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Evidence from two independent backcross experiments supports genetic linkage of microsatellite Hcms8a20, but not other candidate loci, to a major ivermectin resistance locus in *Haemonchus contortus*

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Abstract

Haemonchus contortus is the leading parasitic nematode species used to study anthelmintic drug resistance. A variety of candidate loci have been implicated as being associated with ivermectin resistance in this parasite but definitive evidence of their importance is still lacking. We have previously performed two independent serial backcross experiments to introgress ivermectin resistance loci from two *H. contortus* ivermectin-resistant strains - MHco4(WRS) and MHco10(CAVR) - into the genetic background of the ivermectin-susceptible genome reference strain MHco3(ISE). We have interrogated a number of candidate ivermectin resistance loci in the resulting backcross populations and assessed the evidence for their genetic linkage to an ivermectin resistance locus. These include the microsatellite marker Hcms8a20 and six candidate genes *Hco-glc-5*, *Hco-avr-14*, *Hco-lgc-37* (previously designated *Hco-hg-1*), *Hco-pgp-9* (previously designated *Hco-pgp-1)*, *Hco-pgp-2* and *Hco-dyf-7*. We have sampled the haplotype diversity of amplicon markers within, or adjacent to, each of these loci in the parental strains and fourth generation backcross populations to assess the evidence for haplotype introgression from the resistant parental strain into the genomic background of the susceptible parental strain in each backcross. The microsatellite Hcms8a20 locus showed strong evidence of such introgression in both independent backcrosses, suggesting it is linked to an important ivermectin resistance mutation in both the MHco4(WRS) and MHco10(CAVR) strains. In contrast, *Hco-glc-5*, *Hco-avr-14*, *Hco-pgp-9* and *Hco-dyf-7* showed no evidence of introgression in either backcross. *Hco-lgc-37 and Hco-pgp-2* showed only weak evidence of introgression in the MHco3/4 backcross but not in the MHco3/10 backcross. Overall, these results suggest that microsatellite marker Hcms8a20, but not the other candidate genes tested, is linked to a major ivermectin resistance locus in the MHco4(WRS) and MHco10(CAVR) strains. This work also emphasizes the need for genome-wide approaches to identify mutations responsible for the ivermectin resistance in this parasite.

Keywords: Haemonchus contortus; Nematode; Ivermectin; Anthelmintic; Drug resistance

1. Introduction

Identifying genetic markers of anthelmintic resistance is a key research priority in order to provide tools to monitor and manage its emergence and spread (James et al., 2009; Beech et al., 2011). Anthelmintic resistance is widespread in *Haemonchus contortus*, a parasitic nematode of small ruminants that has been a leading model for the study of resistance to ivermectin, one of the most important drugs used in human and animal helminth control (Kaplan and Vidyashankar, 2012; Gilleard, 2013). There have been many efforts over the last two decades to test specific candidate genes for associations with ivermectin resistance in *H. contortus* (Gilleard, 2013). These candidate genes have been chosen largely on the basis of having potential roles as drug targets or in drug efflux (Gilleard, 2006; Gilleard and Beech, 2007). Although evidence of selection by ivermectin treatment has been suggested for a number of such loci, their roles in resistance have not yet been conclusively demonstrated (Gilleard, 2006; Gilleard and Beech, 2007).

In the study presented here, we examine seven genetic loci previously suggested to be associated with ivermectin resistance in *H. contortus*; a microsatellite marker Hcms8a20 and six candidate genes, *Hco-avr-14, Hco-glc-5, Hco-lgc-37, Hco-pgp-9, Hco-pgp-2* and *Hco-dyf-7*. For each of these loci, we have investigated evidence for genetic linkage to a major ivermectin resistance locus in two ivermectin-resistant strains, MHco4(WRS) and MHco10(CAVR). These strains are commonly used in resistance studies and were originally derived as field populations from different continents (Redman et al., 2008). We have used two previously characterised ivermectin-resistant *H. contortus* populations - MHco3/4.BC and MHco3/10.BC - that were independently derived by serial backcrossing of the MHco4(WRS) and MHco10(CAVR) resistant strains against the MHco3(ISE) susceptible strain, respectively (Redman et al., 2012). For each of the candidate loci, we evaluated the evidence for introgression of haplotypes from the resistant parental strains into the fourth generation

backcross populations in order to determine whether they are located in a region of the genome linked to a major ivermectin resistance locus (Redman et al., 2012).

The basis on which each of the candidate loci have been previously implicated in ivermectin resistance is briefly explained here. The Hcms8a20 microsatellite was the only one out of 18 microsatellite loci that showed evidence of genetic introgression in the two independent backcross experiments undertaken to map ivermectin resistance loci in the MHco4(WRS) and MHco10(CAVR) strains [strains?] (Redman et al 2012). *Hco-avr-14* and *Hco-glc-5* are members of the *H. contortus* glutamate-gated chloride channel (GLC) family (Wolstenholme and Rogers, 2005; McCavera et al., 2007; Laing et al., 2013). Three members of this family in *Caenorhabditis elegans* - *Cel-glc-1, Cel-avr-14* and *Cel-avr-15* - have been shown to be ivermectin targets by genetic mutation and complementation studies (Dent et al., 2000; Ghosh et al., 2012). The *H. contortus* homologue of *Cel-avr-14* - *Hco-avr-14* - was implicated in ivermectin resistance by its ability to rescue ivermectin susceptibility when heterologously expressed in *C. elegans,* suggesting it is an ivermectin target in the parasite (McCavera et al., 2007, 2009; Glendinning et al., 2011). *Hco-glc-5* was implicated by the observation of haplotype frequency changes during experimental passage of an *H. contortus* strain under ivermectin selection (Blackhall et al., 1998a). *Hco-lgc-37* - a gamma aminobutyric acid (GABA)-gated chloride channel renamed from *Hco-hg-1* (Beech et al., 2010a) - was also implicated using the same approach and strains (Blackhall et al., 2003). A subsequent study has also presented evidence that certain alleles of both *Hco-glc-5* and *Hco-lgc-37* were associated with reduced sensitivity of adult feeding and larval movement to moxidectin (Beech et al., 2010b).

Hco-pgp-9 and *Hco-pgp-2* are members of the P-glycoprotein (PGP) trans-membrane transporter family. *Hco-pgp-9* and *Hco-pgp-2* were originally designated as *Hco-pgp-1* and *Hco-pgp-A*, respectively (Xu et al., 1998; Le Jambre et al., 1999), but were subsequently re-named based on the identification of their respective *C. elegans* orthologues (Williamson and Wolstenholme, 2012). This

gene family, known to be involved in ivermectin efflux in mammals (Schinkel, 1999), has been suggested to be involved in ivermectin resistance in parasitic nematodes in a number of studies and reviews (Blackhall et al., 1998b; Xu et al., 1998; Le Jambre et al., 1999, Sangster et al., 1999; Bartley et al., 2009; Williamson and Wolstenholme, 2012). In the case of *Hco-pgp-2*, Blackhall et al. (1998b) used the same methodology and strains as used for *Hco-glc-5* and *Hco-lgc-37*. As with the two previously examined candidate genes, they showed that a single *Hco-pgp-2* haplotype had a significantly higher frequency in the ivermectin selected population compared with the non-selected population (Blackhall et al., 1998b). *Hco-pgp-9* was independently implicated in ivermectin resistance by the observation that populations of *H. contortus/Haemonchus placei* hybrid F1 progeny that survived ivermectin treatment contained a higher frequency of *Hco-pgp-9* alleles derived from the resistant *H. contortus* parental strain than from the susceptible *H. placei* parental strain (Le Jambre et al., 1999).

The most recent candidate gene to be implicated in ivermectin resistance is *Hco-dyf-7,* a gene which encodes a protein required for proper dendritic anchoring and migration of amphid neurons (Heiman and Shaham, 2009). This gene has recently been proposed to have an important, and possibly global, role in ivermectin resistance (Urdaneta-Marquez et al., 2014). Classical genetic mapping and transgenic rescue experiments showed that loss-of-function of the *Cel-dyf-7* gene confers ivermectin resistance in *C. elegans* (Urdaneta-Marquez et al., 2014). In the same study, an orthologue of this *C. elegans* gene was identified in *H. contortus*, *Hco-dyf-7.* A specific haplotype of this *H. contortus* gene, defined by 15 single nucleotide polymorphisms (SNP)s, was shown to increase in frequency during ivermectin selection using the same parasite strains as had been previously used by Blackhall et al. (1998a, 1998b, 2003) for *Hco-glc-5*, *Hco-lgc-37* and *Hco-pgp-2*. In addition, several - although not all - of the SNPs that defined this haplotype were reported to occur more frequently in a panel of

ivermectin-resistant *H. contortus* field populations compared with several susceptible populations (Urdaneta-Marquez et al., 2014).

In summary, there is circumstantial, but not conclusive, evidence for the potential association of a number of different *H. contortus* candidate loci with ivermectin resistance. In this paper, we interrogate two previously validated independent backcross experiments for evidence of genetic linkage of each of these candidate loci to a major ivermectin resistance mutation. Our results support previous evidence that the microsatellite marker Hcms8a20 is linked to a major invermectin resistance locus in the two ivermectin-resistant parental strains used in each backcross. In contrast, our results do not provide similar support for any of the other candidate loci tested.

2. Materials and methods

2.1. Parasite populations and preparation of genomic DNA

The backcross populations used in this study were derived from two independent backcross experiments between the susceptible genome reference strain - MHco3(ISE) (Laing et al., 2013) - and each of two independent ivermectin-resistant strains, MHco4(WRS) and MHco10(CAVR) (Redman et al., 2012). The MHco3(ISE) strain is susceptible to all the major anthelmintic classes and was originally derived from the ISE strain by multiple rounds of inbreeding before the genome project was commenced (Roos et al., 2004; Laing et al., 2013). MHco4(WRS) is derived from the White River Strain (WRS) originally isolated as an ivermectin-resistant field strain from South Africa (Van Wyk and Malan, 1988). MHco10(CAVR) is derived from the Chiswick Avermectin Resistant Strain (CAVR) strain originally isolated as an avermectin-resistant strain from the field in Australia (Le Jambre et al., 1995). Details of the genetic backcrossing strategy have been previously reported in Redman et al. (2012), so only a brief overview is given here. F1 progeny of parental crosses were

134 exposed to ivermectin by oral dosing and the survivors were then backcrossed against the susceptible
135 MHco3(ISE) parental strain. Subsequent backcrossing of drug survivors was repeated for three more
136 generations such that phenotypically resistant fourth generation backcross populations were obtained
137 (MHco3/4.BC and MHco3/10.BC). These populations have an overall genetic background of the
138 susceptible parental MHco3(ISE) strain as previously validated with microsatellite markers. However,
139 they are phenotypically resistant to ivermectin, showing that ivermectin resistance loci have been
140 introgressed from the original resistant parental strains (Redman et al., 2012). There was no evidence of
141 sex linkage of the resistance trait from the backcrossing experiments. Pools of genomic DNA were
142 created by combining lysates from the heads of 20 males and 20 females from each of the three
143 parental populations - MHco3(ISE), MHco4(WRS) and MHco10(CAVR). The DNA lysis protocol has
144 been previously described (Redman et al., 2008). The fourth generation backcrosses were
145 experimentally passaged for one generation to increase worm numbers (Redman et al., 2012). Progeny
146 of this passage (F2s of the fourth generation backcrosses) were selected with 0.1 mg/kg of ivermectin
147 in vivo in a controlled efficacy test (Redman et al., 2012) from which drug surviving females were used
148 to create lysate pools of the MHco3/4.BC and MHco3/10.BC backcrosses assessed in this study. Pools
149 were created by combining genomic DNA (gDNA) of 33 and 26 drug surviving females of the
150 MHco3/4.BC and MHco3/10.BC strains, respectively.

151

152 *2.2. PCR amplification*

153 Test PCR amplifications were carried out on the six candidate genes using primer pairs reported
154 in previously published studies (Blackhall et al. 1998a, b, 2003; Jagannathan et al., 1999; Le Jambre et
155 al., 1999; Urdaneta-Marquez et al., 2014). Only the *Hco-lgc-37* primer pair from Blackhall et al. (2003)
156 amplified sufficiently robustly in all five populations and were used in this study. Geneious Pro version

157 6.1.6 was used to design new primer pairs for the other loci (http://www.geneious.com, Kearse et al.,
158 2012) (Supplementary Table S1). These spanned at least one intron to ensure sufficient polymorphism
159 was obtained in the amplified sequence. Primers were also designed to amplify a non-coding 725 bp
160 fragment 1032 bases away from Hcms8a20, a microsatellite for which there was previous evidence of
161 genetic linkage to an ivermectin resistance locus (Redman et al., 2012). PCRs were performed in 50 µl
162 reactions using 0.5 µL of pooled gDNA template and Phusion High-Fidelity HF DNA Polymerase
163 (New England Biolabs, USA country). The following thermocycling conditions were used: 98ºC for 2
164 min, (98ºC for 10 s, 60ºC for 30 s and 72ºC for 30 s) for 35 cycles, 72ºC for 5 min.

165

166 *2.3. Cloning and sequencing of PCR amplicons*

167 PCR products were gel purified using an e.Z.N.A MicroElute® Gel Extraction Kit (Omega
168 Biotek, USAcountry) and then cloned into the pJET1.2/blunt cloning vector using a CloneJET PCR
169 Cloning Kit (ThermoFisher Scientific, USAcountry). For each cloning plate, corresponding to each
170 population/locus combination, 24 colonies were randomly selected and plasmid DNA purified using
171 the e.Z.N.A Plasmid Mini-Kit I (Omega Biotek) (20 colonies were selected in the case of *Hco-dyf-7*).
172 Plasmid clones were then sequenced using the T7(5'-TAATACGACTCACTATAGGG-3') forward
173 primer on a BigDye Terminator Cycle Sequencing platform (Applied Biosystems, USAcountry). *Hco-*
174 *pgp-9* clones required an additional (reverse) primer to sequence given its longer amplicon length
175 (pJET1.2 reverse primer: 5'-AAGAACATCGATTTTCCATGGCAG-3').

176

177 *2.4. DNA sequence analysis*

All sequences were aligned using the MUSCLE alignment tool (Edgar, 2004) at default settings in Geneious Pro v. 6.1.6. Primer sequences were trimmed and the sequences were re-aligned. Polymorphisms appearing more than once in the sequence data set are expected to be real, whereas polymorphisms that only occur once are possible artefacts due to polymerase-induced errors (Chaudhry et al., 2015; Redman et al., 2015). Consequently, we only considered SNPs occurring more than once in the entire dataset in order to take a conservative approach and ensure only real polymorphisms were considered. Sequence alignments of both the unedited and edited sequences are presented in Supplementary Figs. S1, S2.

The frequency of each haplotype was determined at each locus for each population and this is referred to as the "haplotype profile". Haplotypes were labelled alphabetically for each locus based on their rank frequency observed in the MHco3(ISE) population (Figs. 1 – 3). Remaining haplotypes not observed in MHco3(ISE) were labelled arbitrarily. The number of clones successfully sequenced and the number of different haplotypes observed at each locus were recorded for each strain (Table 1). For each locus, the number of haplotypes shared by the parental strains of a cross, together with the number of haplotypes unique to each parental strain of a cross, was also recorded (Table 1). Pairwise Fst values (calculated in Arlequin v. 3.1 at default settings; Excoffier et al., 2005) were used to quantify the level of similarity between haplotype profiles between parental and backcross populations for each amplicon marker. P-values of less than 0.05 were considered significant.

2.5. Chromosomal assignments for each amplicon marker

Chromosomal synteny is high between *H. contortus* and *C. elegans* (although gene order on a chromosome is generally poorly conserved) (Laing et al., 2011). Consequently, we inferred the chromosomal assignment for each amplicon marker based on the syntenic relationships with the *C.*

elegans orthologues of gene models present on the same *H. contortus* genomic scaffold. The closest 14 gene models on the same genome scaffold as each amplicon marker (a total of 15 gene models including the candidate gene) were assessed for orthology with *C. elegans* genes where possible. Scaffolds containing the Hcms8a20 and *Hco-lgc-37* amplicon markers were small, so all gene models on each scaffold were assessed (1 and 6, respectively). A custom BLAST search (Liebert et al., 2000) (blastn -task dc-megablast -query H.contortus_genes.fa -db C.elegans_CDSs.fa -out outfile) was used to identify orthologues in the most current ensembl.org *C. elegans* CDS database downloaded at ftp://ftp.ensembl.org/pub/release-83/fasta/caenorhabditis_elegans/cds/. Only unambiguous orthologues were used in the analysis of synteny. Identification of a *C. elegans* orthologue was considered unambiguous if the *H. contortus* query gene model identified a *C. elegans* gene model with an e-value of 1e-9 or less and with no other hits on different chromosomes having an e-value within one order of magnitude. *Hco-glc-5*, *Hco-avr-14* and *Hco-pgp-2* are located on chromosome 1, *Hco-lgc-37* on chromosome 3, and *Hco-dyf-7* on the X chromosome (Supplementary Table S2). A chromosomal assignment could not be made for *Hco-pgp-9* due to mixed chromosomal synteny with *C. elegans* orthologues, nor for Hcms8a20 due to the lack of adjacent gene models on the relatively short scaffold on which it was located (27 kb).

[Somewhere in section 2 mention which *P* value was considered significant because you mention *P* values in section 3.]

3. Results

3.1. Amplicon markers for all the candidate loci show a high level of genetic differentiation between the H. contortus parental strains used in the two independent backcross experiments

In order to investigate haplotype introgression between parental genomes during a serial backcross, markers with a high level of genetic differentiation between parental strains are needed. In

this particular case, haplotypes unique to the ivermectin-resistant parental strain, or those present at very different frequencies in the two parental strains, are the most informative for assessing haplotype introgression from each resistant parental strain into the backcross populations. Consequently, we first assessed the haplotype diversity of the seven amplicon markers within and among the parental strains used in the two backcrosses. We PCR-amplified and sequenced between 14 and 24 clones for each marker from the MHco3(ISE), MHco4(WRS) and MHco10(CAVR) parental strains (Table 1). A high level of sequence diversity was observed for all seven loci (Table 1). The mean number of haplotypes for the seven markers was 3.5, 8.33 and 5.83 haplotypes per locus for MHco3(ISE), MHco4(WRS) and MHco10(CAVR) parental strains, respectively (Table 1).

Although some shared haplotypes were present, the majority were not shared between the parental strains used in each backcross (Table 1). Pairwise Fst values were used to quantify the genetic differentiation for each amplicon marker between the parental strains (Table 2). There was a high level of genetic differentiation between the MHco3(ISE) susceptible parental strain and both resistant parental strains, MHco4(WRS) and MHco10(CAVR), for all seven amplicon markers. Pairwise Fst values ranged between 0.078 and 0.533 and all were statistically significantly different from zero at $P < 0.01$ (Table 2).

3.2. The amplicon marker adjacent to microsatellite Hmcs8a20 shows strong evidence of haplotype introgression from the ivermectin-resistant parental strain in both independent backcross populations

We investigated the evidence for haplotype introgression from the resistant parental strains into the two independent backcross populations for an amplicon marker adjacent to microsatellite Hmcs8a20. This marker was PCR-amplified, cloned and sequenced from the MHco3/4.BC and MHco3/10.BC backcross populations and the haplotype profiles compared with those of the parental

247 strains (Table 1). In both of these backcross populations, this marker showed a high degree of genetic
248 differentiation from the MHco3(ISE) susceptible parental strain; Fst values were high and statistically
249 significantly different from zero for both the MHco3(ISE) - MHco3/4.BC and MHco3(ISE) -
250 MHco3/10.BC pairwise comparisons (Fst = 0.383, *P* = 0.000 and Fst = 0.512, *P* = 0.000, respectively)
251 (Table 3). The most frequent haplotypes of this marker in the two backcross populations were
252 haplotype-D in MHco3/4.BC and haplotype-E in MHco3/10.BC at 60.9% and 78.3% frequencies,
253 respectively. These haplotypes were not detected in the MHco3(ISE) susceptible parental isolate but
254 were present in respective resistant parental strains used in each backcross (Fig. 1). These results
255 suggest haplotype introgression from each resistant parental strain into the MHco3(ISE) susceptible
256 parental background during both independent backcrossing experiments.

257

258 *3.3. The amplicon markers within the Hco-glc-5*, *Hco-avr-14*, *Hco-pgp-9 and Hco-dyf-7 genes show no*
259 *evidence of haplotype introgression from the ivermectin-resistant parental strain in either of the*
260 *independent backcross populations*

261 Amplicon markers within the *Hco-glc-5*, *Hco-avr-14*, *Hco-pgp-9* and *Hco-dyf-7* genes were
262 PCR-amplified, cloned and sequenced from the MHco3/4.BC and MHco3/10.BC backcross
263 populations, and the haplotype profiles compared with those present in the parental strains (Table 1).
264 We measured the level of pairwise genetic differentiation for each of these markers between the
265 MHco3(ISE) parental strain and each of the MHco3/4.BC and MHco3/10.BC backcross populations. In
266 all pairwise comparisons, the Fst values were not statistically significantly different from zero (Table
267 3). In contrast to the Hcms8a20 amplicon marker, the most frequent haplotype present in each
268 backcross population was not unique to the resistant parental strain for any of the four candidate
269 ivermectin resistance loci. Furthermore, in all cases, the most frequent haplotype present in backcross

populations was present at a higher frequency in the MHco3(ISE) susceptible parental strain than the resistant parental strain (Figs. 2, 3). These results suggest no evidence of haplotype introgression from either resistant parental strain into the MHco3(ISE) susceptible parental background during either of the backcrossing experiments for these four amplicon markers.

3.4. The amplicon markers within the Hco-lgc-37 and Hco-pgp-2 genes show weak evidence of haplotype introgression from the ivermectin-resistant parental strain in the MHco3/4.BC backcross but not the MHco3/10.BC backcross populations

Amplicon markers within the *Hco-lgc-37* and *Hco-pgp-2* genes were PCR-amplified, cloned and sequenced from the MHco3/4.BC and MHco3/10.BC backcross populations and the haplotype profiles compared with those present in the parental strains (Table 1). We measured the level of pairwise genetic differentiation for each of these markers between the MHco3(ISE) parental strain and each of the MHco3/4.BC and MHco3/10.BC backcross populations. In the case of the MHco3/10.BC backcross, the Fst values were not statistically significantly different from zero for either marker (Table 3). For the MHco3/4.BC backcross, the MHco3(ISE)-MHco3/4.BC pairwise Fst values were statistically significantly different from zero for both the *Hco-lgc-37* and *Hco-pgp-2* markers at 0.220 ($P = 0.004$) and 0.115 ($P = 0.000$), respectively (Table 3). For these two pairwise comparisons, there was also a haplotype present in the backcross populations that was unique to the resistant parental strain with respect to the MHco3(ISE) parental strain; haplotype-I of *Hco-pgp-2* and haplotype-H of *Hco-lgc-37* (Fig. 2). However, these were only present at very low frequencies in each backcross (Fig. 2). These results provide weak evidence for haplotype introgression from the resistant parental strains into the MHco3(ISE) susceptible parental background for these two markers in the MHco3/4.BC backcross but not in the MHco3/10.BC backcross populations.

4. Discussion

The objective of this work was to investigate a number of leading candidate loci for evidence of genetic linkage to a major ivermectin resistance locus in two ivermectin-resistant *H. contortus* strains; MHco4(WRS) and MHco10(CAVR). These two strains have previously been serially backcrossed against the ivermectin-susceptible *H. contortus* genome reference strain MHco3(ISE) with ivermectin selection applied at each generation (Redman et al., 2012). The resulting fourth generation backcross populations (MHco3/4.BC and MHco3/10.BC) have been previously genetically and phenotypically validated (Redman et al., 2012). In that study, 17 out of 18 microsatellite markers were found to have allelic profiles in the MHco3/4.BC and MHco3/10.BC populations that were similar to those of the MHco3(ISE) susceptible parental strain. This demonstrated that the genetic background of these backcross populations was predominantly derived from the MHco3(ISE) susceptible parental strain as expected from the serial backcrossing regime. In the original published study, just one of the 18 microsatellite markers, Hcms8a20, had an allele at high frequency in the backcross populations (following ivermectin treatment) that was derived from the resistant parental strains in both serial backcross experiments (Redman et al., 2012). This result suggested that this microsatellite marker is linked to an important ivermectin resistance locus in both the MHco4(WRS) and MHco10(CAVR) ivermectin-resistant strains.

In the work presented here, we aimed to test the hypothesis that the Hcms8a20 microsatellite marker was linked to an important ivermectin-resistant locus in the MHoc4(WRS) and MHco10(CAVR) strains and, at the same time, test the other leading candidate ivermectin resistance loci from the peer-reviewed literature. Fig. 4 illustrates the conceptual approach we adopted. We examined the haplotype profiles of PCR amplicon markers located within, or close to, our loci of

316 interest in the parental and backcross populations of both serial backcross experiments. If a genetic
317 marker is closely linked to an important ivermectin resistance locus, haplotypes of that marker that are
318 present in the ivermectin-resistant parental strain, and linked to the ivermectin resistance mutation,
319 should be inherited through the backcross due to the ivermectin selection applied at each generation by
320 so called "genetic hitchhiking" (Fig. 4A). Consequently, such haplotypes will be introgressed from the
321 ivermectin-resistant parental strain into the predominantly MHco3(ISE) genetic background during the
322 backcrossing procedure (Fig. 4A). Hence, it is possible to make predictions regarding the haplotype
323 profiles of markers in the backcross populations depending on whether or not they are linked to an
324 important ivermectin resistance mutation (Fig. 4B). The presence and frequency of haplotypes for a
325 marker that is not linked to an important ivermectin resistance locus should be very similar to those
326 found in the MHco3(ISE) parental strain and haplotypes that are unique to the resistant parental strain
327 should be absent (or rare) in the backcross populations. This is because there will be no introgression of
328 haplotypes from the ivermectin-resistant parental strain into the backcross populations in that region of
329 the genome (markers A and C in Fig. 4). Conversely, the presence and frequency of haplotypes for a
330 marker that is linked to an important ivermectin resistance locus should differ significantly from those
331 in the MHco3(ISE) parental strain due to the presence of one or more haplotypes that have been
332 introgressed from the resistant parental strain (markers B and D in Fig. 4). The extent of this difference
333 will depend on how closely linked the marker is to the loci under selection.

334 The haplotype profiles of the amplicon marker located 17.8 kb away from the Hcms8a20
335 microsatellite marker locus clearly fit the predictions for a marker linked to an important ivermectin
336 resistance locus. In both the MHco3/4.BC and MHco3/10.BC backcross populations, the haplotype
337 profiles of the Hcms8a20 amplicon marker were significantly different to the haplotype profile that was
338 present in the MHco3(ISE) susceptible reference strain (Table 3, Fig. 1). This is indicated by the high
339 Fst values observed between both backcross populations and the MHco3(ISE) strain, by far the highest

values for any of the seven loci examined (Table 3). Furthermore, in both backcross populations the most frequent haplotype for the Hcms8a20 amplicon marker was one which was present in each ivermectin-resistant parental strain but absent from the MHco3(ISE) susceptible parental strain; haplotype-D in MHco3/4.BC and haplotype-E in MHco3/10.BC (Fig. 1). This suggests that these haplotypes have been inherited from the resistant parental strains during the serial backcrosses under the influence of the ivermectin selection applied at each generation. Interestingly, a different haplotype has been introgressed from each resistant parental strain in the two independent backcross experiments. This is consistent with the previously published results using the allele profiles of the Hcms8a20 microsatellite marker itself (Redman et al., 2012). In that case, for each separate backcross experiment, a different microsatellite allele, specific to each ivermectin-resistant parental strain, was introgressed into the backcross populations. This is not necessarily surprising since MHco4(WRS) and MHco10(CAVR) were originally derived from different continents - South Africa and Australia, respectively - so the causal ivermectin resistance mutation(s) may well have originated independently in the two strains. In that case, they would be expected to be present on different haplotype backgrounds in each strain.

In contrast to the Hcms8a20 marker, the haplotype profiles of the amplicon markers within the candidate ivermectin resistance genes *Hco-glc-5*, *Hco-avr-14*, *Hco-pgp-9* and *Hco-dyf-7* clearly fit the predictions for markers that are not linked to an important ivermectin resistance locus in the MHco4(WRS) and MHco10(CAVR) resistant strains. For these markers, there was no significant difference in the haplotypes present in either of the independent backcross populations compared with the susceptible MHco3(ISE) population. Furthermore, unlike for the Hcms8a20 amplicon marker, there were no haplotypes unique to either resistant parental strain present in either of the backcross populations for any of these genes. This was also the case for the amplicon markers within the *Hco-lgc-37* and *Hco-pgp-2* genes for the MHco3/10.BC backcross population, suggesting these genes are also

not linked to an important ivermectin resistance locus in the MHco10(CAVR) strain. However, the result for these two markers was less clear cut in the other backcross experiment; there was a statistically significant difference in the haplotypes present in the MHco3/4.BC backcross population compared with the susceptible MHco3(ISE) population. Also, for each of these two markers, there was a haplotype present in the MHco3/4.BC backcross population that was unique to the MHco4(WRS) resistant parental strain (haplotype-I for *Hco-pgp-2* and haplotype-H for *Hco-lgc-37*). However, these haplotypes were present at very low frequencies in the MHco3/4.BC population. Overall, we conclude there is weak but inconclusive evidence of linkage of the *Hco-lgc-37* and *Hco-pgp-2* genes to an important ivermectin resistance locus in the MHco4(WRS), but not the MHco10(CAVR), strain. This more ambiguous result could potentially be due to these genes being distantly linked to an important ivermectin resistance locus on the same chromosome or tightly linked to a locus with a minor effect.

Although the results presented here provide additional evidence that the Hcms8a20 marker is genetically linked to an important ivermectin resistance locus in the MHco4(WRS) and MHco10(CAVR) strains, this is not the case for the other leading candidate ivermectin resistance loci. It is possible that these loci are not associated with ivermectin resistance given they have been implicated largely on the basis of single studies using a small number of populations and often a small number of individuals within each population. Other recent work has also found no evidence of selection for the *Hco-lgc-37*, *Hco-glc-5, Hco-avr-14* and *Hco-dyf-7* loci when comparing *H. contortus* from UK farms with a history of ivermectin treatment with those without (Laing R, Maitland K, Lecová L, Skuce P, Tait A, Devaney E;, In press [either ‘in press’ or ‘unpublished data’]). Alternatively, it may be that these loci are linked to an important ivermectin loci in some strains but not in MHco4(WRS) and MHco10(CAVR). This is possible since the previous published work implicating these candidate loci was not conducted on these particular strains.

Although this and previous work provides strong evidence that the Hcms8a20 microsatellite marker is genetically linked to an important ivermectin resistance locus in the MHco4(WRS) and MHco10(CAVR) strains, it could be still be relatively distant from the causal mutation(s). Only limited genetic recombination will have occurred in four generations of backcrossing. Consequently, the genomic region(s) introgressed into the backcross populations from the resistant parental strains is expected to be large. This is supported by recent genome-wide SNP analysis of the backcross and parental populations that has identified several genomic scaffolds that have a strong signal of introgression, some of which are several Mb in length (Martinelli, A, Rezansoff, A, Doyle, S, Gilleard, J and Cotton, J, unpublished data). The Hcms8a20 marker is located on one of these scaffolds, providing further support for its linkage to an important ivermectin resistance locus (Martinelli, A, Rezansoff, A, Doyle, S, Gilleard, J and Cotton, J, unpublished data). Further interrogation of the *H. contortus* MHco3(ISE) reference genome as the assembly improves will help locate the genomic position of the causal mutations to which the Hcms8a20 locus is linked (http://www.sanger.ac.uk/resources/downloads/helminths/haemonchus-contortus.html).

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Figure Legends

[Each figure legend must 'stand alone', separate from every other figure legend and the main text. Therefore if the information provided in the figure legend is specific to a particular parasite genus/species, the genus/species should be mentioned in the first summary sentence of the figure legend.]

Fig. 1. Frequency histograms of haplotypes of the amplicon marker adjacent to microsatellite Hcms8a20. Haplotypes are labelled alphabetically on the X axis with respective frequencies observed on the Y axis in each population. (A) Haplotype distributions for the MHco4(WRS) x MHco3(ISE) backcross by frequency histograms for MHco3(ISE), MHco4(WRS) and MHco3/4.BC, respectively. (B) Haplotype distributions for the MHco10(CAVR) x MHco3(ISE) backcross by frequency histograms for MHco3(ISE), MHco10(CAVR) and MHco3/10.BC, respectively.

Fig. 2. Haplotype distributions for the candidate loci in the MHco4(WRS) x MHco3(ISE) backcross. Frequency histograms of haplotypes of the six candidate gene loci assessed in this study are shown for the MHco3(ISE), MHco4(WRS) and MHco3/4.BC backcross strains. Haplotypes are labelled alphabetically on the X axis with respective frequencies observed on the Y axis in each population.

Fig. 3. Haplotype distributions for the candidate loci in the MHco10(CAVR) x MHco3(ISE) backcross. Frequency histograms of haplotypes of the six candidate gene loci assessed in this study are shown for the MHco3(ISE), MHco10(CAVR) and MHco3/10.BC backcross strains. Haplotypes are labelled alphabetically on the X axis with respective frequencies observed on the Y axis in each population.

Fig. 4. [?general heading] (A) Schematic representation of the genomes of the resistant and susceptible parental strains and the backcross population. The coloured bars represent the five pairs of chromosomes with the resistant parental strain genetic background represented in red and the susceptible parental genetic background represented in blue. Causal resistance mutations are indicated by *. The positions of four hypothetical genetic markers A, B, C and D are indicated. (B) Predictions for the haplotype profiles of the four hypothetical genetic markers in the backcross population.

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565 [For both supplementary figures, given the larges amounts of data (Alex Loukas has approved this
566 requested change), please consider submitting the data to Mendeley and replace all references to the
567 figures in the manuscript with the relevant urls supplied by Mendeley. See https://data.mendeley.com/]

568

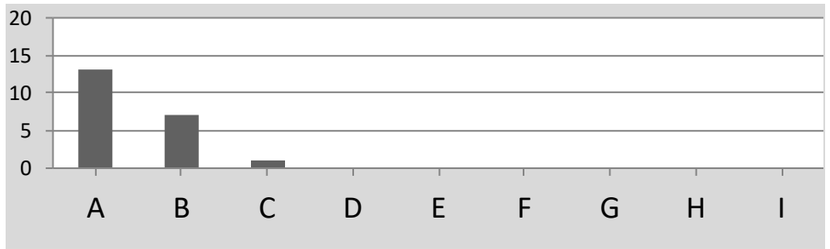
569 **Supplementary Fig. S1.** Sequence alignments for each of the seven loci using unedited sequences (no
570 singleton single nucleotide polymorphisms (SNPs) removed).

571

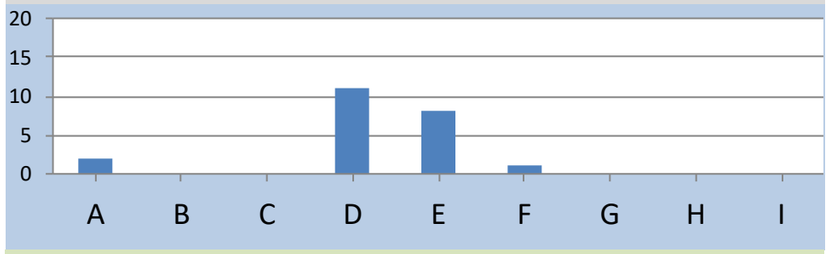
572 **Supplementary Fig. S2.** Sequence alignments for each of the seven loci using edited sequences
573 (singleton single nucleotide polymorphisms (SNPs) removed).

Panel A

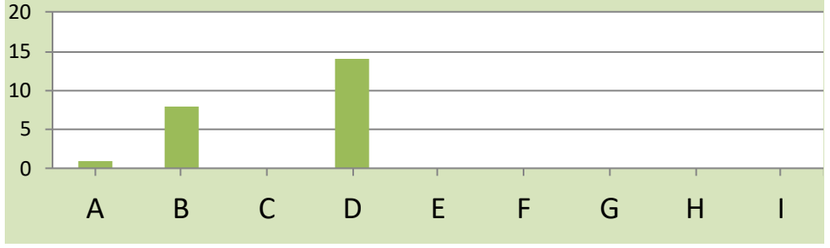
MHco3(ISE)



MHco4(WRS)

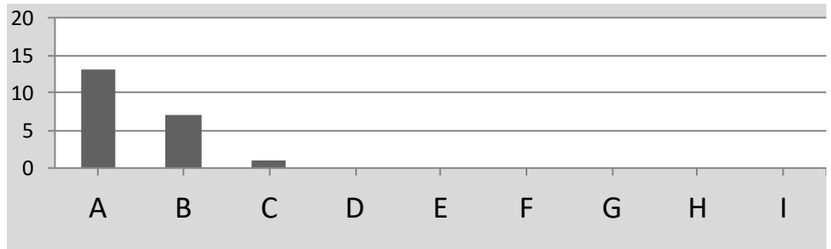


MHco3/4.BC

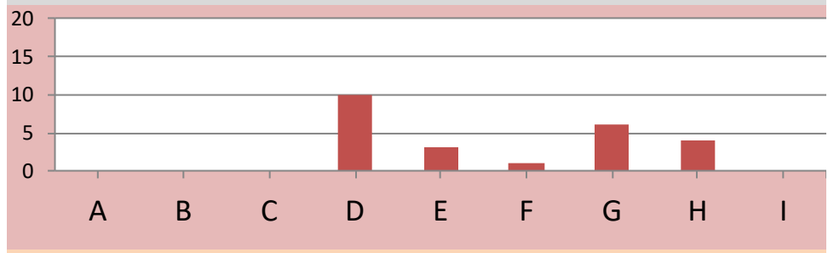


Panel B

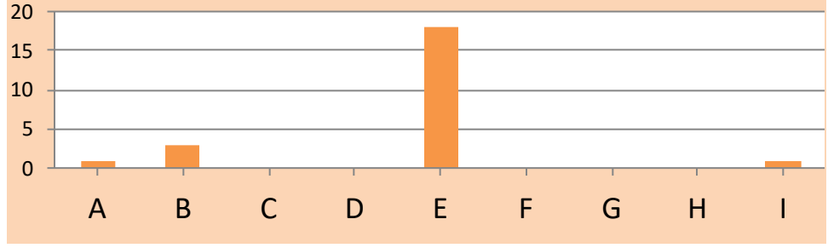
MHco3(ISE)

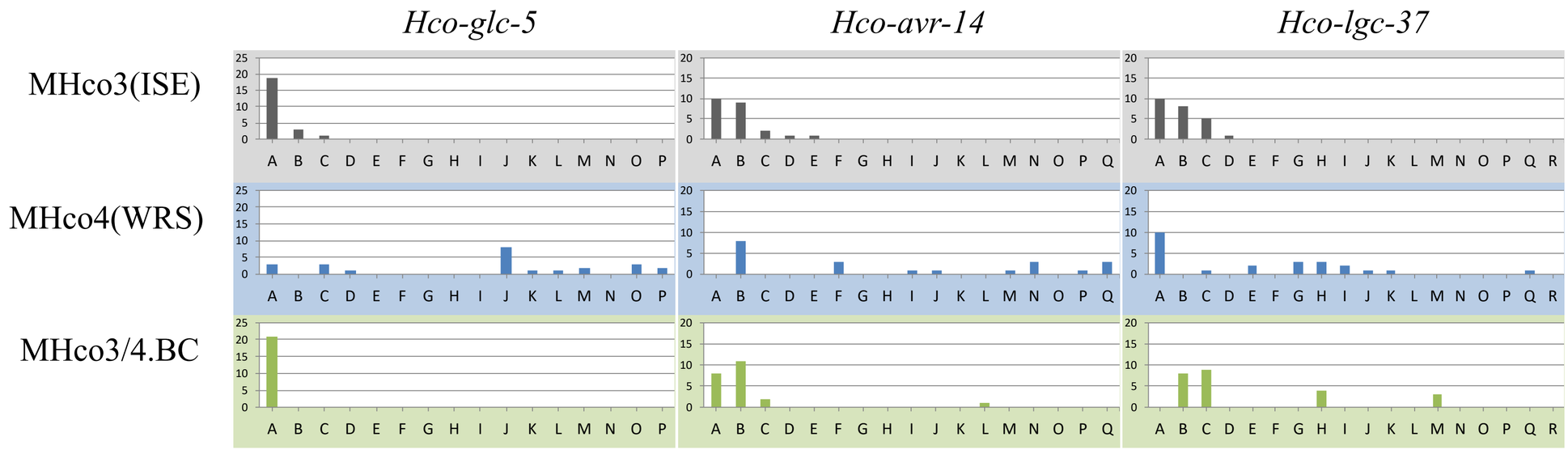


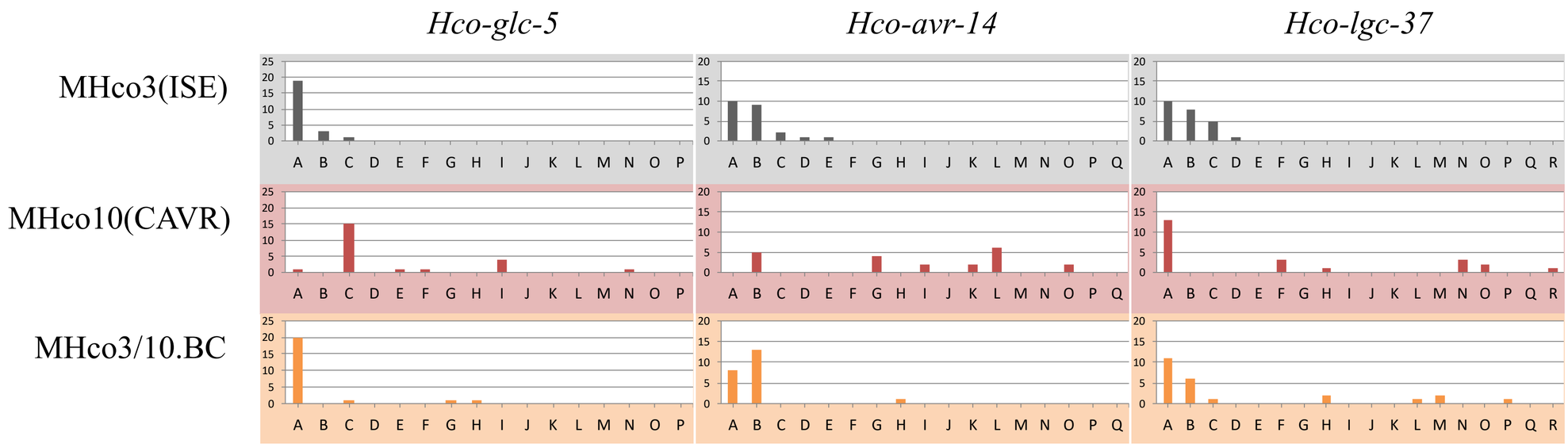
MHco10(CAVR)



MHco3/10.BC

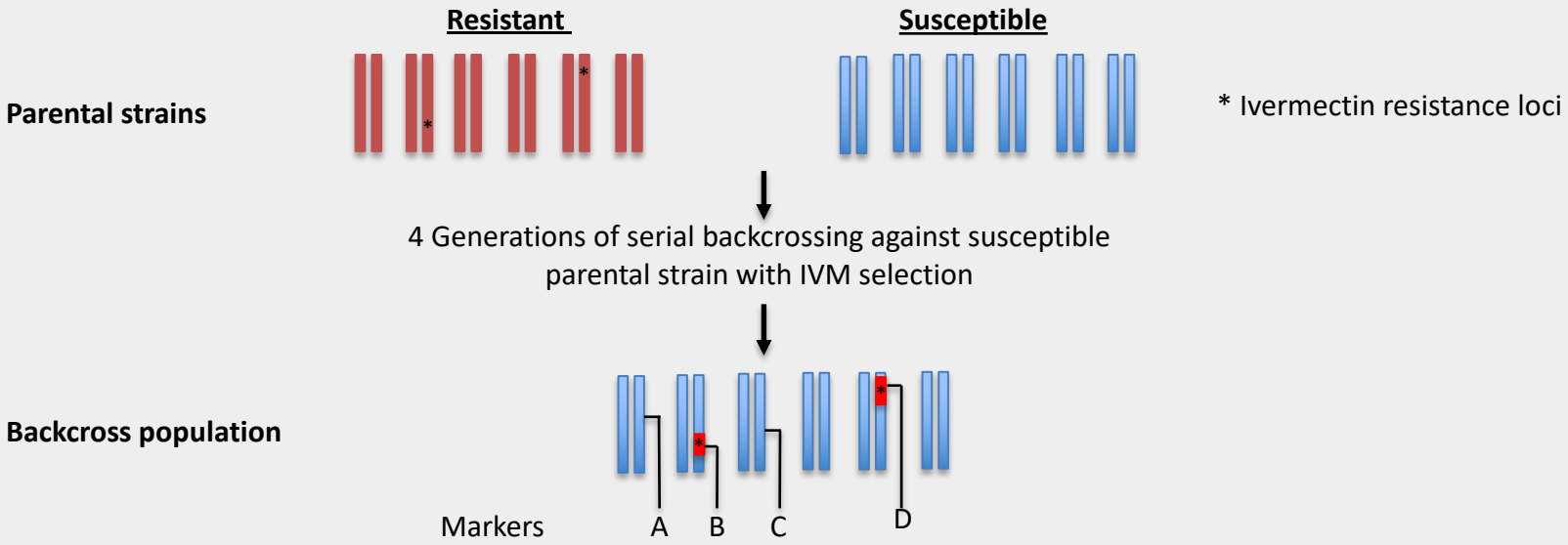



Hco-glc-5
Hco-avr-14
Hco-lgc-37
MHco3(ISE)
MHco4(WRS)
MHco3/4.BC
Hco-pgp-9
Hco-pgp-2
Hco-dyf-7
MHco3(ISE)
MHco4(WRS)
MHco3/4.BC


Hco-glc-5
Hco-avr-14
Hco-lgc-37
MHco3(ISE)
MHco10(CAVR)
MHco3/10.BC
Hco-pgp-9
Hco-pgp-2
Hco-dyf-7
MHco3(ISE)
MHco10(CAVR)
MHco3/10.BC

A

Schematic representation of the genomes of parental and backcrossed populations



B

Predictions for haplotype profiles of genetic markers in backcrossed populations

Hypothetical markers	Overall haplotype frequencies	Presence of haplotypes unique to resistant parental strain
Markers A and C within “non-introgressed” region	Not significantly different from susceptible parental strain	Absent (or rare)
Markers B and D within “introgressed” region	Significantly different from susceptible parental strain	One or more present (moderate or high frequency)

Table 1. Summary of the results of sequencing data for cloned PCR products for each candidate locus from each of the Haemonchus contortus parental and backcross strains**.**

	Strain	Hcms8a20	*Hco-glc-5*	*Hco-avr-14*	*Hco-lgc-37*	*Hco-pgp-9*	*Hco-pgp-2*	*Hco-dyf-7*	Mean
Number of clones sequenced	MHco3(ISE)	21	23	23	24	24	22	16	22.00
	MHco4(WRS)	22	24	21	24	24	13	13	19.83
	MHco3/4.BC	23	21	22	24	23	18	16	20.67
	MHco10(CAVR)	24	23	21	23	23	17	16	20.50
	MHco3/10.BC	23	23	22	24	23	22	14	21.33
Number of distinct haplotypes observed in each strain	MHco3(ISE)	3	3	5	4	5	3	1	3.50
	MHco4(WRS)	4	9	8	9	13	5	6	8.33
	MHco3/4.BC	3	1	4	4	3	5	2	3.17
	MHco10(CAVR)	5	6	6	6	9	5	3	5.83
	MHco3/10.BC	4	4	3	7	7	2	3	4.33
	Total no. of distinct haplotypes	9	16	17	18	25	10	10	16.00
Shared vs. unique haplotypes for MHco3(ISE) and MHco4(WRS) parental strains	Shared	1	2	1	2	2	2	1	1.67
	Unique to MHco3(ISE)	2	1	4	2	3	1	0	1.83
	Unique to MHco4(WRS)	3	7	7	7	11	3	5	6.67
Shared vs. unique haplotypes for MHco3(ISE) and MHco10(CAVR) parental strains	Shared	0	2	1	1	3	1	1	1.50
	Unique to MHco3(ISE)	3	1	4	3	2	2	0	2.00
	Unique to MHco10(CAVR)	5	4	5	5	6	4	2	4.33

Table 2. Pairwise Fst values between the susceptible *Haemonchus contortus* MHco3(ISE) parental strain and MHco4(WRS) and MHco10(CAVR) resistant parental strains. Associated *P* values are shown in parentheses.

Pairwise Comparison	Hcms8a20	*Hco-glc-5*	*Hco-avr-14*	*Hco-lgc-37*	*Hco-pgp-9*	*Hco-pgp-2*	*Hco-dyf-7*
MHco3(ISE)-MHco4(WRS)	0.382 (0.000) [a]	0.340 (0.000) [a]	0.120 (0.000) [a]	0.078 (0.009) [a]	0.163 (0.000) [a]	0.281 (0.000) [a]	0.484 (0.000) [a]
MHco3(ISE)-MHco10(CAVR)	0.358 (0.000) [a]	0.533 (0.000) [a]	0.166 (0.000) [a]	0.108 (0.000) [a]	0.140 (0.000) [a]	0.253 (0.000) [a]	0.367 (0.002) [a]

[a] $P < 0.05$ was considered statistically significant.

Table 3. Pairwise Fst values between the susceptible *Haemonchus contortus* MHco3(ISE) parental strain and MHco3/4.BC and MHco3/10.BC backcross strains. Associated *P* values are shown in parentheses.

Pairwise Comparison	Hcms8a20	*Hco-glc-5*	*Hco-avr-14*	*Hco-lgc-37*	*Hco-pgp-9*	*Hco-pgp-2*	*Hco-dyf-7*
MHco3(ISE)-MHco3/4.BC	0.383 (0.000) [a]	0.095 (0.063)	0.000 (0.676)	0.115 (0.000) [a]	0.055 (0.135)	0.220 (0.004) [a]	0.000 (0.999)
MHco3(ISE)-MHco3/10.BC	0.512 (0.000) [a]	0.000 (0.514)	0.003 (0.225)	0.000 (0.450)	0.025 (0.153)	0.014 (0.371)	0.117 (0.108)

[a] $P < 0.05$ was considered statistically significant.

Supplementary Table S1. Forward and reverse primers and the PCR product length for each amplicon marker are shown. The length of each genome scaffold on which each amplicon marker is located in the most recent version of the *Haemonchus contortus* MHco3(ISE) genome assembly (as of June 2014; made available by James Cotton and Matt Berriman of the Wellcome Trust Sanger Institute (WTSI), UK is indicated.

Locus	Amplicon length (bp)	Forward primer (5'-3') - Exon	Reverse primer (5'-3') - Exon	Size of scaffold (kbp)
Hcms8a20	725	GAAGCGGCTCAGATGATAAAGG -not exonic	TTACCGAGGGACTCCGAATG - not exonic	27
Hco-glc-5	420	CCGGCTCGGGTTACGCT - exon 5	CTTGTCGTGAGTCTGGATTC - exon 3	14,479
Hco-avr-14	490	TCGTAGTGTTGGCGACCAG - exon 10	CAACGGGTAGAACTCCAATG - exon 8	6,538
Hco-lgc-37	270	GGTGATGTCATGGGTGTC - exon 7	TTGCTGCGAATACGAATC - exon 9	94
Hco-pgp-1	790	AAGTACCATAATCCAGTTGTTAC - exon 11	CAGCAAGAGAATTTTCGGATC - exon 14	6,023
Hco-pgp-2	450	ACCTACGCTGTTGCCGGTGC - exon 7	GTGCCGAGAGCTGCTGAGCC - exon 10	12,643
Hco-dyf-7	690	TCTTTCCAGTGGACGAGGTGTCA - exon 8	AGAGGTCGTCCATCAGTGCTTCT - exon 5	11,346

Supplementary Table S2. Chromosomal assignment (in bold) of *Caenorhabditis elegans* orthologues of the gene models adjacent to each candidate *Haemonchus contortus* (*Hco*) gene on their respective genome assembly scaffolds.

Chromosome	*Hco-glc-5*	*Hco-avr-14*	*Hco-lgc-37*	*Hco-pgp-9*	*Hco-pgp-2*	*Hco-dyf-7*
I	**5**	**12**	0	1	**6**	0
II	0	0	0	0	0	0
III	0	0	**3**	3	0	0
IV	0	0	0	**4**	0	0
V	0	0	0	2	0	0
X	0	0	0	0	0	**10**
No homology	10	3	3	5	9	5
Total	15	15	6	15	15	15