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2 *Fasciola hepatica* demonstrates high levels of genetic diversity, a lack of population structure and  
3 high gene flow, possible implications for drug resistance

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15 **Abstract**

16 *Fasciola hepatica*, the liver fluke, is a trematode parasite of considerable economic importance to  
17 the livestock industry and is a re-emerging zoonosis that poses a risk to human health in *F. hepatica*  
18 endemic areas worldwide. Drug resistance is a substantial threat to the current and future control of  
19 *F. hepatica*, yet little is known about how the biology of the parasite influences the development  
20 and spread of resistance. Given that *F. hepatica* can self-fertilise and therefore inbreed, there is the  
21 potential for greater population differentiation and an increased likelihood of recessive alleles, such  
22 as drug resistance genes, coming together. This could be compounded by clonal expansion within  
23 the snail intermediate host and aggregation of parasites of the same genotype on pasture.  
24 Alternatively, widespread movement of animals that typically occurs in the UK, could promote high  
25 levels of gene flow and prevent population differentiation. We identified clonal parasites, with  
26 identical multilocus genotypes (MLGs) in 61 % of hosts. Despite this, 84 % of 1579 adult parasites  
27 had unique MLGs, which supports high levels of genotypic diversity within *F. hepatica*  
28 populations. Our analyses indicate a selfing rate no greater than 2 % suggesting that this diversity is  
29 in part due to the propensity for *F. hepatica* to cross-fertilise. Finally, although we identified high  
30 genetic diversity within a given host, there was little evidence for differentiation between  
31 populations from different hosts, indicating a single panmictic population. This implies that, once  
32 they emerge, anthelmintic resistance genes have the potential to spread rapidly through liver fluke  
33 populations.

34

35 **Keywords**

36 *Fasciola hepatica*; population genetics; anthelmintic resistance; diversity; self-fertilisation; gene  
37 flow; microsatellites

## 38 **1. Introduction**

39 *Fasciola hepatica* is a trematode parasite that causes disease of economic importance in sheep  
40 and cattle (Bennett and Ijpelaar, 2005; Schweizer et al., 2005), with an estimated 250 million sheep  
41 and 350 million cattle at risk worldwide (Hillyer and Apt, 1997). A zoonosis, it is classed by the  
42 World Health Organisation as a neglected tropical disease endemic in human populations in parts of  
43 South America, western Europe and the Caspian (Mas-Coma, 2005; WHO, 2007; 2015). Over the  
44 last 15 to 20 years, the diagnosis of *F. hepatica* infection in European livestock has increased  
45 (VIDA, 2015; Caminade et al., 2015), possibly due to changing climate, changing farming  
46 practices, including animal movement and land use and the emergence of resistance to the drug of  
47 choice, triclabendazole (van Dijk et al., 2010; Fairweather, 2011a; Fox et al., 2011; Caminade et al.,  
48 2015). Resistance of *F. hepatica* to triclabendazole was first reported in sheep in Australia, 1995  
49 (Overend and Bowen, 1995), and is now frequently reported across Europe and South America  
50 (Daniel et al., 2012; Moll et al., 2000; Gaasenbeek et al., 2001; Álvarez-Sánchez et al., 2006;  
51 Mooney et al., 2009; Olaechea et al., 2011; Ortiz et al., 2013). It is considered to be a substantial  
52 threat to the current and future control of *F. hepatica* (Kelley et al., 2016).

53 Population genetic analyses are key to understanding the origin, evolution, and spread of  
54 resistance genes in populations and are thus a vital component of anthelmintic resistance studies  
55 (Gilleard and Beech, 2007). They allow us to identify management factors influencing the  
56 migration of resistance genes, and so help to mitigate against their spread. It is recognised that the  
57 husbandry and management of different farms have the potential to affect the population structure  
58 of parasites (Grillo et al., 2007) by influencing the movement of the definitive host and, therefore,  
59 *F. hepatica* parasites. Additionally, the age and production system for an animal influences the  
60 extent to which it has been exposed to *F. hepatica* on pasture and to what extent it may have been  
61 treated with anthelmintics.

62 A number of aspects of *F. hepatica* biology have the potential to influence genetic diversity and  
63 population structure and therefore impact on the spread of genes, including those responsible for  
64 anthelmintic resistance (Hodgkinson et al., 2013). Firstly, it is known that clonal expansion of *F.*  
65 *hepatica* occurs within the snail intermediate host, *Galba truncatula* (Thomas, 1883; Krull, 1941).  
66 Therefore, there is the potential for multiple metacercariae of the same origin and genotype to exist  
67 on pasture, and parasites with the same multilocus genotype (MLG) have been found within, and  
68 shared between, definitive hosts (Vilas et al., 2012). Secondly, as a hermaphrodite, *F. hepatica* can  
69 self- and cross-fertilise. Self-fertilisation is a form of inbreeding which has the potential to influence  
70 allele frequency in a population. If anthelmintic resistance is a recessive trait, a high level of self-  
71 fertilisation means there is the potential for resistant alleles to spread more rapidly. Thirdly, clonal  
72 expansion in the snail, combined with low levels of infection in the snail population as a whole,  
73 could pose a bottleneck to gene flow and lead to population structuring. Finally, *F. hepatica* has a  
74 wide host range, infecting multiple species of domestic and wild animals (Parr and Gray, 2000;  
75 Vignoles et al., 2001; 2004; Arias et al., 2012). This may allow the flow of genes among livestock  
76 species and maintain a reservoir of genetic diversity in wild animals. In addition, adult *F. hepatica*  
77 in the definitive host can be long-lived (Durbin, 1952), and their reproductive capacity may be  
78 present for many years in untreated animals.

79 An understanding of *F. hepatica* genetic diversity has implications for the development and  
80 validation of new methods of control. Knowledge of the provenance, infectivity, pathogenicity and  
81 resistance status of laboratory isolates is important (Hodgkinson et al., 2013). Laboratory  
82 maintained isolates of *F. hepatica* are frequently used in research, including in drug and vaccine  
83 trials (Fairweather, 2011b), but are not representative of field isolates. For example, the Cullompton  
84 isolate is aspermic and triploid (Fletcher et al., 2004), the Sligo isolate exhibits abnormal  
85 spermatogenesis (Hanna et al., 2008), and the Fairhurst isolate is highly homogenous (Walker et al.,  
86 2007).

87 Previously we have shown that the British *F. hepatica* population naturally infecting sheep  
88 and cattle is diploid, spermic, and predominantly reproduces by sexual reproduction (Beesley et al.,  
89 2015). Here, we present the largest population genetic study to date for *F. hepatica*, involving the  
90 genotyping of 1579 adult parasites. Adult *F. hepatica* samples were collected from three countries;  
91 Scotland, England and Wales from two definitive host species, sheep and cattle; and MLGs were  
92 produced using our panel of microsatellite markers (Cwiklinski et al., 2015a). A proportion of hosts  
93 harboured multiple, genotypically identical parasites. However, overall, we found substantial  
94 genetic variation within populations infecting a given host and high levels of genetic diversity in the  
95 liver fluke population as a whole, but little differentiation between populations infecting sheep and  
96 cattle. Our data indicate a lack of geographic or host species structuring in UK *F. hepatica* and high  
97 gene flow, which could promote the emergence and spread of drug resistance in a population. The  
98 results of this study may be relevant to other areas where widespread movement of livestock is  
99 practised.

100

## 101 **2. Materials and methods**

102

### 103 *2.1 Populations of Fasciola hepatica*

104 Adult *F. hepatica* were recovered from the livers of 44 naturally infected sheep between  
105 November 2012 and April 2013, from two abattoirs (Wales and Central England, UK). Similarly,  
106 parasites were recovered post mortem from 31 cattle livers between October 2013 and January  
107 2014, from an abattoir (Wales, UK). A total of 950 parasites were genotyped from sheep and 629  
108 from cattle (Table 1). The Rapid Analysis and Detection of Animal Related Risks (RADAR),  
109 Animal and Plant Health Agency (APHA) provided information on the origin of cattle livers. Adult  
110 parasites were isolated from the bile ducts and incubated for 2 hr at 37°C in 1 to 2 ml of Dulbecco's

111 Modified Eagle's Media with 120  $\mu\text{gml}^{-1}$  gentamicin and 120  $\mu\text{gml}^{-1}$  amphotericin B to allow  
112 purging of intestinal contents and eggs. Parasites were snap frozen and stored at  $-80^{\circ}\text{C}$ .

113

## 114 *2.2 Preparation of DNA template and microsatellite genotyping*

115 A small section of each parasite, anterior to the ventral sucker, to avoid contamination with  
116 eggs or sperm, was used for DNA extraction. The tissue was divided into small pieces to ensure  
117 efficient lysis. DNA extraction was performed using a DNeasy Blood & Tissue Kit (Qiagen,  
118 Manchester, UK) as per the manufacturer's instructions and DNA was diluted to  $10\text{ ng}\mu\text{l}^{-1}$ .

119 A panel of 15 microsatellites previously validated with 46 adult *F. hepatica* (Cwiklinski et al.,  
120 2015a), was applied to each parasite DNA sample to generate an individual MLG. For efficiency  
121 the methodology was modified for a multiplex approach; the Type-it Microsatellite PCR kit  
122 (Qiagen) was used according to the manufacturer's instructions (Cwiklinski et al., 2015a). The  
123 fifteen loci were grouped as follows: (1) Fh\_1, Fh\_6, Fh\_13, Fh\_15 annealing temperature  $55^{\circ}\text{C}$ ;  
124 (2) Fh\_2, Fh\_3, Fh\_5, Fh\_8, annealing temperature  $57^{\circ}\text{C}$ ; (3) Fh\_9, Fh\_10, Fh\_11, Fh\_14,  
125 annealing temperature  $57^{\circ}\text{C}$ ; and (4) Fh\_4, Fh\_7 and Fh\_12, annealing temperature  $59^{\circ}\text{C}$ . PCR  
126 products were visualised using SYBR Safe DNA stain (Life Technologies) on a 1.5 % agarose gel.  
127 PCR products were diluted 25-fold in HPLC water (Sigma-Aldrich), and sequenced using an ABI  
128 PRISM 3100 Genetic Analyser capillary electrophoresis system (Life Technologies; Cwiklinski et  
129 al., 2015a). Fragment sizes were determined using Peak Scanner v2.0 software (Life Technologies).

130

## 131 *2.3 Population genetic analyses*

132 Allele frequencies were determined using CERVUS 3.0.7 (Kalinowski et al., 2007; available  
133 from [www.fieldgenetics.com](http://www.fieldgenetics.com)) and genotype frequencies were determined using GENEPOP 4.2.1  
134 (Rousset, 2008; available from <http://kimura.univ-montp2.fr/~rousset/Genepop.htm>). Null allele  
135 frequency was determined using CERVUS 3.0.7 (Kalinowski et al., 2007). Loci Fh\_1, Fh\_3, Fh\_4,

136 Fh\_7, Fh\_8 and Fh\_14 were identified as having greater than 5 % frequency of null alleles,  
137 therefore these loci, along with locus Fh\_9 which produced inconsistent traces, were excluded from  
138 the remaining population genetic analyses.

139 Average heterozygosities were determined for each locus using Arlequin 3.5.1.3 (Excoffier and  
140 Lischer, 2010). Unbiased heterozygosity was calculated using GenClone 2.0 (Arnaud-Haond and  
141 Belkhir, 2007). Heterozygosity was determined for each individual parasite based on the proportion  
142 of loci that were heterozygous. Mann-Whitney  $U$  tests were performed using Minitab 17.  
143 GenClone 2.0 (Arnaud-Haond and Belkhir, 2007) was used to identify repeated MLGs (defined as  
144 two, or more, parasites sharing the same MLG) and calculate corresponding  $P_{sex}$  values, which were  
145 adjusted using  $F_{IS}$  values (Parks and Werth, 1993). Animals from the same farms, or that shared  
146 repeated MLGs, were grouped when calculating  $P_{sex}$  values.

147 To determine whether repeated MLGs tended to co-occur in the same host (Gregorius, 2005;  
148 Criscione et al., 2011; Vilas et al., 2012) a contingency table was created as described by Vilas et  
149 al., (2012), and Fisher's exact test with a Monte Carlo simulation (5000 replicates) was performed  
150 using R 3.0.1 (R Core Team, 2013). All parasites were analysed together, and animals known to  
151 come from the same farm were grouped and also analysed with  $p$ -values corrected using a  
152 Bonferroni correction. The presence of repeated MLGs might make alleles appear more common  
153 and affect population genetic structure analyses. Therefore, for the remaining analyses repeated  
154 MLGs were reduced to one instance.

155 Deviations from Hardy-Weinberg equilibrium were calculated using GENEPOP 4.2.1 (Rousset,  
156 2008) using a two-tailed exact test with Markov Chain algorithm (10,000 dememorization, 250  
157 batches, 5000 iterations). To determine the extent of any significant deviation from Hardy-  
158 Weinberg equilibrium,  $F_{IS}$  values (Weir and Cockerham, 1984) were calculated using GENEPOP  
159 4.2.1 (Rousset, 2008).

160 All pairs of loci, with all parasites analysed together, were assessed for linkage disequilibrium  
161 using GENEPOP 4.2.1 (Rousset, 2008). Due to the number of tests,  $p$ -values were corrected and  
162 compared using (i) Bonferroni correction and (ii) false discovery rate correction (Benjamini and  
163 Hochberg, 1995), the latter performed using R 3.0.1 (R Core Team, 2013). To demonstrate the  
164 extent of linkage disequilibrium for any pair of loci with significant  $p$ -values,  $r^2$  values were  
165 calculated. To calculate this value, knowledge of the gametic phase is needed. Since this is  
166 unknown here, the ELB algorithm (Excoffier et al., 2003) was used to infer the gametic phase.  
167 These calculations were performed using Arlequin 3.5.1.3 (Excoffier and Lischer, 2010).

168 Genotypic richness (Dorken and Eckert, 2001) was used to describe genetic diversity,  
169 calculated using GenClone 2.0 (Arnaud-Haond and Belkhir, 2007). When calculating genotypic  
170 richness, animals from the same farms, or that shared the same MLG, were grouped. Mann-Whitney  
171  $U$  tests were performed using Minitab 17.

172  $F_{IS}$  and  $F_{ST}$  values were calculated using GENEPOP 4.2.1 (Rousset, 2008), and confidence  
173 intervals were calculated using FSTAT 2.9.3 (Goudet, 1995; available from  
174 <http://www2.unil.ch/popgen/softwares/fstat.htm>). The rate of self-fertilisation ( $s$ ) was calculated  
175 from the  $F_{IS}$  values using the equation  $F_{IS} = s / (2 - s)$ . Pairwise  $F_{ST}$  values were calculated using  
176 Arlequin 3.5.1.3 (Excoffier and Lischer, 2010). Principle component analysis (PCA) of these values  
177 was performed in R 3.0.1 (R Core Team, 2013), and the package ggplot2 was used to plot results.  
178 GENEPOP 4.2.1 (Rousset, 2008) was used to produce a measure for the average number of  
179 migrants between populations ( $N_m$ ) using the private allele method developed by Slatkin, (1985).  
180 For this calculation, parasites were grouped according to the definitive host from which they  
181 originated.

182 Isolation by distance testing was possible for parasites from cattle only, as farm location was  
183 known. Parasites were grouped into populations dependent upon farm of origin. Isolation by  
184 distance was then tested using GENEPOP 4.2.1 (Rousset, 2008). A Mantel test (5000 permutations)



185 was performed using log transformed geographic distances with the minimum geographic distance  
186 set at 0.0001. Data were plotted in R 3.0.1 (R Core Team, 2013) using the package ggplot2.

187       Structure 2.3.4 (Pritchard et al., 2000; available from  
188 <http://pritchardlab.stanford.edu/structure.html>) was used to detect population structure. To  
189 determine the ancestry of individuals, the admixture model with default settings was chosen. This  
190 allows for an individual to have mixed ancestry. For the allele frequency model, allele frequencies  
191 were correlated among populations with default settings. Burn-in length was set at 200,000 and was  
192 followed by 100,000 Markov Chain Monte Carlo repeats. K was set at 1 to 47 (the number of farms  
193 animals came from) and repeated 20 times. To determine the most appropriate value for K,  $\Delta K$  was  
194 determined using the method proposed by Evanno et al., 2005, and calculated using STRUCTURE  
195 HARVESTER (Earl and vonHoldt, 2012; available from  
196 <http://taylor0.biology.ucla.edu/structureHarvester/>). Data were plotted in R 3.0.1 (R Core Team,  
197 2013) using the packages ggplot2 and gridExtra.

198

## 199 2.4 Ethical Approval

200       Ethical approval was received from the University of Liverpool's Veterinary Research Ethics  
201 Committee (VREC106 and VREC145).

202

## 203 3. Results

204

### 205 3.1 Microsatellite genotyping using a multiplex approach

206       Summary statistics are shown for the microsatellite panel in Table 2. Eight loci (Fh\_2, Fh\_5,  
207 Fh\_6, Fh\_10, Fh\_11, Fh\_12, Fh\_13 and Fh\_15) were used to produce a MLG for all 1579 parasites.  
208 Only locus Fh\_2 showed significant deviation from Hardy-Weinberg equilibrium, however, the  $F_{IS}$   
209 value at this locus was low, so the deviation was considered minor (Table 2). Each pair of loci was

210 assessed for evidence of linkage disequilibrium. Five pairs of loci showed significant  $p$ -values ( $p <$   
211 0.005 using false discovery rate;  $p < 0.00179$  using Bonferroni correction) but low  $r^2$  values  
212 (median = 0.0001, range 0 to 0.33), indicating that the pairs of loci are closer to equilibrium than  
213 disequilibrium.

214

### 215 3.2 Genetically identical (clonal) parasites are common in UK *Fasciola hepatica* infections

216 Given that the life cycle of *Fasciola* spp. involves clonal expansion within the snail host,  
217 and release of genetically identical cercariae onto pasture, we tested whether multiple parasites  
218 within a liver exhibited the same MLG. Overall, 71 % of sheep and 48 % of cattle livers harboured  
219 clonal parasites (this difference was not statistically significant,  $X^2 = 0.588$ ;  $p = 0.4432$ ). A total of  
220 96 parasite genotypes were represented more than once, with the majority, 65 genotypes, shared by  
221 just two parasites. Sixteen of the animals showed evidence of infection with more than two parasites  
222 of the same genotype, with a maximum of 10 clonal parasites reported in one sheep. Figure 1A and  
223 B show the number of unique and repeated MLG (defined as an MLG present more than once)  
224 within each individual sheep and cow. There were a number of animals where multiple different  
225 MLGs were shared by parasites, with a maximum of eight distinct MLGs observed in a single  
226 animal. This happened on two occasions, sheep 80 and sheep 83 (Fig. 1A).

227 Generally, parasites with the same MLG were present within the same animal, and it was  
228 found that repeated MLGs did tend to co-occur in the same host (Fisher's exact test with Monte  
229 Carlo simulation  $p = 0.0002$ ). However, repeated MLGs were also found to be shared between  
230 individual sheep (sheep 2 and 3; sheep 9 and 10; sheep 80 and 81; sheep 82 and 84) and cattle  
231 (cattle 104 and 106), but clonal parasites were not found to be shared by both sheep and cattle. In  
232 total, 16 % of all parasites identified in sheep and cattle lacked a unique MLG and the proportion  
233 was significantly higher in sheep than cattle ( $X^2 = 4.9052$ ;  $p = 0.02678$ ). However, this was not  
234 because parasite burdens in sheep were higher, since burdens for sheep and cattle were not

235 significantly different (Mann-Whitney  $U$  test  $p = 0.5842$ ). In order to determine whether those  
236 MLGs that occurred more than once in an animal represented different reproductive events or were  
237 from the same clonal lineage,  $P_{sex}$  values, the probability that a MLG is derived from a distinct  
238 reproductive event rather than being from a clonal lineage, were calculated. All the  $P_{sex}$  values were  
239 highly significant at  $n = 2$  and overall ranged from  $1.74 \times 10^{-71}$  to  $3.4 \times 10^{-4}$  in parasites from sheep  
240 and from  $2.97 \times 10^{-47}$  to  $2.39 \times 10^{-5}$  in parasites from cattle. This supports the conclusion that the  
241 repeated MLGs represent parasites arising from clonal lineages.

242

### 243 3.3 *Fasciola hepatica* in the UK is genetically diverse

244 Inbreeding and clonal expansion in *F. hepatica* may impact on levels of genetic diversity in  
245 *F. hepatica* populations, hence we genotyped a large number of parasites from multiple sheep and  
246 cattle throughout the UK. The heterozygosity of individual parasites, a measure of genetic variation,  
247 ranged from 0.25 to 1, whilst the mean heterozygosity of all parasites across all loci was 0.752 (SD  
248 = 0.130), suggesting high levels of genetic variation in the overall population. In the majority of  
249 cases, 29 animals, each parasite genotyped had a unique MLG (Fig. 1A and B). Genotypic richness  
250 (R), the measure of genetic diversity that describes the number of distinct MLGs within a  
251 population, was high,  $R = 0.901$ . As with heterozygosity a range of values for R were reported  
252 within individual definitive hosts, 0.343 to 1, however, parasites in the majority of animals showed  
253 a genotypic richness of greater than 0.8 (Fig. 1C). These analyses confirmed that the UK *F.*  
254 *hepatica* population demonstrated high genetic diversity.

255

### 256 3.4 *Fasciola hepatica* from sheep and cattle are not genetically distinct

257 Given that both sheep and cattle can be infected with *F. hepatica* and often co-graze, we  
258 asked whether there is evidence of population structuring between the two hosts. The pairwise  $F_{ST}$   
259 between parasites from sheep and cattle was 0.00145. Although this value was statistically

260 significant ( $p < 0.05$ ) given the large sample size, a value of less than 1% indicates little genetic  
261 differentiation between parasites from sheep and cattle. Furthermore, PCA analysis of pairwise  $F_{ST}$   
262 values between the parasites within each definitive host does not reveal any clustering based on host  
263 species (Fig. 1D). No significant difference in the level of genetic variation and diversity was seen  
264 when parasites from sheep and cattle were assessed separately: heterozygosity across all loci was  
265 0.758 (SD: 0.141) in sheep and 0.745 (SD: 0.118) in cattle (Mann-Whitney  $U$  test  $p = 0.092$ ) and  
266 the genotypic richness across all parasites was 0.890 in sheep and 0.918 in cattle (Mann-Whitney  $U$   
267 test  $p = 0.689$ ). Sheep and cattle share a number of common alleles and genotypes (Table 3) but  
268 private (unique) alleles were also identified for each host species, with 14.7 % and 6.0 % of all  
269 alleles unique to sheep and cattle, respectively. The most common allele at each locus was identical  
270 for both host species, with the exception of loci Fh\_2 and Fh\_4 (Table 3; data not available for  
271 locus Fh\_1). The most common genotypes were also identical at nine loci (Fh\_5, Fh\_7, Fh\_8, Fh\_9,  
272 Fh\_10, Fh\_11, Fh\_12, Fh\_14 and Fh\_15; Table 3). Therefore, parasites from sheep and cattle  
273 showed not only a similar level of genetic variation, but also largely similar alleles and genotypes.  
274 From the evidence presented in this study there does not appear to be structuring of the parasites  
275 from sheep and cattle, and *F. hepatica* infecting the two species of definitive host are genetically  
276 similar.

277

### 278 3.5 High gene flow exists in UK *Fasciola hepatica* populations

279 The extent of gene flow among *F. hepatica* populations was investigated given that  
280 widespread movement of sheep and cattle is commonly practiced in the UK. The evidence from a  
281 number of our analyses indicates that, in the UK, *F. hepatica* represents a single panmictic  
282 population with no geographic structuring. PCA analysis of pairwise  $F_{ST}$  from locations up to 650  
283 km apart showed there was no clustering based on the location of the definitive host (Fig. 1D).  
284 Similarly, there was also no evidence of isolation by distance (exact location information was

285 available for cattle only) since the slope of the regression line was negative, and the  $p$ -value was  
286 non-significant (Fig. 2A). The mean likelihood results from Structure (Pritchard et al., 2000) did not  
287 reach an asymptote which would be expected if the population was structured (Fig. 2B). In addition  
288 the majority of  $\Delta K$  values were low (Fig. 2C) indicating a single population with no structure.  
289 Finally,  $F_{ST}$  analysis between definitive hosts (across all parasites and loci) was 0.0202, which was  
290 low, supporting little genetic differentiation and low levels of population structure. This lack of  
291 genetic differentiation infers high gene flow in the population. When parasites from sheep and cattle  
292 were assessed separately, the  $F_{ST}$  values between sheep and between cattle were very similar:  
293 0.0193 and 0.0207, respectively. Since private alleles were identified,  $N_m$  (the effective number of  
294 migrants) can be used to give an indirect estimate of gene flow. Parasites were grouped based on the  
295 definitive host from which they were collected, giving a mean sample size of 18.99.  $N_m$  across all  
296 loci was 5.59, and since this means the number of migrants per generation into the population is  
297 greater than 2, it is indicative of high gene flow (Slatkin, 1985). Similarly, when parasites from  
298 sheep and cattle were assessed separately,  $N_m$  values were 6.85 and 8.20, respectively. Therefore  
299 both the  $F_{ST}$  and  $N_m$  values support a high level of gene flow in the UK *F. hepatica* population.

300

### 301 3.6 Low levels of self-fertilisation occur in UK *Fasciola hepatica* populations

302 Self-fertilisation will result in loss of genetic diversity within individual parasites, which can be  
303 estimated from Wright's  $F_{IS}$  statistic.  $F_{IS}$  across all loci and parasites was 0.0011, which was not  
304 significantly different from zero (95 % CI: -0.011, 0.013), and indicated a selfing rate no higher  
305 than 2 %.

306

## 307 4. Discussion

308 This study has provided valuable insights into aspects of *F. hepatica* population biology.

309 The fact that the selfing rate was estimated to be no greater than 2 % suggests that self-fertilisation

310 can occur but it is rare in the field. Clonal parasites, with identical MLGs, were identified in 61 %  
311 of definitive hosts, implying that clones are commonly found in *F. hepatica* infections, a finding  
312 that is consistent with earlier studies (17 of 20 animals; Vilas et al., 2012). We found parasites with  
313 identical MLGs were usually in the same host (Fig. 1A and B) and when clonal parasites were  
314 found to be shared between animals, each pair of animals was from the same geographic area and  
315 typically from the same farm. Our findings indicate that, following clonal expansion in the snail,  
316 there is aggregation of infective clonal metacercariae on pasture, with little mixing of parasites prior  
317 to ingestion by the definitive host. The life cycle of *F. hepatica* lends itself to clumped transmission  
318 in several ways. Firstly, a single miracidium infecting a snail produces multiple (e.g. mean 114.9;  
319 SD 80.3; Dreyfuss et al., 1999) genetically identical cercariae. Secondly, snails are known to shed  
320 multiple cercariae at the same time (Hodasi, 1972; Dreyfuss et al., 2006). Thirdly, reported levels of  
321 *F. hepatica* infection in *G. truncatula* in the UK and the Republic of Ireland can be as low as 3 %  
322 (Crossland et al., 1969; Relf et al., 2011). Finally, snail habitats tend to be small (Rondelaud et al.,  
323 2011), which may concentrate metacercariae in small areas of pasture. However, it is important to  
324 appreciate that mortality can occur at every stage of the life cycle (Ollerenshaw, 1959), thus  
325 potentially limiting the survival of clonal parasites. Indeed, the maximum number of clonal adult  
326 parasites in any one host was ten out of the 36 parasites genotyped (Fig. 1A). The fact that  $P_{sex}$   
327 values were significant, indicated that parasites with identical MLGs arose from the same clonal  
328 lineage rather than distinct reproductive events, which would be consistent with the findings of  
329 Vilas et al., (2012). Neither our study nor Vilas et al., (2012) reported parasites with the same MLG  
330 in both sheep and cattle. Whilst it would be expected that sheep and cattle that were known to co-  
331 graze might be more likely to be infected with the same clonal lineage, parasites with the same  
332 composite mitochondrial haplotypes have been reported in sheep and cattle from distinct counties of  
333 Northern Ireland (Walker et al., 2007).

334 Despite the presence of clonal parasites in sheep and cattle, these constituted only 16 % of  
335 the total parasite population under study as the majority of the 1579 parasites analysed had unique  
336 MLGs. Our analysis of the population as a whole indicated that the UK *F. hepatica* population was  
337 highly genetically diverse (Fig. 1C). Undoubtedly, one of the best ways to maintain this diversity is  
338 the capacity for *F. hepatica* to reproduce in the definitive host through meiosis. Our findings on low  
339 selfing rates indicate that cross-fertilisation predominates in *F. hepatica*. Recently, it has been  
340 observed that parasites with higher heterozygosity levels were more likely to establish in the liver  
341 following infection (Zintl et al., 2015) raising the possibility that host selection enhances the  
342 likelihood of cross-fertilisation.

343 Of particular interest here is the fact that we sampled lambs that had grazed for only one  
344 season, yet they displayed highly diverse adult parasite populations, equivalent to those seen in  
345 cattle that had grazed over several seasons; a point which has been alluded to before by Walker et  
346 al., (2007). This suggests that the metacercariae on pasture, to which the lambs were exposed, were  
347 also highly genetically diverse. Clonal expansion and low levels of infection in snails present a  
348 potential genetic bottleneck and raise the question about how *F. hepatica* maintains its genetic  
349 diversity. It is known that, experimentally, snails can be infected with two miracidia four hours  
350 apart (Dreyfuss et al., 2000; Dar et al., 2011) and, in the field, snails have been found to be infected  
351 by more than one miracidium (Rondelaud et al., 2004). If a snail can be simultaneously infected  
352 with multiple miracidia and subsequently shed cercariae of many genotypes, this could drive  
353 genetic diversity. Snail habitats can be difficult to locate, and whilst the level of infection within  
354 snails has been reported to be as low as 0.8 % (Rondelaud and Dreyfuss, 1997), it is possible that  
355 levels of infection in the snail are considerably higher. There is also evidence that *F. hepatica* can  
356 infect snails other than *G. truncatula* (Abrous et al., 1999; Rondelaud et al., 2001; Dreyfuss et al.,  
357 2005; Relf et al., 2009; Caron et al., 2014). Furthermore, given that snails infected with *F. hepatica*  
358 have been found in areas with no ruminant contact (Dreyfuss et al., 2003) wild definitive hosts,

359 such as rabbits and deer, could function as important reservoir hosts in maintaining diversity (Parr  
360 and Gray, 2000; Arias et al., 2012). Another possible way to maintain genotypic diversity is via the  
361 long-term time survival of metacercariae on pasture. Metacercariae have been reported to be both  
362 viable and infective for at least 130 days at 10°C (Boray, 1969), but we have no knowledge of how  
363 long metacercariae survive in the field, yet this has important implications for control. At a practical  
364 level given that efficacy of drugs and vaccines can be compromised by the presence of genetic  
365 diversity, an important understanding of this standing genetic variation is essential to the rational  
366 selection of new vaccine candidates/drug targets for *F. hepatica*.

367         There is the potential for husbandry and management practices to affect the population  
368 structure of parasites (Grillo et al., 2007). Our analysis of the UK *F. hepatica* population showed no  
369 evidence of structuring geographically or amongst parasites from sheep and cattle (Fig. 1D, 2B and  
370 C), indicating panmixia and high gene flow. It has been suggested that movement of the definitive  
371 host is a key factor in maintaining high levels of gene flow in *F. hepatica* (Semyenova et al., 2006;  
372 Bazsalovicsová et al., 2015). Livestock in the UK are frequently moved around and between  
373 countries and it is likely that the movement of livestock in the UK contributes to the high gene flow  
374 observed. Even a small amount of migration can destroy any observed population structure giving  
375 the appearance of panmixia (Wright, 1931); for example moving animals to a new farm could  
376 introduce a new population of parasites as well as exposing the definitive host to a different resident  
377 parasite population. Whilst further analysis of parasites from flocks or herds where animal  
378 movement is restricted, or ideally ‘closed’, may reveal structure not previously detected, panmixia  
379 is not merely a feature of UK *F. hepatica* populations, similar findings have been reported in Spain  
380 and Bolivia (Hurtrez-Boussès et al., 2004; Vázquez-Prieto et al., 2011). The results of this study  
381 may be relevant to other areas where widespread movement, or importation, of livestock is  
382 practised. In support of this, identical mitochondrial haplotypes found between fluke isolated from  
383 the Republic of Ireland and Greece was attributed to importation of animals (Walker et al., 2007). It



384 would be interesting to determine the level of genetic diversity in, and genetic differentiation  
385 between, populations of *F. hepatica* from wild, as opposed to farmed, definitive hosts.

386 Resistance to triclabendazole has been reported widely throughout the UK (Daniel et al.,  
387 2012; Gordon et al., 2012; Hanna et al., 2015). Investigation of triclabendazole resistance in fluke  
388 in laboratories worldwide has resulted in the pursuit of a number of potential candidate genes and  
389 biological pathways (reviewed by Kelley et al., 2016). The precise loci and, therefore, genes  
390 involved are still to be defined, but a genome-wide approach is currently underway to identify the  
391 major genetic determinant of triclabendazole resistance (Hodgkinson et al., 2013). Our findings  
392 have implications for the emergence and spread of anthelmintic resistance. In terms of emergence,  
393 we have shown that there is high standing genetic variation in UK *F. hepatica* populations, which  
394 may include rare genetic variants able to confer resistance to anthelmintics (Gilleard, 2013). This is  
395 consistent with the observation of high levels of coding variation reported within the *F. hepatica*  
396 genome for UK isolates (Cwiklinski et al., 2015b). While the treatment history, and thus  
397 triclabendazole resistance status, of the parasites analysed here was not known; high mitochondrial  
398 diversity has been reported in wild-type parasites that survived treatment with triclabendazole, as  
399 well as the triclabendazole resistant Oberon lab isolate (Walker et al., 2007). Although we have  
400 shown that self-fertilisation is not the norm in UK *F. hepatica* populations, any adult fluke with a  
401 resistant genotype that remains following drug treatment would be able to exploit this aspect of  
402 their biology to reproduce and contaminate the pasture. Thereafter, our results indicate that clonal  
403 expansion within the snail intermediate host, coupled with clumped transmission, could act to  
404 propagate these resistant genotypes within a farm and increase the likelihood of resistant genotypes  
405 mating within a host. In relation to the spread of resistance, in the UK sheep are treated with  
406 anthelmintics against *F. hepatica* more often than cattle and resistance to triclabendazole is more  
407 frequently reported in parasites infecting sheep (Sargison et al., 2010). However, our findings  
408 indicate that drug resistant *F. hepatica* from sheep could be readily be transferred to cattle.

409 Furthermore, since there is no evidence of structuring either geographically or between parasites  
410 from sheep and cattle, this means anthelmintic resistance has the potential to spread around the  
411 country, compounded by the movement of animals and maintained in wildlife reservoirs.

412

### 413 *Conclusion*

414 We have used microsatellite markers to show that *F. hepatica* populations in the field are  
415 genetically diverse and outbred. Thus, despite the ability of *F. hepatica* to self-fertilise within the  
416 definitive host and to clonally multiply within the intermediate host, there is little difference  
417 between the genetic structure of *F. hepatica* and that of any other sexually reproducing parasite.  
418 The fact that some hosts were infected with parasites of identical MLG indicates clumped  
419 transmission to the definitive host, which may be due to aggregation of infective stages on pasture.  
420 Adult *F. hepatica* isolated from naturally infected sheep and cattle in the UK were found to be  
421 highly genetically diverse within the definitive host, but there was little genetic differentiation  
422 between populations. This level of genetic diversity is not a product of grazing over time, since the  
423 genetic diversity of adult parasites infecting lambs grazing for only one season was similar to that of  
424 cattle grazing over several seasons. The genetic diversity reported here implies drug resistance loci  
425 will be recombining freely within the genome, coupled with the high gene flow exhibited by *F.*  
426 *hepatica* populations, this has implications for the emergence and spread of anthelmintic resistance  
427 in *F. hepatica* populations.

428

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647

## 648 **Figure Legends**

649 Fig. 1: Representation of the number of clonal parasites (those with repeated MLGs) found within  
650 each individual sheep (**A**) and cattle (**B**) and shown as a proportion of the total number of parasites  
651 genotyped from each definitive host; numbers on the x-axis are individual animal identifiers; \*  
652 indicates where more than one clone set was found in an individual host, the bar is split to  
653 distinguish the number of parasites within each clone set; ^ indicates where clone sets are shared  
654 between hosts. (**C**) Histogram displaying the genotypic richness values within each definitive host,  
655 separated into sheep and cattle; genotypic richness. Genotypic richness ( $R$ ) is a measure of genetic  
656 diversity and is calculated as  $R = (G - 1) / (N - 1)$  where  $G$  = the number of genotypes identified in  
657 each host and  $N$  = the number of parasites genotyped; each histogram bar is of width 0.05 with the  
658 bar centred over the upper limit. (**D**) Principle Component Analysis for pairwise  $F_{ST}$  values between

659 the parasites of each definitive host. Each data point and its corresponding number represent an  
660 individual animal, and the shape and colour of the symbol represent the location and species of that  
661 animal, respectively.

662 Fig. 2: **(A)** Results of isolation by distance results for cattle parasites. Each point plots the genetic  
663 difference (pairwise test statistic based on  $F_{ST} / [1 - F_{ST}]$ ) against the geographical distance (on a  
664 natural logarithm scale) between each pair of populations. Each population consists of the parasites  
665 on one farm; comparisons are not made between parasites on the same farm. The regression line is  
666 shown and has the following parameters: slope = -0.00129 (95 % CI = -0.00317, 0.00142); intercept  
667 = -0.434;  $p$ -value = 0.2968. Therefore, there is no evidence of isolation by distance as the slope is  
668 negative and the  $p$ -value non-significant. **(B)** Structure (Pritchard et al., 2000) was used to detect  
669 population structure.  $K$  represents the number of populations assumed for each simulation and is  
670 plotted against the mean natural log probabilities. Each simulation was repeated 20 times and error  
671 bars show the standard deviations. **(C)** To determine the most appropriate value for  $K$ ,  $\Delta K$  (the rate  
672 of change in the log probability between successive  $K$  values; Evanno et al., 2005) was determined  
673 using Structure Harvester (Earl and vonHoldt, 2012). The results indicate a single population with  
674 no structure.

Table 1: *Fasciola hepatica* populations collected from sheep and cattle

Species	No. of animals	Demographic information	Median burden (range)	No. of parasites genotyped (median; range per liver)
Sheep <sup>1</sup>	8	Scotland	69	288 <sup>2</sup>
Sheep <sup>1</sup>	5	Wales	(36 –	180 <sup>2</sup>
Sheep <sup>1</sup>	1	England	>200)	36 <sup>2</sup>
Sheep <sup>1</sup>	6	England or Wales	9.5 <sup>3</sup>	216 <sup>2</sup>
Sheep <sup>1</sup>	24	5 farms local to the abattoir in Wales or Central England	(3 – 100)	230 (10.5; 2 – 18)
Cattle	1	England <sup>4</sup>	19	13
Cattle	30	21 farms in Wales <sup>4</sup>	(1 – >230)	616 (18; 1 – 36)

<sup>1</sup>. from lambs (approximately 6 to 12 mths old) that were exposed to *F. hepatica* metacercariae over a period of 3-9 mths in the summer and autumn 2012; <sup>2</sup>. 36 parasites were sampled from each animal; <sup>3</sup>. total enumeration was not performed for six animals; <sup>4</sup>. this information was provided through Rapid Analysis and Detection of Animal-related Risks (RADAR), Animal and Plant Health Agency

Table 2: Summary statistics for the microsatellite panel based on 1579 parasites

Locus	Frequency of null alleles*	No. of alleles exhibited	No. of genotypes exhibited	$H_{obs} / H_{nb}$	$F_{IS}$ **
Fh_1	<b>0.5922</b> <sup>^</sup>	9 <sup>^</sup>	17 <sup>^</sup>	ND	ND
Fh_2	0.0112	28	109	0.823 / 0.843	<b>0.0299</b> <sup>12</sup>
Fh_3	<b>0.1252</b>	7	17	ND	ND
Fh_4	<b>0.0753</b>	16	83	ND	ND
Fh_5	0.0097	39	177	0.852 / 0.867	0.0199
Fh_6	0.0098	30	178	0.885 / 0.903	0.0082
Fh_7	<b>0.1051</b>	11	37	ND	ND
Fh_8	<b>0.2255</b>	16	55	ND	ND
Fh_9	-0.1378	2	3	ND	ND
Fh_10	0.0160	17	75	0.797 / 0.823	0.0327
Fh_11	0.0237	15	68	0.802 / 0.840	0.0442
Fh_12	0.0051	15	66	0.733 / 0.740	0.0061
Fh_13	-0.0058	12	28	0.633 / 0.628	0.0006
Fh_14	<b>0.2794</b>	18	75	ND	ND
Fh_15	0.0064	10	21	0.494 / 0.505	0.0198

\* calculated using CERVUS 3.0.7 (Kalinowski *et al.*, 2007), results in bold indicate greater than 5 % null allele frequency;  $H_{obs}$  = observed heterozygosity;  $H_{nb}$  = unbiased heterozygosity; MLGs = multilocus genotypes; \*\*  $F_{IS}$  values are given to indicate deviations from Hardy-Weinberg equilibrium with those results in bold indicating significant  $p$ -values when using the two-tailed exact test – a Bonferroni and false discovery rate correction were applied <sup>1</sup> = significant when Bonferroni correction applied ( $p = 0.00625$ ) <sup>2</sup> = significant when false discovery rate correction applied; ^ values for locus Fh\_1 were determined for 720 of the parasites from sheep only; ND = not determined

Table 3: Frequency and identity of the most common alleles and genotypes at each locus for parasites from sheep, cattle and all animals

Locus	Most common allele* (frequency)			Most common genotype* (frequency)		
	Parasites from sheep	Parasites from cattle	Parasites from sheep and cattle	Parasites from sheep	Parasites from cattle	Parasites from sheep and cattle
Fh_1	10 (0.32)	ND	ND	1010 (0.26)	ND	ND
Fh_2	08 (0.23)	17 (0.24)	08 (0.22)	0818 (0.098)	0817 (0.11)	0817 (0.095)
Fh_3	08 (0.50)	08 (0.47)	08 (0.49)	0708 (0.35)	0808 (0.29)	0708 (0.32)
Fh_4	19 (0.19)	17 (0.22)	17 (0.20)	1819 (0.080)	1717 (0.086)	1819 (0.073)
Fh_5	27 (0.23)	27 (0.20)	27 (0.22)	2427 (0.083)	2427 (0.085)	2427 (0.084)
Fh_6	15 (0.21)	15 (0.20)	15 (0.21)	1530 (0.056)	1515 (0.048)	1530 (0.049)
Fh_7	13 (0.41)	13 (0.44)	13 (0.42)	1313 (0.22)	1313 (0.24)	1313 (0.23)
Fh_8	12 (0.29)	12 (0.32)	12 (0.30)	1212 (0.16)	1212 (0.18)	1212 (0.17)
Fh_9	07 (0.62)	07 (0.64)	07 (0.63)	0607 (0.65)	0607 (0.56)	0607 (0.62)
Fh_10	09 (0.35)	09 (0.33)	09 (0.34)	0909 (0.12)	0909 (0.14)	0909 (0.13)
Fh_11	13 (0.28)	13 (0.32)	13 (0.30)	1313 (0.096)	1313 (0.13)	1313 (0.11)
Fh_12	10 (0.43)	10 (0.48)	10 (0.45)	1010 (0.19)	1010 (0.25)	1010 (0.21)
Fh_13	08 (0.55)	08 (0.50)	08 (0.53)	0808 (0.31)	0815 (0.31)	0808 (0.28) and 0815 (0.28)
Fh_14	17 (0.24)	17 (0.27)	17 (0.25)	1717 (0.14)	1717 (0.15)	1717 (0.15)
Fh_15	14 (0.64)	14 (0.64)	14 (0.64)	1414 (0.41)	1414 (0.41)	1414 (0.41)

\* alleles are identified by the number of repeats and are in a two-figure format (e.g. 08 indicates the most common allele has 8 repeats of the microsatellite), with genotypes in a four-figure format made up of two alleles (e.g. 0818 indicates the most common genotype is made up of the alleles 08 and 18 having 8 and 18 repeats of the microsatellite respectively)



Figure 1

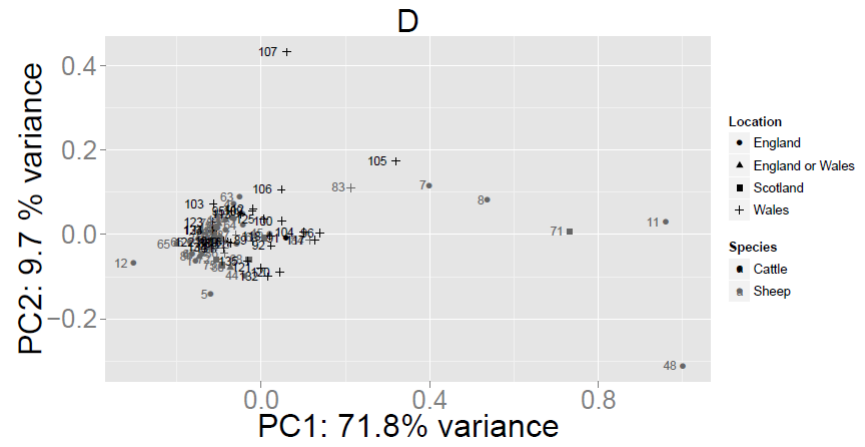
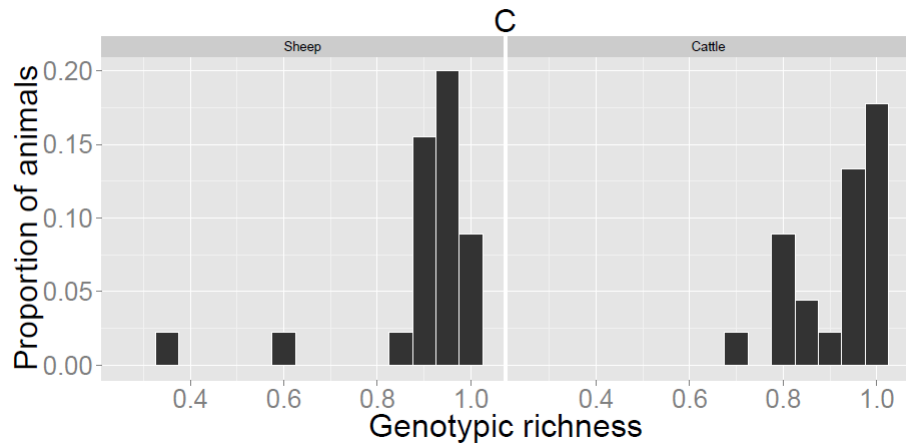
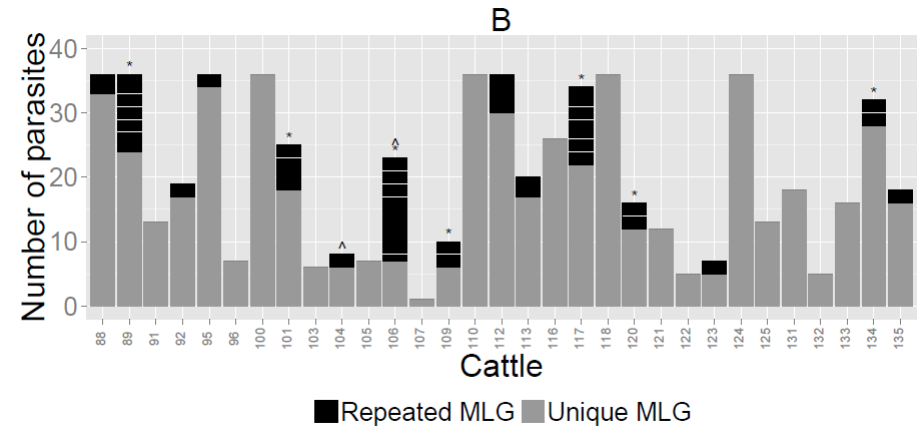
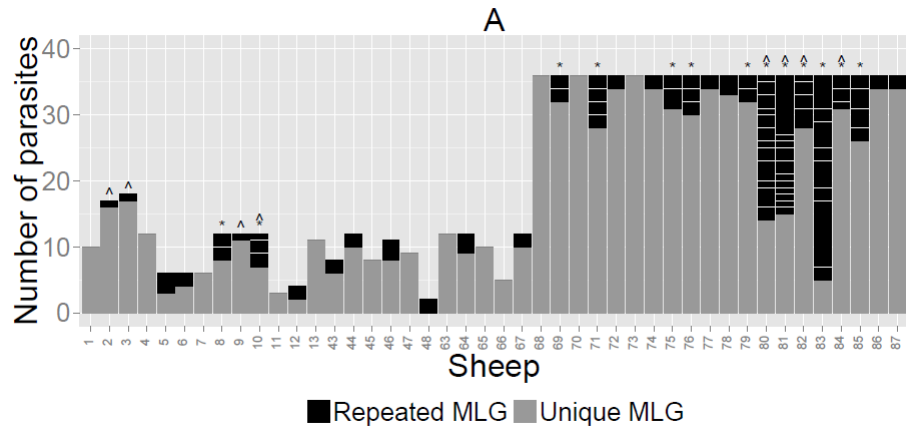


Figure 2

