

PRIMER NOTE

Isolation and characterization of microsatellite markers in the liver fluke (*Fasciola hepatica*)

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Abstract

Six microsatellite markers were isolated from *Fasciola hepatica*, a re-emerging parasite that causes important veterinary and public health problems. In a sample of 52 liver flukes from a region of hyperendemicity (Bolivian Altiplano), five microsatellite were polymorphic. Our results showed that liver flukes present important genetic variability, suggesting a preferential outcrossing reproduction mode for this hermaphroditic parasite.

Keywords: *Fasciola hepatica*, liver fluke, microsatellite

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The liver fluke (*Fasciola hepatica*) is a re-emerging food-borne Trematode that causes important veterinary and public health problems worldwide (see Hurtrez-Boussès *et al.* 2001). The highest human infection rates have been recorded in the Bolivian Altiplano (Esteban *et al.* 1999). In this area of hyperendemicity, the liver fluke has been recently introduced from European strains (see Mas-Coma *et al.* 2001), conjointly with its intermediate host, the freshwater snail *Lymnaea truncatula* (see Meunier *et al.* 2001). Here, we report on the first isolation of microsatellite markers in *F. hepatica*.

Identification and characterization of microsatellite markers were made according to Estoup & Martin (1996). A complete digestion of *F. hepatica* DNA was made with *Sau3A* enzyme. Fragments between 300 and 900 bp served to construct a genomic library (3185 clones). The clones were screened for (CA)₁₀ and (GA)₁₀ probes, marked with Digoxigenin. Positive clones were detected using an anti-Dig labelling kit (Roche Diagnostics). In total, 39 positive clones were sequenced in 4% polyacrylamide gels (8 M urea), using (α -³³P)-ATP (Amersham) with T7 sequencing kit (Pharmacia). We retained five of these sequences that presented microsatellite motifs and suitable flanking regions (see Table 1). Moreover, we screened 122 sequences already published in EMBL or GenBank and retained one of them (EMBL Accession no.: FH222CBP), which presented a suitable microsatellite. Therefore, we used a total of six sequences, for which we defined primers (see Table 1) using PRIMER 3 software

(Rozen & Skaletsky 2000; http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

Adults of *F. hepatica* ($N = 52$) were collected in February 1996, in slaughterhouses of the Bolivian Altiplano. Liver flukes were sampled by dissecting the livers of definitive hosts (sheep, pig and cow) and were immediately stored in ethanol 80%.

DNA extraction was performed using 500 μ L Chelex 10% (Estoup & Martin 1996). DNA extracts were diluted 1/10 and used as the template for microsatellite amplifications. Microsatellite loci were amplified from 6 μ L of diluted DNA for the locus *FH26/1* and 4 μ L DNA for all other loci, in a 20 μ L reaction volume containing 2 μ L buffer 10 \times (Promega), 1.5 mM Mg²⁺ (Promega), 2 mM dNTPs (Invitrogen/Life Technologies), 5–10 pmol of each primer (see Table 1 for exact amount) and 1.3 U of *Taq* DNA polymerase (Promega). Thermocycling, performed using a MJ-Research PTC 100 96-well, consisted of an initial denaturation at 94 °C for 4 min, 30 cycles at 94 °C for 30 s, annealing temperature (see Table 1 for specific annealing temperatures) for 30 s, 72 °C for 30 s and a final elongation step at 72 °C for 10 min. Fluorescently labelled primers for use on an ABI automated sequencer were purchased (Applied Biosystems; Table 1). One microlitre of polymerase chain reaction (PCR) products amplified from each DNA sample was pooled in two groups of loci (*FH1*, *FH25*, *FH26/1* and *FH222CBP* group and *FH15–FH23* group), together with 0.5 μ L of internal size standards (GENESCAN 500 LIZ, Applied Biosystems), and diluted in Hi-Di Formamide (20 μ L QSP) for automatic electrophoresis. Measurements of allele length were automated

Table 1 Characteristics of microsatellite loci of *Fasciola hepatica* and genetic diversity

Locus	Accession no	Repeat array	Primer sequences (5' → 3')	T_a (°C)	Amount of primer (pmol)	Dye	No. of alleles	Allele size (bp)	H_O	H_E	F_{IS} (P-value)
FH1	AJ508374	(TC) ₉	F: TTGGATTAGGTCGTTTCG R: CACCAACCTCTGTTATG	50	8	NED	1	115	—	—	—
FH15	AJ508371	(GT) ₅ AC(GTAT) ₂ GCAT (GTAT) ₂ (GT) ₂ CT(GT) ₉	F: TTCITTCAGCCGAAATTCG R: AATTGTTGCTCCTAACTGG	48	8	VIC	6	229–243	0.307	0.369	0.166 (NS)
FH23	AJ508372	(GTTT) ₄ (GT) ₈ CT(GT) ₆ CT (GT) ₇ (GGT) ₄ (GT) ₆ AA(GT) ₃	F: AGCACGAGAAAATTCAG R: GCGAATTAATACAGCAACC	48	8	6-FAM	4	274–297	0.065	0.591	0.89 (< 0.05)
FH25	AJ508373	(AC) ₈	F: TAGCGTTTTGACTCTAC R: GATTCGGTTAGGATGTTG	51	10	NED	4	276–282	0.404	0.423	–0.090 (NS)
FH26/1	AJ508370	(CA) ₅	F: TGCATGTAGAAGTAACGG R: AATAGATTCACAGGTGTGAC	45	8	VIC	2	122–136	0.250	0.370	0.324 (NS)
FH222CBP	AJ003821	(CA) ₁₇	F: GTGGATCCCACTGTGAGAC R: TGTCCAACCTGCATGACCAT	50	5	6-FAM	12	146–176	0.846	0.867	–0.024 (NS)

T_a = annealing temperature, Amount of primer = the amount of each primer in 20 µL of PCR.

F_{IS} value was estimated according to Weir & Cockerham (1984); P-value after sequential Bonferroni correction (Rice 1989). This probability provides an unbiased estimator of the probability that there is not significant deviation from Hardy-Weinberg equilibrium. H_O , H_E , F_{IS} and P-values were computed using FSTAT 2.9-3.2 software (Goudet 1995).

with GENESCAN software (Applied Biosystems). Each liver fluke was genotyped at the six studied loci.

Five of the six studied loci are polymorphic, with on average (\pm SD) 5.6 (\pm 3.8) different alleles (Table 1). There is no significant linkage disequilibrium between any pair of loci. Observed and expected heterozygosities are high (mean H_O = 0.374 \pm 0.291; mean H_E = 0.524 \pm 0.212). Deviations from Hardy-Weinberg equilibrium are nonsignificant, except at locus *FH23* where there is a significant deficit in heterozygotes (Table 1). Such a divergence at one locus may be due to the presence of null alleles at this locus. Assuming the existence of a null allele at locus *FH23*, our results support a panmictic reproduction mode in liver fluke.

The demonstrated genetic variability of the liver fluke should be taken into account in future prospects of treatments and vaccines, because some variants may be able to escape the host immune system and/or treatments.

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