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Determination of ribosomal internal transcribed spacer 2 (ITS2) interspecific markers in *Fasciola hepatica*, *Fascioloides magna, Dicrocoelium dendriticum* and *Paramphistomum cervi* (Trematoda), parasites of wild and domestic ruminants

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Summary

The species-specific ribosomal internal transcribed spacer 2 (ITS2) markers were designed for PCR-based molecular differentiation of *Fasciola hepatica, Fascioloides magna*, *Dicrocoelium dendriticum* and *Paramphistomum cervi*, liver and stomach flukes of domestic and free living ruminants. Complete ITS2 sequences were obtained for *D. dendriticum* and *P. cervi*, for the later species, ITS2 structure was determined for the first time. Intraspecific variation within geographically distant populations was found to be either very low (*F. hepatica*; *D. dendriticum*) or even absent (*F. magna*; *P. cervi*). ITS2 regions with the absence of intraspecific polymorphisms but with interspecific sequence heterogeneity were applied for design of species-specific primers. The specificity of developed primers was tested on genomic DNA isolated from adult individuals of studied fluke species. Application of the primers is of particular value for molecular differentiation of morphologically hardly distinguishable *F. hepatica*, *F. magna* and *P. cervi* eggs after coprological examinations.

Keywords: liver and stomach flukes; ribosomal internal transcribed spacer; molecular markers; intraspecific variation

Note: Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers HM026461 and HM026462.

Introduction

Parasitic infections are generally considered to be the most prevalent and important health problems of grazing ruminants. Domestic and free-living ruminants are often infected by various intestinal, stomach or liver helminths, among which digenetic species of *Fasciola, Fascioloides, Dicrocoelium,* and *Paramphistomum* genera often induce similar clinical signs (Dorchies, 2006; Sanabria *et al*., 2009). Infections are most frequently documented in domestic ruminants such as sheep, goat, and cattle (Pybus, 2001; Otranto & Traversa, 2003; Anuracpreeda *et al*., 2008), and in a variety of free living ruminants like cervids, bison, chamois and camel (Pybus, 2001).

Common liver fluke, *Fasciola hepatica* (Fasciolidae), has a cosmopolitan distribution and occurs in bile ducts of its definitive host. Fasciolosis of domestic ruminants is often accompanied by clinical signs including weight loss, diarrhoea, decreased milk yield and occasionally death (Pritchard *et al*., 2005), while infections in cervids are usually without any clinical signs (Lang, 1977). Besides, *F. hepatica* causes also epidemics of human fasciolosis in several world regions (Mas-Coma, 2005).

Another serious liver fluke of the family Fasciolidae*, Fascioloides magna* (giant liver fluke or large American fluke), belongs with its maximal length up to 8-10 cm to the largest flukes ever. While other liver flukes are localized in bile ducts, *F. magna* occurs in pairs or groups within fibrous capsules directly in the liver parenchyma of infected animals (Špakulová *et al*., 2003). Infections of ruminants are characterized by excessive wandering of juvenile flukes into various abdominal and pleural organs what may cause death of the host (Pybus, 2001).

Dicrocoeliosis is mainly caused by *Dicrocoelium dendriticum* (Dicrocoeliidae), which is also known as lancet fluke or small liver fluke. The parasite affects bile ducts and gall

bladder of definitive hosts. Although the pathogenicity of *D. dendriticum* is usually low, dicrocoeliosis can lead to weight loss, anaemia, oedema and reduced milk production, with potentially fatal consequences in rare cases (Otranto & Traversa, 2003).

The stomach fluke, *Paramphistomum cervi* (Paramphistomidae), is the main parasite of fore stomach (rumen and reticulum) of domestic ruminants and cervids (Anuracpreeda *et al*., 2008). Most infections of adult flukes are harmless although heavy infections can cause a chronic ulcerative rumenitis with atrophy of ruminal papillae (Rolfe *et al*., 1991). In acute infections, a massive number of immature flukes migrate through the intestinal tract causing acute parasitic gastroenteritis with high morbidity and mortality rates particularly in young animals (Sanabria & Romero, 2008).

Adults of each of the fluke species can be well recognized by distinct species-specific morphological characters (Erhardová-Kotrlá, 1971; Otranto & Traversa, 2003). However, their eggs and intramolluscan larval stages are considered to be hardly distinguishable; particularly eggs of *F. hepatica*, *F. magna* and *P. cervi* have very similar size, shape and internal structures (Oberhauserová *et al*., in press), therefore, their differentiation during coprological examinations cannot rely on egg morphology. In such cases, molecular approaches employing PCR-based techniques using species-specific primers constitute the accurate methods for species determination.

It is generally accepted that the ribosomal DNA clusters (rDNA) coding structural components of ribosomes are particularly useful for genetic studies to demonstrate substantial intra- and interspecific variability because they are highly repeated and contain variable regions flanked by more conservative regions (Hillis & Dixon, 1991). Especially the internal transcribed spacers (ITS1 and ITS2) were proved to be useful as species markers (Minchella *et al*., 1997) since intraspecific variation within ITS is usually low, whereas consistent interspecific differences exist between species. Ribosomal DNA sequences are regarded as particularly useful for discrimination of digenean trematode species (Nolan & Cribb, 2005).

The aim of this work was to design species-specific ribosomal ITS2 markers for PCR-based differentiation of *F. hepatica, F. magna*, *D. dendriticum* and *P. cervi*, liver and stomach flukes of ruminants. ITS2 regions which were absent from intraspecific polymorphisms but were characterized by interspecific sequence heterogeneity were applied for design of species-specific primers. The specificity of designed primers was tested on genomic DNA isolated from adult individuals of all studied species.

Materials and Methods

Material

For current molecular analysis, adult individuals of *F. hepatica*, *F. magna*, *D. dendriticum* and *P. cervi* were used (Tab. 1). The flukes were isolated from hunted or slaughtered definitive hosts, rinsed in PBS buffer (pH 7.4) and fixed in 96 % ethanol.

DNA isolation

The genomic DNA of *F. hepatica*, *D. dendriticum* and *P. cervi* adults was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, diluted in 50 µl deionized water and stored in -20 °C. The genomic DNA of *F. magna* was isolated from 20 mg of adults' tissue incubated in extraction buffer (10 mM TRIS-HCl, pH 7.5; 10 mM EDTA; 50 mM NaCl; 2 % sodium dodecyl sulphate) supplemented with 20 mM dithiothreitol and 900 µg proteinase K at 56 °C with gentle shaking for 12 – 18 hours. After lysis, phenol:chlorophorm:isoamylalcohol (25:24:1) extraction and ethanol precipitation (100 % ethanol; 3 M sodium acetate; pH 4.8) were performed. Genomic DNA was diluted in 50 µl deionized water and stored in -20 °C.

PCR amplification and sequencing of complete ribosomal ITS2

The complete ITS2 structure was studied in three specimens of both *D. dendriticum* and *P. cervi*; for *P. cervi* the ITS2 sequence was originally determined. To evaluate the overall level of intraspecific variation, ITS2 sequences of geographically distant *F. hepatica, F. magna* and *D. dendriticum* populations, retrieved from GenBank (see Tab. 2), were also considered for comparative purposes. For PCR amplification of the complete ribosomal ITS2 spacer

Table 1. Origin of adult flukes of *Fasciola hepatica, Fascioloides magna, Dicrocoelium dendriticum* and *Paramphistomum cervi* used in the study

Fluke species	Host	Location of parasite	Locality
Fasciola hepatica	cattle (*Bos taurus* f. *domestica*) slaughtered	bile ducts	Košice, Slovakia
Fascioloides magna	red deer (*Cervus elaphus*) hunted	liver cysts	Bodíky, Slovakia
Dicrocoelium dendriticum	sheep (*Ovis aries*) slaughtered	bile ducts	Krivany, Slovakia
Paramphistomum cervi	red deer (*Cervus elaphus*) hunted	rumen	Bodíky, Slovakia

of *D. dendriticum* and *P. cervi*, the 5.8S-2 (5´-GTCGATGAAGAGCGCAGC-3´; Králová-Hromadová *et al*., 2003), and ITS-2 (5´-AGGAGGCGAATCACTAT-3´; Cunningham, 1997) primers with annealing positions in the 5.8S and 28S rDNA, respectively, were used. The total volume of amplification mixture was 20 µl and contained 10-20 ng of genomic DNA, 20 pmol of each of the 2 primers, 0.2 mM of each of the deoxynucleotide triphosphate (Applichem, Darmastadt, Germany), 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA) with corresponding reaction buffer and 1.5 mM $MgCl_2$. The PCR run as follows: 5 min at 94 °C as the initial step, followed by 30 cycles 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. The final step was 5 min at 72 °C. The PCR-amplified products were vizualised on the 1 % agarose gel, purified using the Wizard PCR purification kit (Promega) and sequenced using 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). The sequence alignment was performed using ClustalW (Thompson *et al*., 1994). The boundaries of ITS2 spacer were determined as previously described in Králová-Hromadová *et al*. (2008).

Design of species-specific primers

ITS2 sequences of distant geographic populations of four fluke species (see Tab. 2) were aligned in order to determine levels of intra- and interspecific polymorphism. Regions displaying no intraspecific variation and at the same time specific for particular fluke species were used for design of species-specific primers (for more details see Results and Discussion).

Table 2. ITS2 sequences of *Fasciola hepatica, Fascioloides magna, Dicrocoelium dendriticum* and *Paramphistomum cervi* analyzed in the current study

Fluke species	GenBank accession number	Origin	References
F. hepatica	DQ683546*	Austria	unpublished
	AJ272053*	Spain	Mas-Coma *et al*., 2001
	AM707030	Spain	Alasaad *et al*., 2007
	AM709498-500	Spain	Alasaad *et al*., 2007
	AM709609-22	Spain	Alasaad *et al*., 2007
	AM709643-49	Spain	Alasaad *et al*., 2007
	AJ557567*	France	Huang *et al*., 2004
	FJ593632	Turkey	Erensoy *et al*., 2009
	FJ467927	Turkey	Erensoy *et al*., 2009
	FJ459806	Turkey	Erensoy *et al*., 2009
	EF612481	Iran	Lotfy *et al*., 2008
	EU391412-24	Iran	unpublished
	AJ557568*	China	Huang *et al*., 2004
	EF612479-80	Egypt	Lotfy *et al*., 2008
	AM850107	Niger	unpublished
	AM900370	Niger	Ali *et al*., 2008
	GQ231546-47	Tunisia	Farjallah *et al*., 2009
	AB010974*	Uruguay	Itagaki & Tsutsumi, 1998
	AB207148*	Australia/Ireland	Itagaki *et al*., 2005
	EU260058	Australia	Le *et al*., 2008
F. magna	EF534992*	Slovakia	Králová-Hromadová *et al*., 2008
	EF534993*	Czech Republic	Králová-Hromadová *et al*., 2008
	DQ683545*	Austria	unpublished
	EF534995*	USA	Králová-Hromadová *et al*., 2008
	EF612487	USA	Lotfy *et al*., 2008
	EF051080*	USA	Bildfell *et al*., 2007
	EF534994*	Canada	Králová-Hromadová *et al*., 2008
D. dendriticum	AB367789	Japan	unpublished
	AB369980	Japan	unpublished
	AB369981	Japan	unpublished
	DQ379986	Italy	Maurelli *et al*., 2007
	HM026461	Slovakia	current work
P. cervi	HM026462	Slovakia	current work

* - sequences applied in design of *F. hepatica*- and *F. magna*-specific primers (Králová-Hromadová *et al*., 2008)

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                                                                   DD_ITS2_SPEC_F →
FH    GTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTTTTGCCAGCTGGCGTGATCTCCTCTATGAGTAATCATGT--GAGGTG
FM    CTTACAAACTATCACGACGCCCAAATAGTCGTGGCTTGGGTTTTGCCAGCTGGCGGGATCTCCTCTATGAGTAATCA-AT--GAGGTG
DD    CTTACAAACTATCACGACGCCCAATAAGTCGTGGCTTGGATTTTGCCAGCTGGC----TTTACTCCCCAGTCGGAAACGTCAGGGGTG
PC    CTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGAATCTGCCAGCTGGCGTGATTTCCTCTGTGGTTCGCCACGT--GAGGTG

                                          PC_ITS2_SPEC_F →
FH    CCAGATCTATGGCGTTTCCCTAATGTATCCGGATGCACCCTTGTCTTGGCAGAAAGCCGTGGTGAGGTGCAGTGGCGGAATCGTGGTT
FM    CCAGATCTATGGCGTTTCCCTAATGTATCCGGATGCACCCTTGTCCTGGCAGAAGGCCGTGGTGAGGTGCGGTGGCGGAATCGTGGTT
DD    TCAGATCTATGGCGTTATCCTAATGTATCCGGATACA--CACACCTAGTTATCAG-ACAGGTGGAGATGTGTCTACGGAGTCGTGGCT
PC    CCAGATCTATGGCGTTTTCCTAATGTCTCCGGACACAACCGCGTCTTGCTGGTAGCGCAGACGAGGGTGTGGCGGTAGAGTCGTGGCT

                   FM_ITS2_SPEC_F →                             FH_ITS2_SPEC_F →
FH    TAATAATCGGGTTGGTACTCAGTTGTCAGTGTGTTTGGCGATCCCCTAGTCGGCACACTTATGATTTCTGGGATAATTCCATACCAGG
FM    TAATAAACGGGTTGGTAACCAGTTATCGTTGTGTTGGGCGATCCCCTAGTCGGCACATTTACGATTTCTGGGATGATCCCATACCAGG
DD    CAGT--ACTA--T-----TTATGCGCGCT-CTGTAGAACATCTCGTGTGGTGAAT--TCCGAAATACGGCCTTCTAATCCTGAC----
PC    CAGTGAACTG--TAATGGTAGCACGCTCTGCTGTTGTGCCTTTGTTAGTGTAACTGGCTTGAGATGCTATTGCCGTCCGTCTA-ATCA
                                                              ← DD_ITS2_SPEC_R

                                                                     ← FH_ITS2_SPEC_R
FH    CACGTTCCGTCACTGTCACTTTGTCATTGGTTTGATGCTGAACTTGGTCATGTGTCTGATGCTATTTTCATATAGCGACGGTACCCTT
FM    CACGTTCCACTACTGTCGCTTTATCGTCGGTTTGATGCTAGGCTTGGTCATGTATCTGATGCTACTGTTGTTTAAAGACGGTATCCTT
DD    ----------------------------------------------------------------------------------------
PC    TGATCACCTACTGTGGTGTTCTGTTAC-------------------------------------------------------------
       ← PC_ITS2_SPEC_R                                                ← FM_ITS2_SPEC_R
FH    CGTGGTCTGTCTTC
FM    CGTGATCAGTCTTC
DD    --------------
PC    --------------
```

Fig. 1. The ITS2 sequence alignment of *Fasciola hepatica* (FH), *Fascioloides magna* (FM), *Dicrocoelium dendriticum* (DD) and *Paramphistomum cervi* (PC). Underlined nucleotides in bold – positions of intraspecific variations. Nucleotides in grey field – sequence homology determined for four species. Nucleotides in boxes – species-specific primers.

Results and Discussion

The novelty of the current work lies in the original determination of ITS2 sequence structure of *P. cervi*. The ITS2 spacer was 286 bp long and no intraspecific polymorphism was detected among the three individuals studied. The ITS2 spacer of *D. dendriticum* herein sequenced was 241 bp long and all three sequenced individuals had identical ITS2 structure. Comparison of our data with ITS2 sequences of *D. dendriticum* retrieved from GenBank (Tab. 2) revealed the presence of three polymorphic sites (Fig. 1). The sequences were deposited in the GenBank under the accession numbers HM026461-62.
Specific primers for *P. cervi* and *D. dendriticum* were originally designed in the present study. They are localized in the selected ITS2 regions, for which no intraspecific variation and high interspecific sequence divergence were characteristic. Positions and orientations of primers are given in Figure 1; sequences of primers and size of amplified specific region are listed in Table 3.
Specific primers for *F. hepatica* and *F. magna* were already described in our previous work (Králová-Hromadová *et al*., 2008). *F. hepatica*-specific primers were designed according to the available sequence data on *F. hepatica* (see Tab. 2, accessions with*) and revealed four polymorphic sites within the ITS2 spacer (Králová-Hromadová *et al*., 2008) (Fig. 1). Since then, additional ITS2 sequences of worldwide fluke populations from Europe, Africa, Australia and Asia have been deposited in the GenBank. GenBank accession numbers, references, and countries of origin of all *F. hepatica* ITS2 sequences, linked with the current analysis, are given in Table 2. Since no additional polymorphic sites were determined within the ITS2 spacer of all so far available *F. hepatica* populations, previously designed *F. hepatica*-specific primers (Králová-Hromadová *et al*., 2008) were repeatedly used in the current work (Fig. 1; Tab. 3).
For *F. magna*, ribosomal ITS2 structure was determined to be 100 % identical for Slovak, Czech, Canadian and American individuals (Králová-Hromadová *et al*., 2008). Furthermore, also ITS2 sequences of giant liver fluke from Austria and USA (for details see Tab. 2) revealed no intraspecific polymorphisms. Therefore, position and structure of *F. magna*-specific primers (Králová-Hromadová *et al*., 2008) remained the same (Fig. 1; Tab. 3).
Specificity of primers of four flukes was tested on the genomic DNA of adult individuals. The primers annealed only in DNA of particular species (Fig. 2) and did not amplify DNA of remaining taxa (data not shown). The size of amplified fragments of all species is indicated in Table 3.

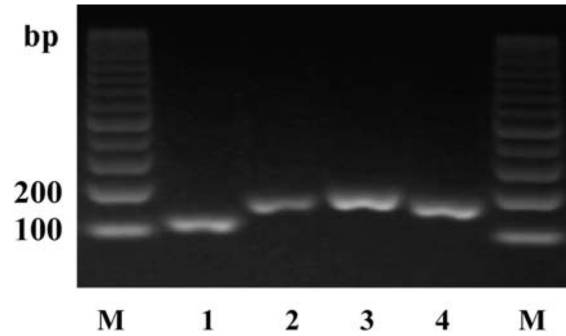

Figure 2. PCR amplification of *Fasciola hepatica* (FH), *Fascioloides magna* (FM), *Dicrocoelium dendriticum* (DD) and *Paramphistomum cervi* (PC) using respective species-specific ITS2 primers. M – molecular marker 100 bp ladder (Fermentas, Vilnius, Lithuania). Lane 1 – FH; 2 – FM; 3 – DD; 4 – PC.

Table 3. Ribosomal ITS2 primers for species-specific identification of *Fasciola hepatica* (FH), *Fascioloides magna* (FM), *Dicrocoelium dendriticum* (DD) and *Paramphistomum cervi* (PC)

Forward primer	Reverse primer	Amplified region
FH_ITS2_SPEC_F * 5´-CTTATGATTTCTGGGATAATT-3´	FH_ITS2_SPEC_R * 5´-CCGTCGCTATATGAAAA-3´	112 bp
FM_ITS2_SPEC_F * 5´-ACCAGTTATCGTTGTGTTG-3´	FM_ITS2_SPEC_R * 5´-CCGTCTTTAAACAACAG-3´	152 bp
DD_ITS2_SPEC_F § 5´-CCCCAGTCGGAAACGTCA-3´	DD_ITS2_SPEC_R § 5´-GATTAGAAGGCCGTATTTCGGA-3´	176 bp
PC_ITS2_SPEC_F § 5´-CGCGTCTTGCTGGTAGCGCAGAC-3´	PC_ITS2_SPEC_R § 5´-GTAACAGAACACCACAGTAGGTGATCA-3´	161 bp

* - primers designed in our previous study (Králová-Hromadová *et al*., 2008)
§ - originally designed primers

In the present work, ribosomal ITS2 molecular markers were designed for discrimination of liver and stomach flukes of ruminants. Intraspecific variation within geographically distant populations was detected to be either very low (*F. hepatica*; *D. dendriticum*) or even none (*F. magna*; *P. cervi*). On the other hand, interspecific heterogeneity was sufficient to define the regions which were used for design of species-specific primers providing only specific products during PCR amplification. The specificity of previously developed (*F. hepatica*; *F. magna*; Králová-Hromadová *et al*., 2008) and newly described (*D. dendriticum*; *P. cervi*; current data) primers was successfully tested on genomic DNA isolated from adults as well as on DNA isolated from eggs of all studied fluke species (Oberhauserová *et al*., in press). Ribosomal ITS markers are promising tool for molecular discrimination of morphologically hardly distinguishable *F. hepatica*, *F. magna* and *P. cervi* eggs after coprological examinations.

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