned amplicons, a different clone was sequenced for each strand.

For directly sequenced PCR products, site polymorphisms were recorded only when both alternative nucleotide peaks were present in all sequencing reactions representing both DNA strands. If the heights of the alternative nucleotide peaks at polymorphic sites were not equal, the height of the minor peak was required to significantly exceed background

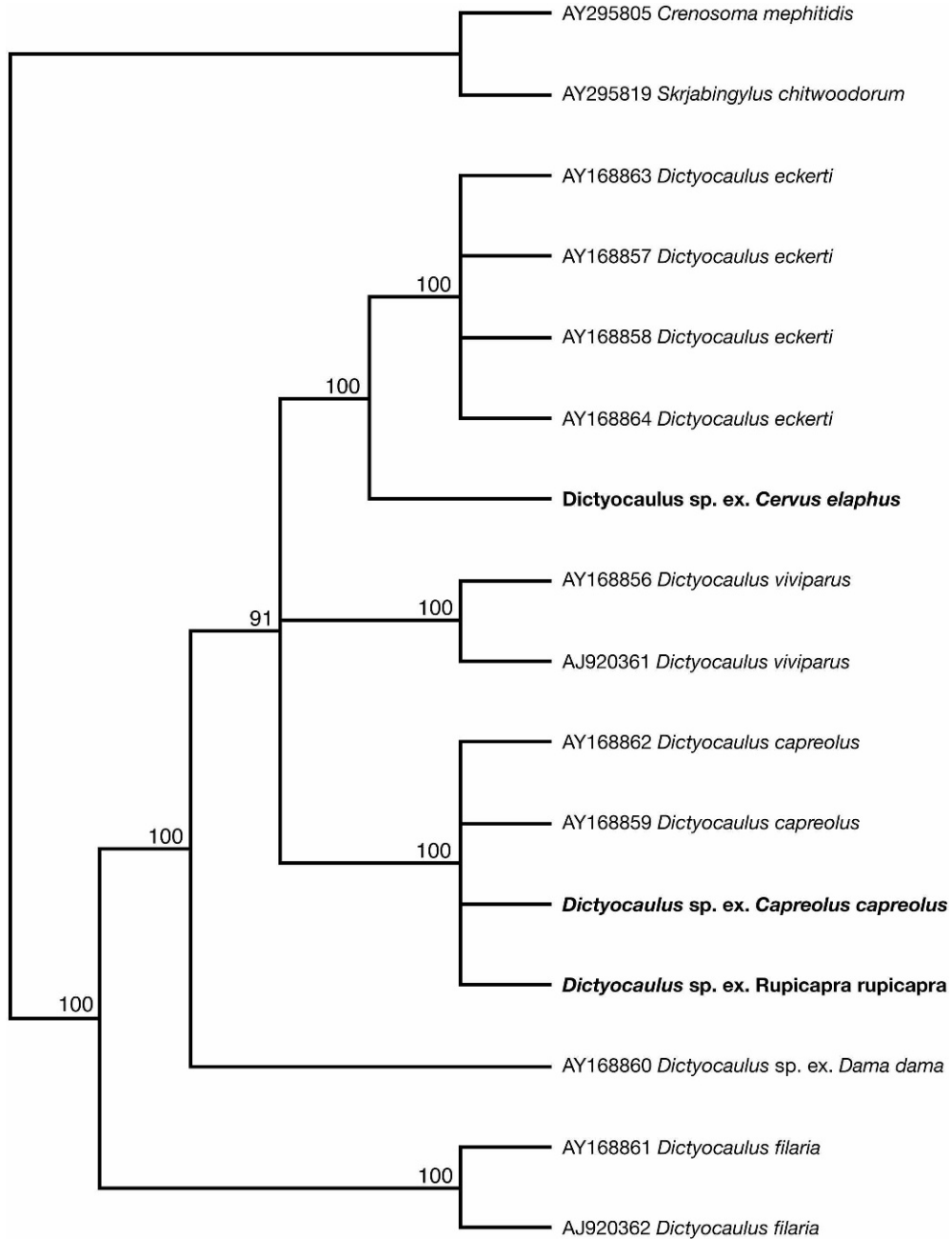


FIGURE 2. Bayesian tree depicting phylogenetic relationships among *Dictyocaulus* spp. inferred from SSU rDNA sequences. Posterior probabilities are mapped onto the tree.

terminations, and to comprise $\geq 25\%$ of the major peak, in order to be scored as a polymorphism. For cloned rDNA, sequence differences between clones were recorded as polymorphisms. CodonCode Aligner (Version 1.5.1, CondonCode Corporation, Dedham, Massachusetts) and Phred base calling were used for assembly of contigs. Sequences produced for this study have been deposited in GenBank (Table I). Completed sequences were aligned with other available *Dictyocaulus* spp. sequences from GenBank using Clustal-X version 1.53b (Thompson et al., 1997). Phylogenetic analysis of SSU rDNA sequences, using parsimony analysis, was performed using PAUP*4.0b10 (Swofford, 1998). Phylogenetic trees were viewed and printed using TreeView version 1.6.6 (Page, 1996). Sequences from 2 metastrongyloid species (*Skrjabinigylus chitwoodorum* Hill, 1939 and *Crenosoma mephitidis* Hobmaier, 1941) were used as outgroups to root the trees (Chilton et al., 2006). Portions of the 5' and 3' end of the alignment, for which a sequence was not available for each

representative taxon, were excluded from the analyses. There were 2 portions of the alignment that could not be reliably aligned; these included bases 610–617 and 1276–1299. Thus, these 2 regions were also excluded from the phylogenetic analyses. Sequences were analyzed, and most parsimonious trees were found using the branch-and-bound search option of PAUP*. Relative reliability of clades was assessed using 2,000 replicates of bootstrap resampling with parsimony inference. Clades with bootstrap support greater than 60% were mapped on the strict consensus of the most parsimonious trees recovered. A second analysis of the data was conducted using the program MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and using the same alignment and outgroups. The standard deviation of split frequencies was used to assess if the number of generations completed was sufficient; the chain was sampled every 200 generations over a total of 2 million generations. Burn-in (20%) was determined empirically by examination of the log likelihood values of the chains.

TABLE II. Pairwise absolute number of differences calculated for the combined large-subunit ribosomal RNA gene sequences (5' and 3' ends) for *Dictyocaulus* spp. from Spain. The absolute distance matrix, excluding gaps, is shown on the top right portion of the matrix. Differences including gaps are represented in the bottom left portion of the matrix.

| Sequence | 1 | 2 | 3 | 4 | 5 |
|---|-----|-----|-----|-----|-----|
| 1. AMO39753 <i>Dictyocaulus viviparus</i> | – | 48 | 57 | 58 | 210 |
| 2. <i>Cervus elaphus</i> isolate | 142 | – | 47 | 47 | 178 |
| 3. <i>Capreolus capreolus</i> isolate | 292 | 299 | – | 1 | 144 |
| 4. <i>Rupicapra rupicapra</i> isolate | 297 | 296 | 10 | – | 144 |
| 5. AMO39754 <i>Dictyocaulus filaria</i> | 263 | 302 | 413 | 423 | – |

RESULTS

The alignment of the SSU rDNA sequences revealed, with the exception of a single base, identical sequences for the isolate from *C. elaphus* and 4 sequences identified as *D. eckerti* in GenBank and obtained from various ruminant hosts (GenBank AY168863, AY168857, AY168858, and AY168864). The single exception was a substitution of a C in the Spanish isolate for a T in *D. eckerti* (position 929 in the multiple alignment). Two other SSU sites that appeared polymorphic (G or A, designated R) were seen in the Spanish red deer *Dictyocaulus* sp. The 2 SSU rDNA *Dictyocaulus* sp. sequences from *R. rupicapra* and *Ca. capreolus* were identical to one another and also matched 2 GenBank sequences from *A. alces* (AY168862) and *Ca. capreolus* (AY168859), except that there were 3 polymorphic “R” sites for the 2 Spanish isolates that were referred to as either A or G in the GenBank sequences.

The SSU rDNA alignment for all available *Dictyocaulus* spp., and the 2 metastrongyloid outgroups, yielded 98 parsimony-informative characters. The analysis yielded 3 equally parsimonious trees of 135 steps, with a consistency index of 94.8%. The majority-rule consensus of these trees depicted *D. viviparus* as sister to *D. eckerti* plus *Dictyocaulus* from Spanish red deer. Within the monophyletic genus *Dictyocaulus*, bootstrap parsimony analysis (Fig. 1) showed very weak support for a clade including *D. viviparus* and *D. eckerti*. The *D. capreolus* sequences, and the *Dictyocaulus* representing *R. rupicapra* and *C. capreolus* from Spain, formed a strongly supported monophyletic group. A *Dictyocaulus* sp. sequence (AY168860) from the fallow deer, *Dama dama*, was not included with the *D. eckerti*, *D. viviparus*, or *D. capreolus* clades. *Dictyocaulus filaria* was the sister taxon to a clade containing the other *Dictyocaulus* spp.

Bayesian analysis of the SSU rDNA alignment yielded a tree with a topology very similar to that found by parsimony analysis. There was no posterior probability support for the inclusion of *D. viviparus* with the *D. eckerti* clade (Fig. 2), a result reflecting the low parsimony bootstrap support for this grouping. The Bayesian posterior tree also excluded the *Dictyocaulus* sp. isolate of roe deer

from the clade containing *D. capreolus*, *D. viviparus*, and *D. eckerti*.

The 2 LSU rDNA sequences produced for each of the nematodes from Spain were combined and aligned with the only 2 *Dictyocaulus* LSU sequences previously deposited in GenBank, namely those of *D. viviparus* and *D. filaria*. The alignment revealed the absence of a region between bases 354 and 621, in which most bases in the *R. rupicapra* and *C. capreolus* *Dictyocaulus* isolates were absent (230 out of 267), compared to *Dictyocaulus* sp. from Spanish *C. elaphus* and published sequences of *D. viviparus*, *D. filaria*, and the 2 metastrongyloid sequences that were compared. The *C. elaphus* isolate also included unique nucleotide substitutions that, altogether, yielded considerable genetic distance from the other isolates (Table II). Between the isolates from *C. capreolus* and *R. rupicapra*, there was a difference at base 1002 (A in the former, G in the latter), as well as an insert of TACGA at bases 1004 to 1009 in the *C. capreolus* nematode that was absent in the lungworm from *R. rupicapra*. In addition, there was an insert of TATA at base 1107–1110 in the *R. rupicapra* lungworm that was absent in the *C. capreolus* nematode and in the other species (Table III). No phylogenetic tree was produced for these LSU sequences because too few sequences were available for comparative analysis.

DISCUSSION

The LSU rDNA sequence acquired from the *C. elaphus* lungworm could not be compared to sequences representing *D. eckerti*, as these are not yet available. However, the SSU rDNA sequences acquired from *Dictyocaulus* spp. in this host indicate a close affinity to the sequences of *D. eckerti*, which has been reported in *C. elaphus* (Gibbons and Khalil, 1988; Höglund et al., 2003). Similarly, the SSU rDNA gene sequence from *C. capreolus* is also most similar to that of *D. capreolus*; our finding of a putative *D. capreolus* from Spanish roe deer is also consistent with the recently reported type host for this species in Sweden (Gibbons and Höglund, 2002).

The LSU rDNA sequence obtained from the *C. elaphus* lungworm is more similar to that of *D. viviparus* than it is to the corresponding sequences acquired from *C. capreolus* and *R. rupicapra* isolates, a pattern consistent with the SSU rRNA gene sequences with respect to the sister group relationship inferred for *D. viviparus* and the *C. elaphus* isolate. A different sister group relationship, namely one between *D. capreolus* and *D. eckerti*, was shown by Höglund et al. (2003) using SSU rDNA gene sequences. However, bootstrap support for the *D. viviparus*–*D. eckerti* relationship in the present study was weak, suggesting that SSU data are insufficient for distinguishing between these alternatives. In contrast, there was strong support, in the present study, for a clade consisting of all sampled *Dictyocaulus* except for *D. filaria*,

TABLE III. Sequence differences for large-subunit ribosomal RNA sequences from 2 portions of the alignment.

| Sequence | pos. 1000 | pos. 1104 |
|--|-----------------|-----------|
| AMO39753 <i>Dictyocaulus viviparus</i> | CTAGTAG--GATTGG | ATC-ACACG |
| <i>Cervus elaphus</i> isolate | ----- | AT----ACG |
| <i>Capreolus capreolus</i> isolate | CTAGTAC--GA---- | ATA----CG |
| <i>Rupicapra rupicapra</i> isolate | CTGG----- | ATATATACG |
| AM039754 <i>Dictyocaulus filaria</i> | CTAGTAGCGGATTGG | A-----CA |

with the latter species representing the sister taxon to this larger group of *Dictyocaulus* spp. Sequences from additional specimens of *D. eckerti*, including LSU data, would be of value in further characterization of this species and in resolving the phylogenetic relationship between *D. viviparus*, *D. capreolus*, and *D. eckerti*.

The differences in the LSU rDNA sequences found between the isolates from *C. capreolus* and *R. rupicaprae*, in contrast to the identical nature of the SSU rDNA gene sequences in both isolates, indicate that the SSU rRNA gene may be too highly conserved in some closely related species to be of value in distinguishing between them. If the *Dictyocaulus* sp. in our Spanish roe deer sample is *D. capreolus*, then the differences seen in LSU rRNA gene sequences are suggestive of an undescribed species in *R. rupicapra* that is closely related to *D. capreolus* from roe deer. Alternatively, the *Dictyocaulus* spp. from Spanish roe deer and chamois may represent undescribed species. Here, too, there is a need for comparable LSU rDNA sequences from reliably identified *D. capreolus*.

The higher degree of variation seen in the sequences acquired from the LSU rDNA gene from the 3 *Dictyocaulus* spp. in the present study indicates that variable regions of this locus are valuable for distinguishing between different species in this genus. *Dictyocaulus* spp. are often reported from wildlife, based on recovery of larvae in feces, in the absence of post mortem examination of adult nematodes. Furthermore, adults identified as different species have been described from the same hosts. For example, the *Dictyocaulus* sp. has often been reported in the chamois as *D. filaria* (e.g., Gebauer, 1932; Hörning and Wandeler, 1968). However, there are reports of other species from this host, including *D. viviparus* (Stefancikova, 1999). The identity of larval stages could be verified using the partial LSU rDNA sequences described in the current study. The sequences that we acquired from Spanish ruminants are readily distinguishable from *D. filaria* and also reveal differences between the closely related species from *C. capreolus* and *R. rupicapra*.

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