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ORIGINAL PAPER

Genetic variability among *Fasciola hepatica* samples from different host species and geographical localities in Spain revealed by the novel SRAP marker

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Abstract A collection of 483 samples representing *Fasciola* from six naturally infected host species and 16 localities in Spain, previously identified morphologically and genetically as *Fasciola hepatica,* was characterized by a novel genetic marker, namely sequence-related amplified polymorphism (SRAP), aiming to reveal genetic variability within *F. hepatica* in Spain. Visualization of amplification fragments was carried out on 6% denaturing polyacrylamide gels, followed by staining with 0.1% $AgNO_3$ solution. Ten SRAP primer combinations were tested—six of them turned out to be polymorphic. Thirty-four representative *F. hepatica* samples from six host species and 16 geographical localities showed polymorphic banding patterns using SRAP primer combinations and were grouped into four major clusters using the unweighted pair-group method with arithmetic averages, indicating the existence of genetic variability within the examined *F. hepatica* samples. These four clusters were not related to particular host species and/or geographical origins of the samples. The results of the present study revealed that SRAP markers were useful in revealing sufficient polymorphism in *F. hepatica* samples from Spain and had implications for studying the population genetic structure of the Spanish *F. hepatica*. To our knowledge, this is the first application of SRAP marker to study genetic variation in parasites of human and animal health significance.

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Introduction

Fasciola hepatica is the common liver fluke of a wide range species of animals and has a global geographical distribution (Spithill and Dalton 1998; Mas-Coma et al. 2005). In Spain, *F. hepatica* occurs in both domestic and wild hosts, including European rabbit (*Oryctolagus cuniculus*), hare (*Lepus granatensis*), donkey (*Equus asinus*), horse (*Equus caballus*), pig and wild boar (*Sus scrofa*), fallow deer (*Dama*

dama), cattle (*Bos taurus*), domestic sheep (*Ovis aries*), goat (*Capra hircus*), Iberian ibex (*Capra pyrenaica*), red deer (*Cervus elaphus*), European mouflon (*Ovis aries*), chamois (*Rupicapra pyrenaica*), roe deer (*Capreolus capreolus*), and man (Cordero del Campillo et al. 1994; Lavín et al. 1995; Pérez et al. 2006; Alasaad et al. 2007, 2008). The prevalence in domestic animals is high, up to 85.1% and 83.3% in cattle and ovine, respectively, under field conditions from Galicia (Northwest Spain; Sánchez-Andrade et al. 2000; Paz-Silva et al. 2003) but less important in wildlife, 0.7% in Iberian ibex (Alasaad et al. 2008).

Despite the substantial economic losses caused by *Fasciola*, estimated at US$ 2 billion per year worldwide (Spithill and Dalton 1998), and the vast distribution of this cosmopolitan parasite (Mas-Coma et al. 2003), little attention was given to study the morphometric and/or genetic variation of *F. hepatica* from different host species and/or geographical localities (Valero et al. 2001a, b; Periago et al. 2006; Alasaad et al. 2007).

Genetic approaches employing a range of molecular makers have proven useful for parasite identification and studies of genetic variability among parasite populations (Gasser 1999; Prichard and Tait 2001; Gasser 2006; Bildfell et al. 2007; Zhu et al. 2007). Previous studies using nuclear ribosomal DNA markers, mitochondrial DNA markers, and isoelectric focusing banding patterns of whole-body protein have demonstrated the existence of genetic variability among different populations of *F. hepatica* (Lee et al. 1992; Lotfy et al. 2002; Huang et al. 2004; Morozova et al. 2004). A recent study has showed a slight sequence variation in the second internal transcribed spacer (ITS-2; 1/362, 0.3%) among *Fasciola* samples from different host species and geographical localities in Spain and that Spanish *F. hepatica* examined in that study differed from *Fasciola* from elsewhere by two nucleotides in the ITS-2 (Alasaad et al. 2007). Another study concluded that there were some genetic variations in *F. hepatica* by studying the 28S rDNA (Vara-Del Río et al. 2007).

Sequence-related amplified polymorphism (SRAP) is a novel and efficient genetic marker system, revealing genetic variation in open reading frames among related organisms (Li and Quiros 2001). Because of its simplicity and efficiency, SRAP has been used in the construction of genetic maps (Li and Quiros 2001), comparative genetic studies (Li et al. 2003), and examination of genetic diversities (Riaz et al. 2001; Ferriol et al. 2003) in many plant species. It has also been used to study genetic variations in seaweeds (Qiao et al. 2007). Nevertheless, to our knowledge, there were no reports using this marker in the field of parasitology before the present study. The objective of the present study was to investigate the genetic variability among *F. hepatica* from different host species and geographical localities in Spain using the novel SRAP marker.

Table 1 *Fasciola hepatica* samples and host animals used in this study and their host origins and geographical locations in Spain, together with 34 representative *F. hepatica* samples which are used for cluster analysis

Codes	Host species and codes	Geographical locations and codes			No. of animals	No. of specimens	No. of representative specimens and codes
FhDd1	Fallow deer (*Dama dama*)	Dd	Cuenca	1	13	36	2 FhDd1 (1) & FhDd1 (2)
FhCe1	Red deer (*Cervus elaphus*)	Ce	Cuenca	1	8	20	2 FhCe1 (1) & FhCe1 (2)
FhOa1	European mouflon (*Ovis aries*)	Oa	Cuenca	1	5	29	1 FhOa1
FhEc2	Horse (*Equus caballus*)	Ec	Valencia	2	4	8	1 FhEc2
FhOa3	Ovine (*Ovis aries*)	Oa	País Vasco	3	5	36	2 FhOa3 (1) & FhOa3 (2)
FhBt2	Bovine (*Bos taurus*)	Bt	Valencia	2	1	4	1 FhBt2
FhBt4	Bovine (*Bos taurus*)	Bt	Barcelona	4	2	3	2 FhBt4 (1) & FhBt4 (2)
FhBt5	Bovine (*Bos taurus*)	Bt	Mallorca (Balearic Islands)	5	3	16	2 FhBt5 (1) & FhBt5 (2)
FhBt6	Bovine (*Bos taurus*)	Bt	Tenerife (Canary Islands)	6	3	24	2 FhBt6 (1) & FhBt6 (2)
FhBt7	Bovine (*Bos taurus*)	Bt	Ávila	7	1	5	1 FhBt7
FhBt8	Bovine (*Bos taurus*)	Bt	Lugo	8	8	43	2 FhBt8 (1) & FhBt8 (2)
FhBt9	Bovine (*Bos taurus*)	Bt	Cádiz	9	9	44	2 FhBt9 (1) & FhBt9 (2)
FhBt10	Bovine (*Bos taurus*)	Bt	Oviedo	10	10	35	2 FhBt10 (1) & FhBt10 (2)
FhBt11	Bovine (*Bos taurus*)	Bt	Castellón	11	5	11	2 FhBt11 (1) & FhBt11 (2)
FhBt12	Bovine (*Bos taurus*)	Bt	Segovia	12	6	32	2 FhBt12 (1) & FhBt12 (2)
FhBt13	Bovine (*Bos taurus*)	Bt	Logroño	13	5	33	2 FhBt13 (1) & FhBt13 (2)
FhBt14	Bovine (*Bos taurus*)	Bt	Pamplona	14	5	34	2 FhBt14 (1) & FhBt14 (2)
FhBt15	Bovine (*Bos taurus*)	Bt	La Rioja	15	7	36	2 FhBt15 (1) & FhBt15 (2)
FhBt16	Bovine (*Bos taurus*)	Bt	Valladolid	16	8	34	2 FhBt16 (1) & FhBt16 (2)

Fig. 1 Spain map showing approximate sites for sample collection. *Codes next to the sites* represent the sample codes in Table 1

Materials and methods

Parasite collection and identification

Between 2002 and 2006, 483 adult trematodes were collected from the liver of 108 naturally infected animals; all of them were local animals, representing six species and 16 geographical locations in Spain. Individual flukes were washed extensively in physiological saline, identified morphologically as *F. hepatica* according to existing keys and descriptions (Yamaguti 1958) and then fixed in 70% ethanol until extraction of genomic DNA. The identity of 18 representative flukes was confirmed by sequencing the internal transcribed spacers of nuclear ribosomal DNA (Alasaad et al. 2007). The codes, host species, and geographical origins of the fluke samples used in the present study are listed in Table 1 and Fig. 1.

Extraction and purification of genomic DNA

Genomic DNA was extracted from a portion of individual adult trematodes treated with sodium dodecy1-sulfate or proteinase K (Zhu et al. 2002), column-purified using Wizard^{TM} DNA Clean-Up System (Promega), and then eluted into 65 μl H_2O according to the manufacturer's recommendations. All the DNA samples were stored at -20°C until further use.

SRAP procedure

The SRAP technique described by Li and Quiros (2001) was followed with slight modifications. Ten different primer combinations were employed, using five forward primers and five reverse primers. Each 25 μl polymerase chain reaction (PCR) mixture consisted of 1.0 μl genomic DNA, 2.5 μl PCR buffer, 200 μM of dNTPs, 4 mM of $MgCl_2$, 0.5 μM of primer, 1.25 unit of *Taq* polymerase (TaKaRa), and sterile double-distilled water. Samples were subjected to the following thermal profile for amplification in a thermal cycler (Biometra): 5 min of initial denaturation at 94°C, then five cycles of three steps: 1 min of denaturation at 94°C, 1 min of annealing at 35°C, 1 min of elongation at 72°C, followed by further 35 cycles with annealing temperature being increased to 50°C, with a final elongation step of 5 min at 72°C. Separation of amplified fragments was accomplished on 6% denaturing acrylamide gels [acrylamide-bisacrylamide (19:1), 1×TBE] at 90 V for 2.5 h. The gel was stained with 0.1% $AgNO_3$ solution and then photographed by digital camera. Primer banding patterns which were difficult to score and those primers that failed to amplify consistently were excluded. Consequently, only six primer combinations out of ten were selected and employed to amplify 483 *F. hepatica* samples (Table 2, Fig. 2).

Data analysis

Photographs of denaturing acrylamide gels showing different banding patterns were digitalized and analyzed. SRAP fragments were scored for presence (the presence of the specific allele, coded "1") or absence (the absence of the specific allele, coded "0") in each sample. The distance matrix and dendrogram were constructed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.1 software package. An unweighted pair-group

Table 2 Sequence information of the ten primers used for SRAP analysis

Forward primers (17 bp)	Reverse primers (18 bp)
ME1 5′-TGAGTCCAAACCGG ATA-3′	EM1 5′-GACTGCGTACGAATT AAT-3′
ME2 5′-TGAGTCCAAACCGG AGC-3′	EM3 5′-GACTGCGTACGAATT GAC-3′
ME3 5′-TGAGTCCAAACCGG AAT-3′	EM4 5′-GACTGCGTACGAATT TGA-3′
ME4 5′-TGAGTCCAAACCGG ACC-3′	EM5 5′-GACTGCGTACGAATT AAC-3′
ME5 5′-TGAGTCCAAACCGG AAG-3′	EM6 5′-GACTGCGTACGAATT GCA-3′

method with arithmetic averages (UPGMA) dendrogram was constructed. FIND module (part of the NTSYS package) was used to identify all trees that could result from different choices of tied similarity of dissimilarity values. To test the robustness of the tree topology, the trees were compiled by Coordinate Open Neural info-Space of European Network e-centers (part of the NTSYS package).

Results and discussion

A total of ten different combinations of SRAP primers, using five forward primers and five reverse primers, were evaluated on seven representative flukes to test the amplification efficiency of each primer pair, and representative results are shown in Fig. 2. Six primer combinations displayed better polymorphisms and, thus, were chosen for the amplification of 483 *F. hepatica* samples. These six primer combinations were ME1/EM3, ME2/EM1, ME3/EM6, ME4/EM1, ME4/EM6, and ME5/EM4 (Table 2). The number of fragments amplified from *F. hepatica* samples using these primer combinations ranged from 12 to 20, with an average of 15 polymorphic bands per primer combination, which is consistent with results of previous studies (Li and Quiros 2001; Budak et al. 2004; Ferriol et al. 2004). A total of 51 main polymorphic bands were observed, ranging in size from approximately 100 to 2000 bp. Thirty-four *F. hepatica* samples, representing the 483 *F. hepatica* samples from six host species and 16 geographical localities examined in this study, displayed representative polymorphic banding patterns using SRAP primer combinations (Table 2, Fig. 3), and hence were used for cluster analysis.

The UPGMA clustering algorithm grouped the 34 representative *F. hepatica* samples into four clusters based on SRAP profiles, with the exception of four *F. hepatica* specimens, namely FhCe1(1), FhBt14(2), FhBt4(2), and FhOa3(1). *F. hepatica* samples from different host species and/or geographical localities tended to cluster in the same group, and *F. hepatica* samples from the same host species, e.g., FhOa3 (1) and FhOa3 (2); FhCe1 (1) and FhCe1 (2), and/or from the same geographical location, e.g., FhBt12 (1) and FhBt12 (2); FhEc2 and FhBt2, were grouped into different clusters (Fig. 4).

The possible explanation for such a low level of intraspecific variation among *F. hepatica* samples from different host species and/or geographical localities in Spain

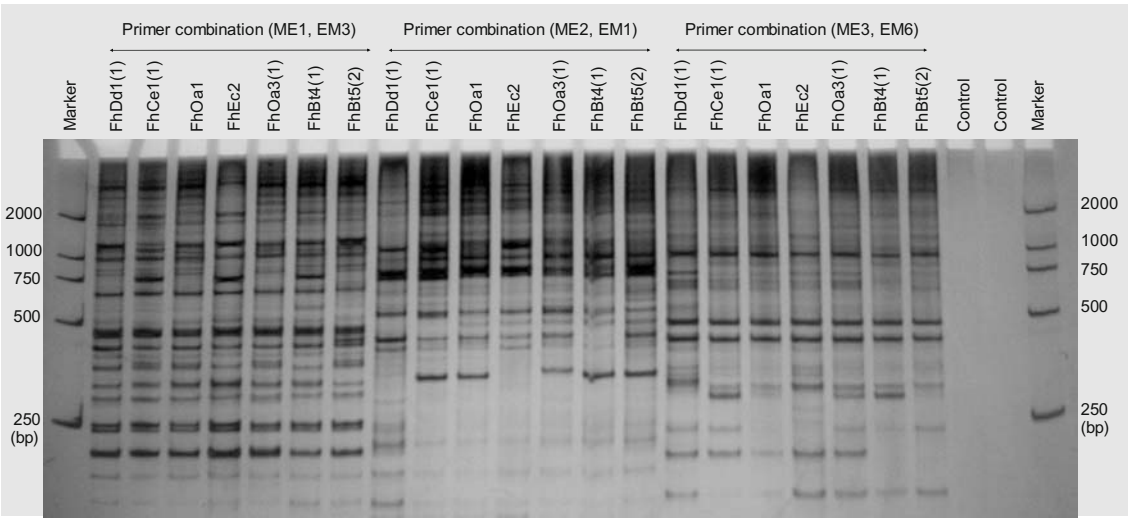

Fig. 2 Representative gel showing SRAP profiles produced for seven representative *Fasciola hepatica* samples used to test the amplification efficiency of SRAP primers by primer combinations ME1/EM3, ME2/EM1 and ME3/EM6 (cf. Table 2), respectively. '*Control*' represents no-DNA control. '*Marker*' represents a DNA size marker 2000 (ordinate values in bp). *Codes* in this figure represent the sample codes in Table 1 and Fig. 1

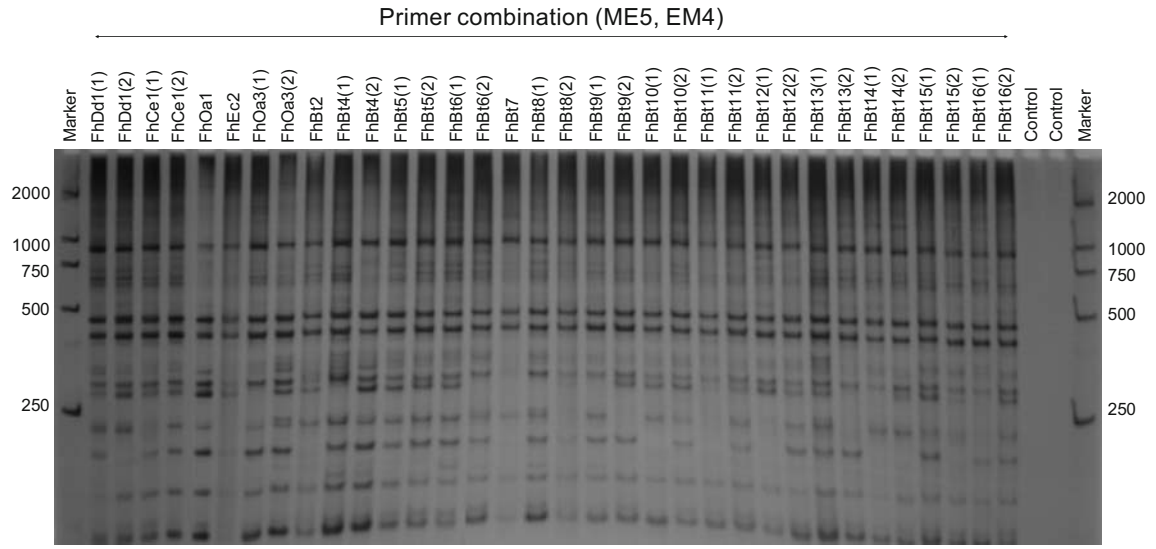

Fig. 3 Representative SRAP profiles produced for 34 representative *Fasciola hepatica* samples using primer combination ME5–EM4. '*Control*' represents no-DNA control. '*Marker*' represents a DNA size marker 2000 (ordinate values in bp). *Codes* in this figure represent the sample codes in Table 1 and Fig. 1

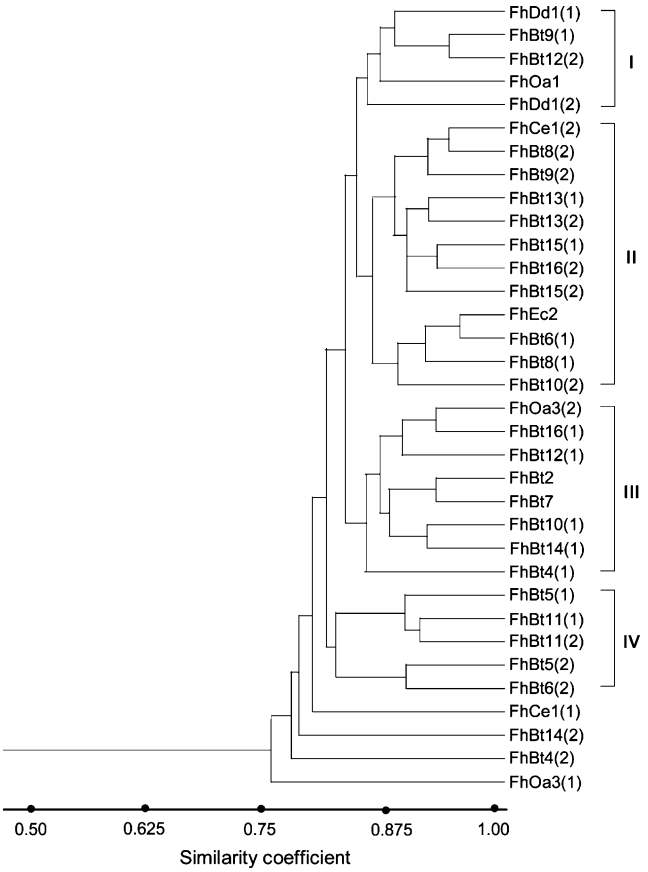

Fig. 4 Dendrogram of 34 representative *Fasciola hepatica* samples from different host species and geographical localities in Spain constructed on the basis of genetic similarity analysis using six SRAP primer combinations. *Codes* in this dendrogram represent the sample codes in Table 1 and Fig. 1. The resulting four clusters are labelled as *I*, *II*, *III*, and *IV*

may be that the examined sequences belonged to the coding regions of the genomes of *F. hepatica* samples and these regions did not mutate much due to their functional constraints (Crow and Simmons 1983; Crow 2000). This may be related to the lifecycle of *Fasciola*, which probably acts against speciation processes, decreasing at the same time their genetic variability and, thus, allowing *Fasciola* to infect new host species (Alasaad et al. 2007; Vara-Del Río et al. 2007).

As expected, SRAP marker was highly polymorphic and inexpensive technique to establish the genetic variability among *Fasciola* samples from different host species and geographical localities. Nevertheless, as this novel marker targets the whole genomic DNA and some of the resulting bands are so large in size, working with such marker requires genomic DNA samples of good quality.

In conclusion, the present study demonstrated that genetic variability in the coding regions of the genomes of 483 *F. hepatica* samples from six host species and 16 geographical locations in Spain was low, indicating that there seemed no genetic association between *F. hepatica* and their hosts and/or geographical locations in Spain. This study also demonstrated that the SRAP is a useful genetic marker for the studies of genetic variability in parasite populations, and may find broad applications in parasitology.

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