Analysis of genetic variability of *Fasciola hepatica* populations from different geographical locations by ISSR-PCR

D. ROBLES-PÉREZ[1], P. GARCÍA-GARCÍA[2], J. M. MARTÍNEZ-PÉREZ[1], F. A. ROJO-VÁZQUEZ[1,3] *and* M. MARTÍNEZ-VALLADARES[3]*

[1] *Faculty of Veterinary Medicine, University of León, Campus de Vegazana, 24071 León, Spain*
[2] *Faculty of Biological and Environmental Sciences, University of León, Campus de Vegazana, 24071 León, Spain*
[3] *Instituto de Ganadería de Montaña (CSIC-ULE), Finca Marzanas, 24346 Grulleros, León, Spain*

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SUMMARY

Inter-simple sequence repeats markers were used to determinate the genetic variability of *Fasciola hepatica* populations recovered from sheep and cattle from Spain (Sp1, Sp2, Sp3 and Sp4), UK (Eng), Ireland (Ir) and Mexico (Mex). Twenty five primers were tested but only five produced 39 reproducible bands, being 71·79% polymorphic bands. This percentage ranged from 10·26% in Sp4 to 48·72% in Sp1, and per host between 28·21 and 48·72% in sheep and between 10·26 and 38·46% in cattle. This relatively low range of genetic diversity within populations, with a mean of 34·40%, implies that a large proportion of variation resided among populations. The population differentiation (Gst = 0·547) indicated that 54·7% of variation is due to differences between populations and 45·3% due to differences within population. The Nei's distance ranged between 0·091 and 0·230 in sheep and between 0·150 and 0·337 in cattle. The genetic relationships between populations and individuals were shown by a UPGMA dendrogram and a principal coordinate analysis; both grouped all populations separately from Sp4, a population of from the Midwest of Spain with the lowest level of diversity. Small genetic distances were observed between Eng and Ir, on the one hand, and Sp1, Sp2, Sp3, from the Northwest of Spain, together with Mex, on the other.

Key words: *Fasciola hepatica*, ISSR-PCR, genetic variability, polymorphism, population genetic structure.

INTRODUCTION

Fasciolosis is a worldwide distributed parasitic disease affecting a wide range of mammalian species, mainly ruminants, but also humans. In animals it causes significant reductions in productive parameters such as a decrease of body weight, milk production and liver condemnation at abattoirs. The causative agent, *Fasciola hepatica*, is a helminth parasite which can be found infecting animals in many countries throughout Europe, America and Oceania. In Africa and Asia *F. hepatica* overlaps with the species *Fasciola gigantica*. The prevalence of fasciolosis is increasing in certain parts of the world due to different causes such as climate change (Rojo-Vázquez *et al*. 2012) and/or man-made environmental modifications (Martínez-Valladares *et al*. 2013*b*).

The knowledge of the amount of the genetic variability is of crucial importance because since Darwin it is known that evolution and adaptation depends on the existence of heritable variability within a species to generate the differences between ancestral and descendant populations (Charlesworth and Charlesworth, 2009). On the other hand, the distribution of the genetic diversity into and between populations allow us to infer the level of genetic flow, the effective population size, the mating system or the inbreeding level, among other characteristics (Allendorf *et al*. 2013). These data can be used to construct models in order to predict the infection capacity of specific genotypes (Vázquez-Prieto *et al*. 2014). Therefore, the study of genetic variability of strains infecting the farm animals is essential to improve the understanding of parasite epidemiology and the control disease.

Up to date, several methods have been carried out to characterize genetically *F. hepatica* and determine its variability. Among them, Semyenova *et al*. (2003) have described the random amplified polymorphic DNA (RAPD), which is based on the use of a single oligonucleotide with an arbitrary sequence that hybridizes with DNA; however, this technique has low specificity and reproducibility. Also Walker *et al*. (2007) used restriction fragment length polymorphisms (RFLP) to determine the genetic variability of different isolates of *F. hepatica*. There are different studies in which other techniques were described such as polymerase chain reaction (PCR) amplification of simple sequence repeat (SSR) (Hurtrez-Bousses *et al*. 2004), detection of sequence-related amplified polymorphism (SRAP) (Alasaad *et al*. 2008; Li *et al*. 2009), use of allozyme markers (Vázquez-Prieto *et al*. 2011) or sequence analysis of

* Corresponding author: Instituto de Ganadería de Montaña (CSIC-ULE), Finca Marzanas, 24346 Grulleros, León, Spain.
E-mail mmarva@unileon.es

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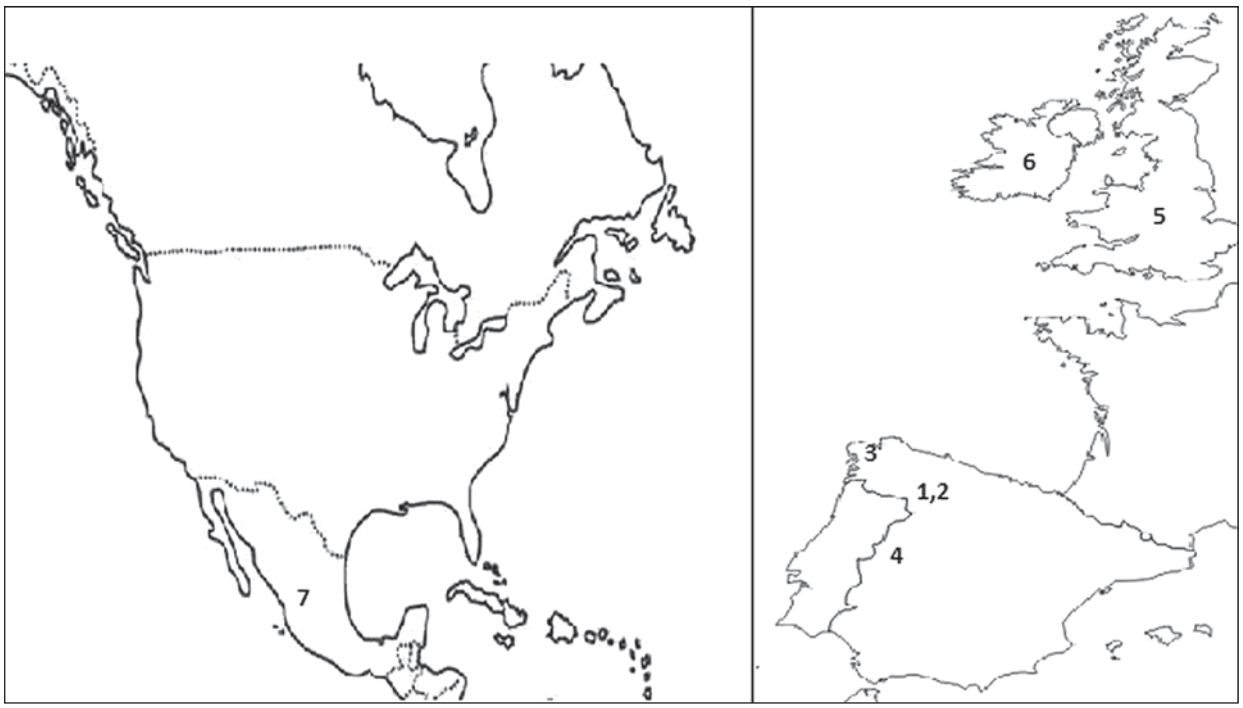

Fig. 1. Locations of different populations of *F. hepatica*. 1: Sp1. 2: Sp2. 3: Sp3. 4: Sp4. 5: Eng. 6: Ir. 7: Mex.

genomic and mitochondrial gene markers (Teofanova *et al*. 2011; Farjallah *et al*. 2013; Martínez-Valladares and Rojo-Vázquez 2014).

Alternatively, Zietkiewicz *et al*. (1994) developed a PCR based on the amplification of inter-microsatellites markers. Inter-simple sequence repeats (ISSRs) are a type of DNA marker which involves the use of microsatellite sequences, repetition of a di-, tri- or tetranucleotide, as primers in the PCR (Gupta *et al*. 1994; Zietkiewicz *et al*. 1994). The resulting bands are sequences of genome between two identical microsatellite primers with an opposite orientation on the DNA strand (Zietkiewicz *et al*. 1994). The ISSR technique is known to generate a large number of polymorphic bands. Moreover, this technique overcomes the technical limitations of RFLP and RAPD.

ISSR-PCR has already been used for others authors to study the genetic variability in parasites such as *Trichinella* (Fonseca-Salamanca *et al*. 2006), *Trypanosoma evansi* (Njiru *et al*. 2007) and *Schistosoma japonicum* (Zhao *et al*. 2009). However, the present study is the first report that uses this technique to study the genetic variation of different populations of *F. hepatica* from different geographical locations.

MATERIALS AND METHODS

Fasciola hepatica *populations and DNA extraction*

A total of 40 *F. hepatica* adults were collected from the livers of infected hosts belonging to the following geographical locations (Fig. 1): Sp1 (Toral de los Guzmanes, León; Spain), Sp2 (Torina, León; Spain), Sp3 (La Coruña; Spain), Sp4 (Cáceres; Spain), Eng (Shrewsbury/South Gloucester strain, Ridgeway Research Ltd Company; UK), Ir (Ireland) and Mex (México). The number of adults used per location is shown in Table 1. Individual flukes were washed extensively in physiological saline, identified morphologically to genus and species according to existing keys and descriptions (Yamaguti, 1958).

Total genomic DNA was extracted from a single piece of adult using the commercial kit SpeedTools Tissue DNA Extraction Kit (*Biotools*). DNA samples were resuspended in 60 μL of buffer BBE (5 mM Tris/HCl, pH 8·5) and stored at −20 °C until use.

ISSR-PCR amplification

For ISSR-PCR analysis, 25 primers randomly selected from the University of British Columbia primer set #9 (UBC) (Table 2) were screened using seven pools of DNA from each population. Only the five primers which produced clear and reproducible bands (UBC846, UBC848, UBC855, UBC856 and UBC881) were selected (Fig. 2). Then, the ISSR-PCR was carried out with all individual samples of each population and with the five selected primers to construct a matrix in function of the presence or absence of bands.

The reaction mixture (20 μL) contained 0·4 μL Taq polymerase, 0·2 mM deoxynucleotide triphosphate (dNTP's), 1·5 μM buffer + $MgCl_2$, 0·5 μM primers and 1 μL DNA diluted ten times of each individual of each population. The thermocycler used (Bio-Rad) was set to 2 min at 95 °C, followed by 40 cycles each of 30 s at 95 °C, 30 s at annealing temperature indicated in Table 2 for each primer

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Table 1. Number and percentage (%) of polymorphic bands (PB), number of alleles per locus (A), effective number of alleles per locus (Ae) and Nei's genetic diversity (H) per population

Isolate	Species	Number of adults	PB (%)	A	Ae	H
Sp1	Sheep	7	19 (48·72%)	1·487 ± 0·506	1·273 ± 0·355	0·163 ± 0·193
Sp2	Sheep	7	18 (46·15%)	1·462 ± 0·505	1·315 ± 0·403	0·177 ± 0·213
Sp3	Cow	5	11 (28·21%)	1·282 ± 0·456	1·217 ± 0·371	0·120 ± 0·199
Sp4	Cow	5	4 (10·26%)	1·102 ± 0·307	1·040 ± 0·128	0·028 ± 0·087
Eng	Sheep	6	11 (28·21%)	1·282 ± 0·456	1·196 ± 0·357	0·109 ± 0·189
Ir	Cow	5	15 (38·46%)	1·385 ± 0·493	1·241 ± 0·355	0·140 ± 0·194
Mex	Cow	5	12 (30·77%)	1·308 ± 0·468	1·196 ± 0·335	0·114 ± 0·185
Mean	–	–	12·9 (34·40%)	1·330 ± 0·456	1·211 ± 0·329	0·122 ± 0·180

Table 2. Primers randomly selected from UBC (University of British Columbia) primer set #9

Primer name	Sequence 5′ – 3′	T_m (°C)
UBC801	ATATATATATATATATT	20
UBC803	ATATATATATATATATC	20
UBC805	TATATATATATATATAC	20
UBC807	AGAGAGAGAGAGAGAGT	40
UBC812	GAGAGAGAGAGAGAGAA	40
UBC823	TCTCTCTCTCTCTCTCC	46
UBC824	TCTCTCTCTCTCTCTCG	49·1
UBC825	ACACACACACACACACT	49·1
UBC826	ACACACACACACACACC	53
UBC827	ACACACACACACACACG	53·7
UBC834	AGAGAGAGAGAGAGAGYT	50
UBC835	AGAGAGAGAGAGAGAGYC	40
UBC836	AGAGAGAGAGAGAGAGYA	40
UBC846	CACACACACACACACART	53
UBC847	CACACACACACACACARC	46
UBC848	CACACACACACACACARG	50
UBC853	TCTCTCTCTCTCTCTCRT	53·7
UBC854	TCTCTCTCTCTCTCTCRG	50
UBC855	ACACACACACACACACYT	59·1
UBC856	ACACACACACACACACYA	50
UBC857	ACACACACACACACACYG	56·7
UBC866	CTCCTCCTCCTCCTCCTC	59·1
UBC871	TATTATTATTATTATTAT	30
UBC881	GGGTGGGGTGGGGTG	63
UBC900	ACTTCCCCACAGGTTAACACA	63

In bold letters, the primers selected for the study. Y=C/T. R=A/G.

and 90 s at 72 °C and finally an extension step at 72 °C for 10 min. The amplification products were analysed by electrophoresis in 1·5% agarose TBE (Tris base, boric acid and EDTA) gel, stained with GelRed and visualized by 'Image Lab' statistical software (Bio-Rad) to determinate the reproducible bands in each individual.

All amplifications were carried out in triplicate with the aim to confirm the results.

Data analysis

A matrix was constructed from amplified fragments in function of the presence (1) or absence (0) of bands. The fragments showing differences among samples were referred to as polymorphic bands (Li *et al.* 2009). By the software POPGENE (1·32) (Yeh *et al.* 1997) several variability parameters were estimated, such as the number and percentage of polymorphic bands, the number of alleles per locus (A), the effective number of alleles per locus (Ae), the Nei's genetic diversity (H), the total genetic diversity (Ht), the intrapopulation genetic variability (Hs) and the interpopulation differentiation (Gst). For the analysis of interpopulation relationships, Nei's distances were calculated and the resulting matrix was used to construct a dendrogram by UPGMA cluster analysis. Also, a bootstrap analysis (using 1000 replicates) was carried out by Phylip software package v3.69 (Felsenstein 2005) to confirm the robustness of the results. In order to visualize the genetic relationships among all samples, the Jaccard similarity index was estimated between all pairs, and a principal coordinate analysis (PCA) was performed by means of GenAlEx 6·501 (Peakall and Smouse, 2012).

RESULTS

With the aim to determine the intra-specific variability of *F. hepatica*, five ISSR primers from UBC (UBC846, UBC848, UBC855, UBC856 and UBC881) were selected. These primers generated 13, 6, 5, 7 and 8 bands, respectively, 39 in total (average of 7·8 bands per primer), ranging from approximately 200 to 1000 bp (Fig. 2). Of these, 28 were polymorphic bands (71·79%) considering all the parasite populations. Per location, the number and percentage of polymorphic bands ranged from 10·26% in Sp4 to 48·72% in Sp1. Other parameters related with the variability within populations (A, Ae and H) are shown in Table 1.

The structure of variability among the studied adults from different populations was estimated by the parameters Ht, Hs and Gst (total, intrapopulations and interpopulations variability, respectively). The results were: Ht = 0·267, Hs = 0·121 and Gst = 0·547.

Data generated from ISSR-PCR, in function of the presence (1) or absence (0) of bands, resulted in

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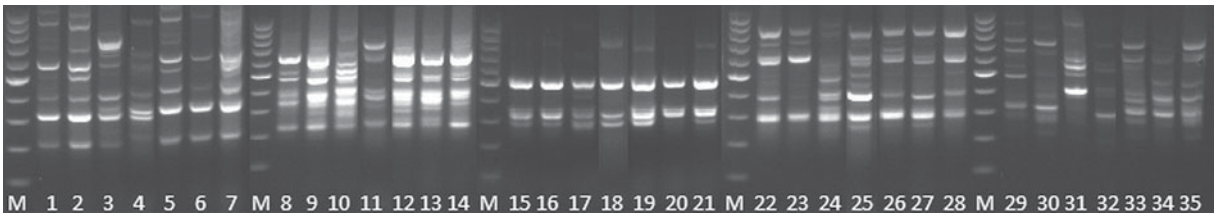

Fig. 2. ISSR-PCR with the selected primers. UBC846 (lanes: 1–7), UBC848 (lanes: 8–14), UBC855 (lanes: 15–21), UBC856 (lanes: 22–28) and UBC881 (lanes: 29–35). For each primer, seven pools of DNA from each location were used in the following order: Sp1, Sp2, Eng, Sp4, Sp3, Ir and Mex. M, marker.

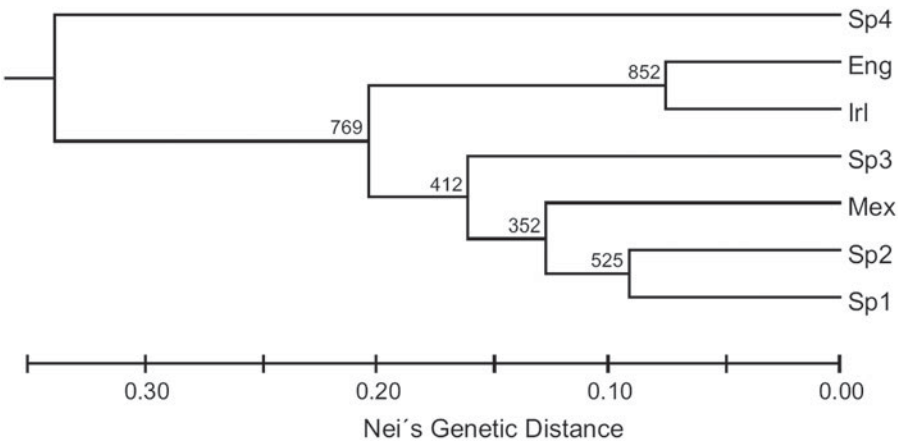

Fig. 3. Dendrogram constructed by the unweighted pair group method analysis (UPGMA), showing the genetic relation between the populations of the study. The numbers next to the nodes indicate the bootstrap values using 1000 replicates.

a matrix of Nei's genetic distances. This was used to construct the UPGMA dendrogram (Fig. 3). According to the dendrogram, the adults from the Midwest of Spain (Sp4) were clearly different from the rest of samples. Within the cluster where most of the populations were present, small genetic distances were observed between some geographically close populations, Eng and Ir, on the one hand, and Sp1, Sp2, Sp3, from the Northwest of Spain, jointly with Mex, on the other (Fig. 3). We also analysed the Nei's distance taking into account the host of each sample, and this case the values ranged from 0·091 to 0·230 in sheep and from 0·150 to 0·337 in cattle.

Additionally, the genetic relationships between all *F. hepatica* adults were determined by a PCA after calculating the Jaccard similarity index. As it is shown in Fig. 4, all adults from Sp4 were situated separately in a different group. Moreover, there is no differentiation between the adults from the populations Sp1, Sp2, Sp3 and Mex.

DISCUSSION

The ISSR-PCR has been described as an alternative for polymorphism studies (Zietkiewicz *et al*. 1994). When ISSR markers are compared to others of similar characteristics, such as RAPDs or SRAPs, some advantages are noticeable. Thus, RAPDs reproducibility is considered poor, and they need a significant laboratory work to standardize protocols in order to obtain reliable results (Teofanova *et al*. 2012), while ISSRs are quite reproducible due to the use of longer primers allowing for higher annealing temperatures than those of RAPDs (Kojima *et al*. 1998). On the other hand, SRAPs show less polymorphism than ISSRs due to the fact that SRAPs focus on genic regions. To detect as much genetic variability as possible, SRAPs are usually analysed in denaturing acrylamide gels, and in many cases the resulting banding pattern is very complex and difficult to score (Alasaad *et al*. 2008; Li *et al*. 2009).

In this study we have used the ISSR-PCR technique for the first time in order to estimate the genetic diversity of *F. hepatica* adults from different geographical locations. The importance of population genetic structure studies lies in achieving an efficient management of the parasitic disease, specially nowadays, when the prevalence of the infection by *F. hepatica* is increasing in certain parts of the world (Rojo-Vázquez *et al*. 2012; Martínez-Valladares *et al*. 2013*b*) and resistant isolates to anthelmintics are appearing in different countries (Moll *et al*. 2000; Mooney *et al*. 2009; Martínez-Valladares *et al*. 2010, 2013*a*; Olaechea *et al*. 2011).

After carrying out the ISSR-PCR in 40 adult specimens from seven different locations, some parameters related with the genetic variability within each isolate have been calculated. In this case,

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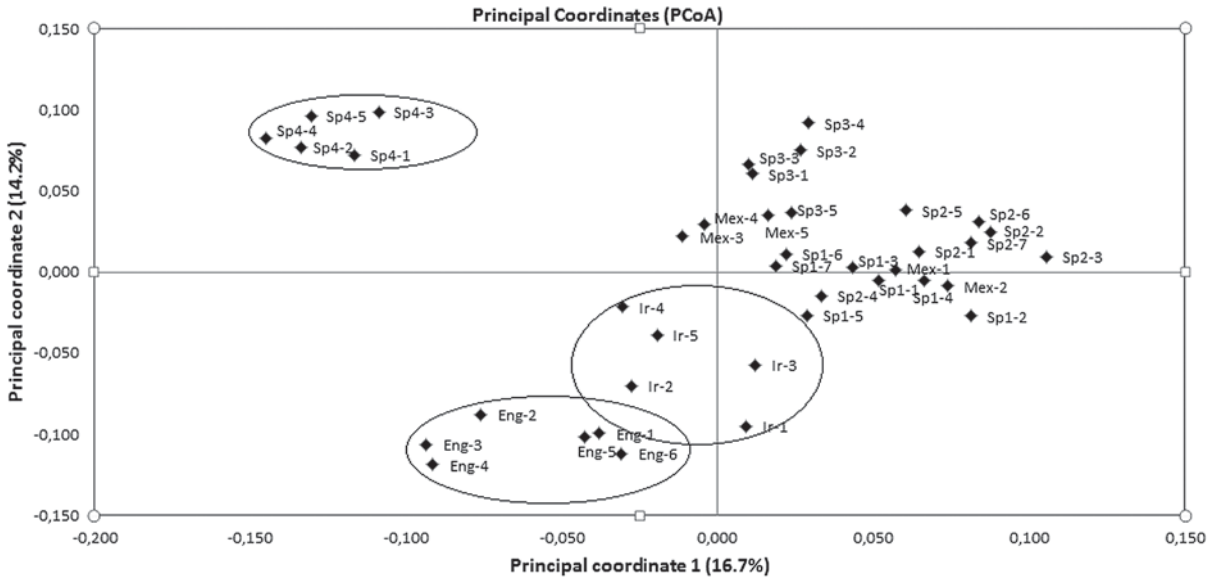

Fig. 4. PCA plot (PC1 *vs* PC2) based on the Jaccard similarity index among all samples.

the total number of polymorphic bands reported in the present study was high (71·79%), meaning that this technique can yield a significant number of polymorphic markers that can be useful in *Fasciola* population studies. However, when individual populations are considered, the level of polymorphic bands is quite lower, ranging between 10·26% in Sp4 and 48·72% in Sp1. This relatively low range of genetic diversity within populations, with a mean of 34·40%, implies that a large proportion of variation resided among populations. Similar results were published by Zhao *et al*. (2009) when the ISSR-PCR was used to study the genetic variability of three populations of *S. japonicum* and found a total of 74·77% of polymorphic bands, ranging from 19·63 to 64·49%. However, when these authors compared two different species, *S. japonicum* and *S. mansoni*, the total percentage of polymorphic bands was significantly higher, 98·13%. On the other hand, the value of A, or the number of alleles per locus, was also the highest in Sp1 and the lowest in Sp4. The value of Ae considers not only the real number of alleles in the populations (A), but also whether they present an even distribution. When A and Ae present the same values, the population has reached the maximum genetic variability (heterozygosity) taking into account its allelic diversity. In this study, all populations showed values of Ae lower than A, which indicates that the populations have not reached the maximum variability possible because one the alleles was more frequent than the other in the polymorphic loci. According to all these parameters, Sp4 presents the lowest level of diversity. No individuals with repeated multilocus genotypes were found in any population, thus there was no need to consider the presence of clones and the effects they have on the genetic variability parameters.

In our study, the population differentiation or Gst was 0·547, indicating that 54·7% of the genetic variation is due to differences between populations *vs* to 45·3%, due to differences between individuals in each population. This result points out a high level of genetic structuration, which is also reflected in the values of Nei's genetic distances, ranging from 0·091 to 0·337. The population Sp4 is the main contributor to the high value of population differentiation because of its unique characteristics; when the analysis was carried out excluding this population, the Gst value lowers to 0·308.

In any case, this result contrasts with that obtained by Vázquez-Prieto *et al*. (2011), which studying three Spanish populations from cattle using allozyme markers, reported a level of population differentiation of 0·006, and a range of Nei's genetic distances between 0·003 and 0·010. A low level of population differentiation (0·078) was also found by Morozova *et al*. (2002) using RAPDs markers. These important differences between studies could be due to the fact that the samples of the study carried out by Vázquez-Prieto *et al*. (2011) were collected within a specific region of the Northwest of Spain (Galicia), and all the samples of Morozova *et al*. (2002) were isolated from to the same Ukrainian cattle population.

On the other hand, Vilas *et al*. (2012) also studied *F. hepatica* populations from cattle and sheep in this same region of Spain (Galicia), than Vázquez-Prieto *et al*. (2011), although in this case sampling a higher number of populations and using allozyme and microsatellite markers at the same time. The range of variation of Nei's genetic distance calculated between populations was 0·023–0·094 in cattle and 0·033–0·156 in sheep and the Fst (equivalent to Gst) values were 0·087 and 0·170 for cattle and sheep, respectively. These authors suggested that parasites

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of sheep appear to show significantly more structured variation at the infrapopulation level. However, the range of Nei's distance found in our study for sheep (0·091–0·230) was lower than for cattle (0·150–0·337), between 0·091 and 0·230, showing less structured variation. In addition, in their study the expected heterozygosity (H) was 0·411 and 0·360 on average in cattle and sheep, respectively, while in our study, it ranged between 0·109 and 0·177 (excluding Sp4). The higher level of genetic diversity in the study of Vilas *et al*. (2012) can be mainly attributed to the fact that the analysed markers cannot be considered as a random sample of loci: firstly, the chosen isozymatic loci were those previously known as polymorphic in their populations, and secondly, they used microsatellite markers which usually are highly polymorphic detecting many alleles per locus; whereas the ISSR-PCR detected two alleles based on the presence or absence of band, and all the loci were scored, whether or not polymorphic.

Using SRAP markers, Alasaad *et al*. (2008) studied the *F. hepatica* genetic variability by analysing Spanish samples from several different hosts and geographical localities and described a low level of genetic variation in *F. hepatica*. This observation may be because the SRAP markers target sequences belonged to coding regions of the genome and these regions have lower mutation rates due to their functional constraints (Crow, 2000). The authors also related this observation to the cycle of *Fasciola* spp., which would act against speciation processes, decreasing at the same time their genetic variability and allowing the infection for new host species (Vara-Del Río *et al*. 2007). Supporting this hypothesis, Alasaad *et al*. (2008) could not find any association between *F. hepatica* genetic characteristics and their hosts and/or region.

In the present study, both UPGMA (Fig. 3) and PCA analysis (Fig. 4) grouped populations into similar clusters, which showed a fair accordance. Both methods showed a clear genetic differentiation of Sp4, and a clustering of the samples related to their geographical origin. Thus, the three samples from the North West of Spain (Sp1, Sp2 and Sp3), and those from Ireland (Ir) and Great Britain (Eng) cluster in the same groups. The genetic relationships between samples from Sp1, Sp2 and Sp3 are less clear, as can be inferred from the low bootstrap values of the groups shown in Fig. 3 (a method to establish the robustness of the clusters), and from PCA analysis (Fig. 4) that showed intermixed some of the samples from this populations. Similarly to Alasaad *et al*. (2008), but in contrast with Vilas *et al* (2012), no association between *F. hepatica* genetic characteristics and their hosts have been found in this study.

From the grouping analysis, the position of two populations, Sp4 and Mex, deserves further comments. There are several reasons that could explain the fact that the samples from Sp4 are genetically different from the rest of the Spanish populations. Regarding the strain Sp4 located in Cáceres (in the Midwest of Spain), it is possible that the strain had entered into Spain from the North of Africa during the period of Moorish rule with infected cattle. We are aware of the native Spanish population of cows that have African origin (Beja-Pereira *et al*. 2006), as it is confirmed by mitochondrial studies. On the other hand, the genetic differentiation of Sp4 can be associated with a reproductive isolation of the strain due to the low density of the parasites in that area, restricting the possibilities of exchanging genetic material with other strains.

On the order hand, the results of the PCA show that there is no differentiation between the adults from Sp1, Sp2, Sp3 and Mex, in agreement with the low bootstrap values calculated in the UPGMA analysis between the Spanish and Mexican populations. This similarity between samples from different locations can be explained by a common origin, since cattle and sheep were repeatedly introduced in America during the colonization leading to a rapid spreading of the parasite from Spain to Mexico by means of infected animals.

Concluding remarks

In the present study, we have used for the first time the ISSR-PCR to study the genetic diversity of *F. hepatica* populations from different countries – Spain, UK, Ireland and Mexico. This technique provided us with a high number of polymorphic markers and showed that the genetic diversity is structured, being the intrapopulation variability at the same level that the interpopulation differentiation. All populations grouped separately from one population from de Midwest of Spain. Within the cluster where most of the adults are present, small genetic distances were observed between some geographically close populations, UK and Ireland, on the one hand, and the rest populations from Spain and Mexico, on the other.

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