Parasitol Res (2007) 101:1251–1255
DOI 10.1007/s00436-007-0629-1

ORIGINAL PAPER

Characterization of the 28S and the second internal transcribed spacer of ribosomal DNA of *Dicrocoelium dendriticum* and *Dicrocoelium hospes*

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Received: 5 May 2007 / Accepted: 4 June 2007 / Published online: 1 July 2007
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Abstract Isolates of *Dicrocoelium dendriticum* (*n*=150) from sheep and cattle bred in southern Italy and isolates (*n*=5) of *D. hospes* from a *Bos indicus* from Senegal were characterized genetically. The 28S region and the second internal transcribed spacer (ITS-2) plus flanking 5.8S and 28S sequences (ITS-2+) of ribosomal DNA (rDNA) were amplified by polymerase chain reaction and sequenced from individual flukes. Regarding the 28S rDNA, sequences of 568 and 581 bp were obtained for *D. dendriticum* and *D. hospes*, respectively. No intraspecific variation was observed between the 28S rDNA of all the *D. dendriticum* specimens studied and the *D. dendriticum* 28S rDNA sequence present in GenBank™. However, intraspecific variation was observed in the 28S rDNA of the *D. hospes* specimens compared to the sequence present in GenBank™. Regarding the ITS2+ rDNA, sequences of 402 and 428 bp were obtained for *D. dendriticum* and *D. hospes*, respectively; both sequences were deposited in GenBank™. Variations intra- and interpopulation were observed for *D. dendriticum*, whereas 100% identity was observed in all the ITS2+ sequences of *D. hospes*. With respect to the interspecific variations, the ITS-2+ of *D. dendriticum* and *D. hospes* differed in 33 positions. The findings of the present study showed an ITS2+ sequence variability (8.2–8.5%) between *D. dendriticum* and *D. hospes*, thus demonstrating the utility of this sequence to discriminate the two species.

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Introduction

Dicrocoeliosis is caused by several species of *Dicrocoelium* Dujardin, 1845 (Trematoda, Digenea), which live in the bile ducts and gall bladder of domestic and wild ruminants (sheep, goats, cattle, buffaloes, roedeer, and camels) and occasionally affect rabbits, pigs, dogs, horses, and humans (Otranto and Traversa 2003). The most important species of this genus are the following: *Dicrocoelium dendriticum* Rudolphi, 1819, *D. hospes* Loss, 1907, *D. chinensis* Tang and Tang, 1978, and *D. suppereri* Hinaiday, 1983 (syn. *D. orientalis* Sudarikov and Ryjikiov, 1951). *D. dendriticum* is found in America, Asia, North Africa, and Europe; *D. hospes* is mainly found in Africa; *D. chinensis* is found in Asia, and *D. suppereri* is found in the old USRR and Austria (Manga-González et al. 2001; Otranto and Traversa 2002).

D. dendriticum is the most widespread liver fluke found in cattle and sheep in southern Italy (Cringoli et al. 2002), as well as in other Mediterranean countries; its occurrence is related to dry and calcareous or alkaline soils, which represent favorable biotopes for its intermediate hosts (Manga-González et al. 2001). In fact, it has a very complex life cycle because it involves numerous species of land mollusks and ants as first and second intermediate hosts, respectively. The economic and health significance of dicrocoeliosis is due to the direct losses occasioned by the confiscation of altered livers and also to the indirect ones caused by the digestive disorders derived from the

hepatobiliary alterations, such as decreased animal weight, growth delay, and reduced milk production (for a review, see Manga-González et al. 2001). Diagnosis of dicrocoeliosis is usually based on egg detection in feces of live infected animals or on adult detection in liver on postmortem examination (Campo et al. 2000).

Several authors have made studies on the variability range and frequency of occurrence of different morphological types of *D. dendriticum* (Macko and Birova 1989), concluding on the possibility of interpretation of *D. hospes* as an intrapopulation of *D. dendriticum*. In addition, genetic variability of adult *D. dendriticum* specimens has been observed using random amplified polymorphic DNA (Sandoval et al. 1999; Manga-González and Gonzalez-Lanza 2005).

However, the findings of a recent study showed marked differences in the ultrastructure of the spermatozoon of *D. dendriticum* and *D. hospes*, constituting additional data supporting the specific identity of these two species (Agostini et al. 2005).

Based on the above debates, the aim of the present paper was to perform molecular studies on two regions of the ribosomal DNA (rDNA) of several *D. dendriticum* specimens from various hosts and locations in southern Italy. In particular, the second internal transcribed spacer (ITS-2) rDNA—a reliable genetic marker already used for molecular systematic studies of platyhelminthes (Adlard et al. 1993; Bowles et al. 1995; Gasser and Chilton 1995; Jousson et al. 1998; Rinaldi et al. 2005)—and the 28S rDNA were utilized. In addition, molecular differences (based on 28S rDNA and ITS-2) between several specimens of *D. dendriticum* and five specimens of *D. hospes* were also studied.

Materials and methods

Dicrocoelium collection

Adults of *D. dendriticum* (n=150) were collected from livers of naturally infected ruminants, namely, sheep (n=26) and cattle (n=10), slaughtered at abattoirs located in the Campania and Calabria regions of southern Italy. Five adults of *D. hospes* from a *Bos indicus* from Senegal were kindly provided by the University of Dakar. The flukes were washed with physiological saline solution and stored in 70% ethanol solution before further DNA extraction.

Genomic DNA extraction

DNA was extracted from individual adult worms using spin columns of QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as specified by the manufacturer. This method has been found to be the best among five methods for DNA isolation from adult *D. dendriticum* (Capuano et al. 2007). DNA concentration and purity were determined spectrophotometrically by measuring their absorbance at 260 and 280 nm. DNA samples were then kept at −20°C until used for polymerase chain reaction (PCR).

DNA amplification by PCR

Two different sets of 50 µl PCR mixes were prepared to amplify a 650-base-pair target of the 28S rDNA and the ITS-2+ rDNA regions, respectively, for both the *Dicrocoelium* species.

Each mix of PCR was made up using 15 mM of Tris-HCl (pH 8.0), 50 mM of KCl, 6–12 ng of adult fluke DNA, 0.2 mM of the four nucleoside triphosphate (dNTPs, Takara, Japan), and 2.0 mM of MgCl2. The partial region of the 28S rDNA was amplified using 0.2 µM of each primer (Sigma, USA) described by Marcilla et al. (2002) and 1.5 U of TaqGold DNA Polymerase (Applied Biosystems, USA), performing a hotstart PCR in a GeneAmp PCR System 2700 (Applied Biosystems, USA) under the following conditions: after an initial step at 95°C for 10 min, the mixture was subjected to amplification cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 5 min. ITS-2, plus the partial flanking rDNA regions 5.8S and 28S (a total region also known as ITS-2+), was amplified using 12.5 pmol of each primer (MWG Biotech, Germany) described by Itagaki et al. (2003) and 2.5 U of TaqGold DNA Polymerase under the following conditions: 95°C for 10 min, 35 cycles at 94°C for 1 min, 53°C for 90 s, 72°C for 1 min, and finally, 72°C for 10 min. Eight microliters of PCR products were electrophoresed on a 1% agarose gel containing 1× tris borate ethylenediamine tetraacetic acid (EDTA; 100 mM Tris-HCl, pH 8.0; 90 mM boric acid, 1.0 mM EDTA, Invitrogen, USA) and ethidium bromide (0.5 µg/ml), visualized and photographed under UV, then analyzed with the GelPro 3.1 software (MediaCybernetics, USA).

Nucleotide sequences analysis

For each marker analyzed, the 150 amplicons of *D. dendriticum* and the five amplicons of *D. hospes* were purified using QIAquick PCR Purification Kit (QIAGEN, Germany) as specified by the manufacturer. Sequencing reactions were performed with both primers used for the PCR on a GeneAmp 2700 using the BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA), and the results were analyzed on a 310 automated DNA sequencer (Applied Biosystems, USA).

The obtained 28S rDNA sequences were compared with those of *D. dendriticum* and *D. hospes* already present in

the GenBank™ database (accession no. AF 151939 and AY 251233, respectively). In addition, the ITS2+ sequences of *D. dendriticum* and *D. hospes* were compared. All the comparison and alignments were conducted using BLAST system (basic local alignment tool) and ClustalW (http://www.ebi.ac.uk/clustalw/).

Results

28S

DNA amplification of the 28S rDNA produced a 650-bp fragment for all the *Dicrocoelium* specimens, although different numbers of nucleotides were defined in both amplicons for each species.

From each 28S rDNA amplicon of *D. dendriticum*, a sequence of 568 bp was obtained; whereas after sequencing of the 28S rDNA of *D. hospes*, a total of 581 nucleotides were obtained. All the 28S rDNA partial sequences of *D. dendriticum* showed a total (100%) identity with the 28S sequence already deposited in GenBank™ (accession no. AF 151939). However, an intraspecific variation was observed in the 28S rDNA of the five *D. hospes* specimens compared to the sequence already deposited in GenBank™ (accession no. AY 251233). Specifically, four *D. hospes* specimens showed 11 single base substitutions (point mutations), whereas one specimen showed five single base substitutions (Table 1).

The 28S rDNA sequences of *D. hospes* described in this study are now available from the GenBank™ database under the following accession nos. EF102024 and EF102025.

Interspecific variations were observed between the 28S of *D. dendriticum* and either the 28S of *D. hospes* EF102024 (nine variant nucleotides) and the 28S of *D. hospes* EF102025 (three variant nucleotides; Table 2).

ITS2+

DNA amplification of the ITS2+ rDNA produced a 450-bp fragment for all the *Dicrocoelium* specimens; however, after sequencing the ITS2+ region of *D. dendriticum*, a total of 402 nucleotides were obtained; whereas a total of 428 nucleotides were obtained for the ITS2+ region of *D. hospes*.

Variations of intra- and interpopulation were observed for *D. dendriticum* analyzing the ITS-2+ sequences. In particular, 15 samples showed a T/A substitution in position 215 and a A/G substitution in position 300; four samples showed only a T/A substitution in position 215; nine samples showed a C/A substitution in position 267.

The ITS2+ sequences of *D. hospes* were confirmed in all the analyzed samples (100% identity).

The ITS2+ sequences described in this study are now available from the GenBankTM database under the following accession nos. DQ 379986 and EF102026, for *D. dendriticum* and *D. hospes*, respectively. With respect to interspecific variations, the ITS2+ sequences of *D. dendriticum* and *D. hospes* differed in 33 positions: 26 single substitutions and seven single base deletions. The point mutations were either purine substitutions (A by G and G by A; n=11), or pyrimidine substitution (C by T and T by C; n=8), or purine/pyrimidine substitutions (G by T, A by T; n=5), or pyrimidine/purine (T by G, T by A, C by G, C by A; n=2; Fig. 1).

Interspecific variations were also observed between the *D. dendriticum* variants (isolate groups 2, 3, 4) and *D. hospes*, as summarized in Table 3.

Table 1 Intraspecific variation in the *D. hospes*28S rDNA sequence already deposited in GenBank™ (accession no. AY251233) and the two *D. hospes* 28S rDNA sequenced in the present study (GenBank™ accession n EF102024 and EF102025)

Nucleotide	*D. hospes* (AY251233)	*D. hospes* (EF102024)	*D. hospes* (EF102025)
53	T	C	T
86	A	G	G
89	G	A	A
235	C	T	T
360	G	A	A
408	G	T	G
409	T	C	T
426	T	G	T
442	T	C	T
448	C	A	A
462	T	C	T

Table 2 Interspecific variation in the 28S rDNA sequences of *D. dendriticum* (GenBank™ accession no. AF151939), *D. hospes* (GenBank™ accession no. EF102024), and *D. hospes* (GenBank™ accession no. EF102025)

Nucleotide	*D. dendriticum* (AF151939)	*D. hospes* (EF102024)	*D. hospes* (EF102025)
53	T	C	T
86	A	G	G
89	G	A	A
360	G	A	A
408	G	T	G
409	T	C	T
426	T	G	T
442	T	C	T
462	T	C	T

```
Dd              ------------------------------------TAATGTGAACTGCATACTGCTTTG 24
Dh(EF102026)    TTTGTGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTG 60
                                                    ************************

Dd              AACATCGACATCTTGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCG 84
Dh(EF102026)    AACATCGACATCTTGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCG 120
                ************************************************************

Dd              AGGGTCGGCTTACAAACTATCACGACGCCCAATAAGTCGTGGCTTGGATTTTGCCAGCTG 144
Dh(EF102026)    AGGGTCGGCTTATGAACTATCACGACGCCCAATAAGTCGTGGCTTGGATTTTGCCAGCTG 180
                ************  **********************************************

Dd              GCTTTACTCCCCAGTCGGAAACGTCAGGGGTGTCAGATCTATGGCGTTATCCTAATGTAT 204
Dh(EF102026)    GCTTCAATCCCCAGACGAGAATGTCAGGGGTGTCAGATCTATGGCGTTATCCCAATATAT 240
                **** * ******* **  ** ****************************** *** ***

Dd              CCGGATACACWCACCTAGTTATCAGACAGGTGGAGATGTGTCTACGGAGTCGTGGCTCAG 264
Dh(EF102026)    CCTGATGCACACATCTGGATAATACACAGATGGAGATGTGTCAACGGAGTCGTGGCTCAG 300
                ** *** *** ** ** * **  * **** ************ *****************

Dd              TAMTATTTATGCGCGCTCTGTAGAACATCTCGTGTRGTGAATTCCGAAATACGGCCTTCT 324
Dh(EF102026)    TG-TAATTATGCGCGCTCTGTAAAACATCTCG-----TGAATTCCGAAATATGACCTTCT 354
                *  ** **************** *********     ************** * ******

Dd              AATCCTGACCTCGGATCAGACGTGATTACCCGCTGAACTTAAGCATATCACTAAGCGGAG 384
Dh(EF102026)    AATCCCGACCTCGGATCAGACGTGATTACCCGCTGAACTTAAGCATATCACTAAGCGGAG 414
                ***** ******************************************************

Dd              GAAAAGAAACCTAACCAA 402
Dh(EF102026)    GAAAAGAAAC-TAAC--- 428
                ********** ****
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Fig. 1 Sequence alignment (5'→3') of ITS-2 plus flanking 5.8S and 28S sequences (*bold*) of *D. dendriticum* (GenBank™ accession no. DQ379986) and *D. hospes* (GenBank™ accession no. EF102026). Conserved nucleotides are indicated with *asterisks*. Gaps are indicated with *hyphens*

Discussion

Molecular tools, usually DNA sequencing, might provide an alternative approach to identify closely related parasites (reviewed in Nolan and Cribb 2005). Among all target sequences used for phylogenetic studies and polymorphism analyses, rDNA is a powerful tool to demonstrate substantial intra- and interspecific variability of distinct parasite species because it includes regions with varying rates of evolution from highly conserved (18S, 5.8S, and 28S) to highly variable (transcribed and nontranscribed or intergenic spacer regions), such as the ITS-1 and the ITS-2 regions, by far the most frequently studied regions for the exploration of species boundaries in digeneans (Hillis and Dixon 1991).

In the present study, the DNA technology was used to characterize two regions of the rDNA, namely, the ITS-2 and the 28S, from several *D. dendriticum* specimens from various hosts and locations in southern Italy and from five *D. hospes* specimens from Africa. Although previous papers observed a high variability in the parasitic species of *D. dendriticum*, both upon genetic (Sandoval et al. 1999) and morphoanatomic parameters (Birova and Macko 1987; Macko and Birova 1989), the ITS-2 intraspecific variation was less than 1.0% (ranging from 0.25 to 0.50%) in the *D. dendriticum* specimens studied.

A limitation of this study is the small number of samples used for *D. hospes*; however, although we could not rigorously argue on rDNA variation for this parasite and on sequence divergence between it and *D. dendriticum*, we tried to compare the sequences obtained. This comparison showed an interspecific variation of the ITS-2+ nucleotidic composition ranging between 8.2 and 8.5% between *D. dendriticum* and *D. hospes*, thus demonstrating an interesting variability in the ITS-2 region.

Table 3 Variability between the single polymorphic group of *D. dendriticum* and the unique ITS-2+ sequence identified in *D. hospes* "population"

D. dendriticum	*D. hospes*[e]	Variability(%)
Isolates group 1 (n=122)[a]	Isolates group 1	8.2
Isolates group 2 (n=15)[b]	Isolates group 1	8.5
Isolates group 3 (n=9)[c]	Isolates group 1	8.2
Isolates group 4 (n=4)[d]	Isolates group 1	8.5

[a] One hundred and twenty-two samples whose sequence was deposited in GenBank™ (accession no. DQ379986)
[b] Fifteen samples with a T215A substitution and a A300G substitution
[c] Nine samples showing only a C/A substitution in position 267
[d] Four samples showing only a T/A substitution in position 215
[e] The five samples of *D. hospes* analyzed

Similar studies performed on other digenean species have showed similar results: the ITS-2 sequence divergence between *Fasciola hepatica* and *F. gigantica* was 2.8% and between *F. hepatica* and *Fascioloides magna* was 13.2% (Adlard et al. 1993); in addition, ITS-2 sequence divergence between *Calicophoron daubneyi* and *C. calicophorum* was 2.8% and between *C. daubneyi* and *C. microbothrioides* was 2.6% (Rinaldi et al. 2005).

Even if it would be strengthened if we would be able to obtain other samples of *D. dendriticum* and mostly of *D. hospes* from other sources from other parts of the world, we believe that our findings will be useful for the identification of these liver flukes in Italy, Africa, and elsewhere.

Acknowledgments We wish to thank Prof. Bâ Cheikh (University of Dakar, Senegal) and Dr. Silvia Agostini, Prof. Carlos Feliu, and Dr. Jordi Miquel (University of Barcelona, Spain) for helping us in obtaining the *D. hospes* specimens.

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