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A method for single pair mating in an obligate parasitic nematode

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ABSTRACT

Parasitic nematode species have extremely high levels of genetic diversity, presenting a number of experimental challenges for genomic and genetic work. Consequently, there is a need to develop inbred laboratory strains with reduced levels of polymorphism. The most efficient approach to inbred line development is single pair mating, but this is challenging for obligate parasites where the adult sexual reproductive stages are inside the host, and thus difficult to experimentally manipulate. This paper describes a successful approach to single pair mating of a parasitic nematode, *Haemonchus contortus*. The method allows for polyandrous mating behaviour and involves the surgical transplantation of a single adult male worm with multiple immature adult females directly into the sheep abomasum. We used a panel of microsatellite markers to monitor and validate the single pair mating crosses and to ensure that the genotypes of progeny and subsequent filial generations were consistent with those expected from a mating between a single female parent of known genotype and a single male parent of unknown genotype. We have established two inbred lines that both show a significant overall reduction in genetic diversity based on microsatellite genotyping and genome-wide single nucleotide polymorphism. There was an approximately 50% reduction in heterozygous SNP sites across the genome in the MHco3.N1 line compared with the MoHco3(ISE) parental strain. The MHco3.N1 inbred line has subsequently been used to provide DNA template for whole genome sequencing of *H. contortus*. This work provides proof of concept and methodologies for forward genetic analysis of obligate parasitic nematodes.

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1. Introduction

Parasitic nematodes are amongst the most important pathogen groups causing diseases that threaten the quality of life in humans worldwide (Prichard et al., 2012). Approximately 2 billion people, mostly living in impoverished regions where sanitation is poor, are affected by soil-transmitted helminthiases. These diseases result in an array of clinical effects, ranging from gastrointestinal disorders to anaemia, reduced physical fitness, decreased cognitive function and poor growth (De Silva et al., 2003). The control of human soil-transmitted helminthiases is underpinned globally by the use of anthelmintics in mass drug administration (MDA) programmes (Supali et al., 2013; McCarty et al., 2014; Harris et al., 2015). Nematode parasites are also important causes of production limiting diseases in ruminant livestock (Nieuwhof and Bishop, 2005), being particularly relevant in already impoverished subtropical regions (Besier et al., 2016). The use of anthelmintic drugs had led to the selection of drug resistance, which is now widespread in many parasites of grazing livestock. Concerns are now emerging regarding similar problems for the MDA programs being used to control human helminths.

There is heritable variation in traits such as virulence, host specificity, environmental adaption and drug resistance, which are important constraints to sustainable helminth control in both humans and animals (Criscoine et al., 2009). There is a huge potential to use genetic crossing and mapping approaches in parasitic helminths to identify both single and quantitative trait loci underlying phenotypes of interest (Chevalier et al., 2014). For example, genetic crossing experiments are more powerful in identifying loci associated with anthelmintic resistance than laboratory selection, or the study of specific candidate genes chosen largely on the basis

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of experimental work implicating them as encoding drug targets, or molecules involved in drug efflux (Rezansoff et al., 2016). However, although genomic resources are rapidly advancing with genome projects being undertaken for an increasing number of species, we still lack the basic tools and techniques to undertake genetic crossing and mapping in parasitic nematodes. Undertaking genetic crosses requires genetically and phenotypically divergent, and preferably near-isogenic, parental lines. Developing these for nematode parasites is challenging since they cannot be maintained in vitro throughout their life cycle, hence experimental models depend upon the infection of the parasites' mammalian hosts. High levels of host specificity make these models intractable for human parasites and necessitate the development of animal models. A ruminant nematode parasite, *Haemonchus contortus*, is currently the most important model system for the study of anthelmintic drug resistance as well being a key tool for anthelmintic drug and vaccine discovery research (Gilleard, 2013). A draft genome sequence has recently been published (Laing et al., 2013) and assembly and annotation improvements are ongoing (Laing et al., 2016). In common with a number of other parasitic nematode species, the high level of genetic polymorphism has made high quality assemblies difficult to produce (Gilleard and Redman, 2016). Hence, the development of inbred parasitic nematode lines with reduced levels of sequence polymorphism is an important goal, both for genome assembly and for undertaking genetic crossing and mapping approaches.

Parasitic nematodes are dioecious, sexually reproducing, diploid organisms, hence the conceptually simplest method of generating inbred lines is by single pair matings. However, there are no published reports of genetically validated single pair matings for any obligate parasitic nematode species to date. This paper describes successful single pair mating for *H. contortus* following direct transplantation of a single sexually immature adult male with multiple sexually immature adult females of the MHco3(ISE) strain into the abomasum of a recipient parasite-free sheep. The method exploits the ease of establishment of parasite populations in a host, while accounting for polyandry (Redman et al., 2008a). Genetic and genomic characterisation of the parental and derived inbred lines demonstrate that the procedure significantly reduced genetic polymorphism of the MHco3(ISE) reference genome strain. This provides proof of concept of single mating in obligate parasitic nematodes, opening up new avenues of genetic approaches to study the biology of these important organisms.

2. Materials and methods

2.1. Parasite material

Cryopreserved L_3 of the ISE *H. contortus* strain (Otsen et al., 2000, 2001) were obtained from Dr. Fred Borgsteede (Central Veterinary Institute, Lelystad, Netherlands) by J.S. Gilleard. The strain was subsequently maintained at the Moredun Research Institute, UK by serial passage through donor sheep and renamed MHco3(ISE) to distinguish it from versions of ISE strain used in other laboratories (Redman et al., 2008b). This strain was adopted as the original reference strain for the *H. contortus* genome project being undertaken at the Wellcome Trust Sanger Institute, UK (Laing et al., 2013).

2.2. Genetic crossing by surgical transplantation

The overall experimental scheme is shown schematically in Fig. 1. To produce sexually immature adults for surgical transfer, a 4 month-old 'worm-free' donor lamb was orally dosed on day 0 with approximately 10,000 MHco3(ISE) *H. contortus* L_3. The donor lamb was euthanased on day 14 p.i. and the contents of its abomasum were collected. A single sexually immature male and 32 sexually immature female *H. contortus* L_4 were then surgically transferred, within 2 h of recovery from the donor lamb, into the abomasum of a 4 month-old recipient lamb (lamb A, Fig. 1). In addition, a single sexually immature male and 20 sexually immature females were surgically transferred to two other lambs (lambs B and C, Fig. 1). The faecal trichostrongyle egg counts (FEC) of the three recipient lambs were monitored daily from days 14 to 21 (1–7 days post transplantation) using a standard salt floatation method with a minimum detection threshold of 1 egg per gram of faeces (epg) (Christie and Jackson, 1982). All three lambs had positive egg counts by day 18 that increased to 20 epg on day 21.

2.3. Collection of progeny from single female adult nematodes following mating with a single male

The three recipient lambs were euthanased 7 days after surgical transfer (day 21). The single, transplanted male worms could not be recovered from any of the three recipient lambs at autopsy but 12, 6 and 12 of the transplanted female worms were recovered from the abomasa of recipient lambs A, B and C, respectively. All recovered female worms were immediately picked into sterile PBS and transferred individually into separate wells of 24 well plates each containing 1 ml of warm RPMI 1640 cell culture medium (Gibco, UK) and incubated in 5% CO_2 at 37 °C for 8 h to promote egg shedding. They were then transferred to a 24 °C incubator for 36 h to permit hatching of any fertilised eggs. Although all of the recovered female worms shed several hundred eggs, the development and hatching rate was extremely low. Consequently, only four female MHco3 *H. contortus* produced L_1 broods of sufficient size (minimum $n = 100$) to allow their molecular and phenotypic characterisation and subsequent propagation of another generation. These four females were arbitrarily named N1 (recovered from recipient lamb A), N2, N3 and N4 (recovered from recipient lamb B), respectively. DNA lysates were prepared from approximately half of the L_1 in each brood (F_1 progeny of the single parent mating) and from the head of each adult female parent. The remaining L_1 were retained for coproculture development to the infective L_3 stage, to allow infection of more animals to produce the next filial generation.

2.4. Larval coproculture

L_1 were either transferred onto a disc of cotton filter paper placed in a petri dish containing 5 ml of an OP50 *Escherichia coli* culture in Luria broth/streptomycin, or inoculated into 10 g of faeces collected from a known 'worm-free' donor lamb that had been sequentially treated with 5 mg/kg of fenbendazole and 7.5 mg/kg of levamisole 5 days previously. The filter paper/*E. coli* and larval coprocultures were then placed individually in perforated plastic bags and incubated in a closed laboratory incubator at 24 °C for 7 days. L_3 were then recovered from the larval cultures using a Baermann method (MAFF, 1986), transferred in tap water into tissue culture flasks and stored at 8 °C for 3 weeks before they were used to infect donor lambs. Fifteen, 20, three and zero L_3 were recovered from the coprocultures of the broods of the adult female MHco3 individuals, N1, N2, N3 and N4.

2.5. Propagation of inbred lines resulting from single pair matings

In order to propagate filial lines, two 6–7 month old 'worm-free' lambs were orally infected with 15 MHco3.N1.F_1 L_3 (lamb D, Fig. 1) and 20 MHco3.N2.F_1 L_3 (lamb E, Fig. 1). The FECs of these two lambs were monitored from 14 days p.i. Mean daily FECs were 1.2 (S.D. 1.0) epg (lamb D), and 22 (S.D. 16) epg (lamb E) between

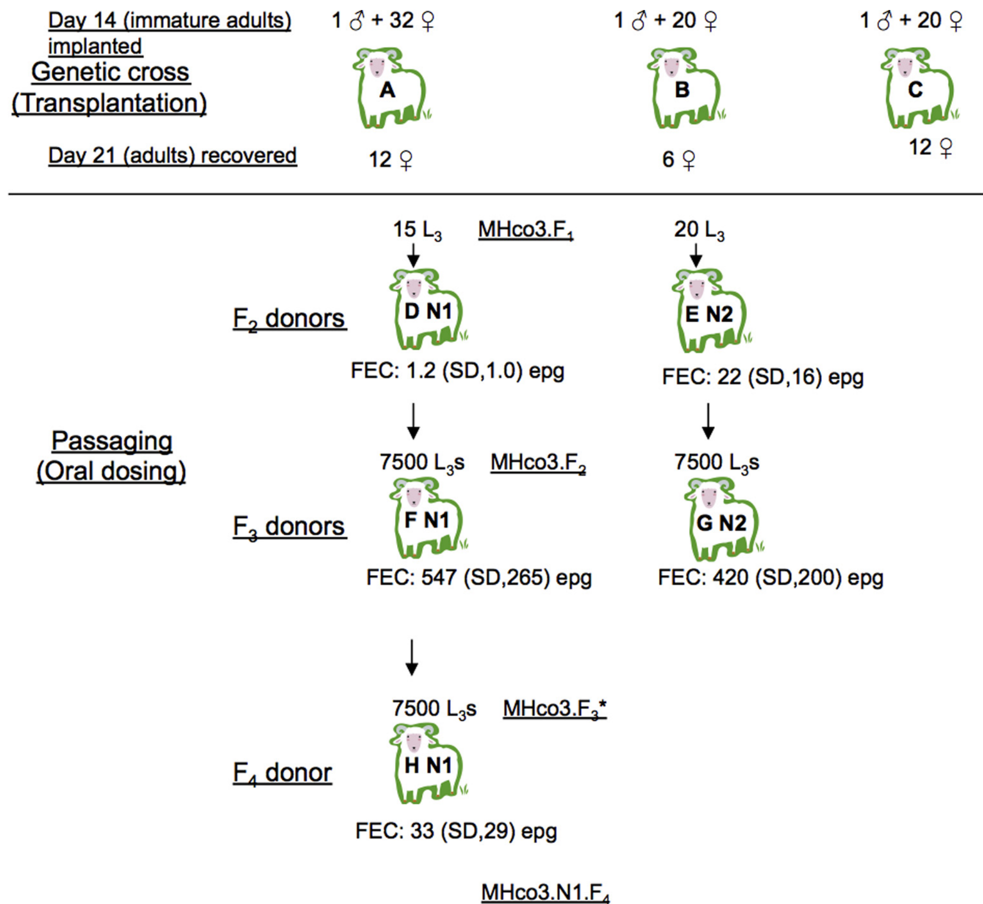

Fig. 1. Genetic crossing and passaging approach to inbreed the MHco3(ISE) standard genome strain of *Haemonchus contortus*. Schematic representation of experimental aim and summary of nomenclature. Genomic DNA extracted from MHco3.N1.F3 adults and used for genome sequencing (Laing et al., 2013) is indicated by an asterisk. FEC, faecal egg count; epg, eggs per gram of faeces.

21 and 60 days p.i. (Fig. 1). In order to propagate the next filial generation, this process was repeated using L_3 derived from eggs (F_2 progeny) recovered from lambs D and E (Fig. 1). Two different 8–9 month-old 'worm-free' lambs were infected separately with 7,500 MHco3.N1.F_2 (lamb F, Fig. 1) and MHco3.N2.F_2 L_3 (lamb G, Fig. 1). The mean daily FECs between 20 and 60 days p.i. of lambs F and G were 547 (S.D. 265) epg and 420 (S.D. 200) epg, respectively. An additional round of passage was undertaken for the MHco3.N1 line only. Samples of 7,500 and 5,000 MHco3.N1.F_3 L_3 derived from eggs recovered from lamb F were used to infect a 10 month-old lamb to produce a MHco3.N1.F_4 (lamb H, Fig. 1) line. The mean daily FECs of this lamb between 20 and 60 days p.i. was 33 (S.D. 29) (Fig. 1).

Freedom of contamination of the *H. contortus* populations by other nematode species was periodically tested during the development of the inbred lines by fluorescent agglutinin staining (Palmer and McCombe, 1996) and examination of larval morphology (Van Wyk and Mayhew, 2013). The lambs were maintained for between 2 and 5 months before they were euthanased and any surviving *H. contortus* were recovered from their abomasa, counted and stored in 70% ethanol.

2.6. DNA lysate preparation

Individual worm DNA lysates were prepared from female heads, L_1 (F_1) and sodium hypochlorite-exsheathed L_3 (F_2, F_3 and F_4) in a volume of 25 µl using standard techniques (Redman et al., 2008b). Bulk worm preparations of 500 ex-sheathed L_3 were made for F_2, F_3 and F_4 generations. Female head lysates (1 µl of a 1:30 dilution) or a 1:10 dilution of L_1 lysates was used as PCR template. Dilutions of lysate buffer without template, made in parallel, were included as negative controls for all PCR amplifications. All DNA lysates were subjected to a previously published ITS-2 rDNA PCR assay, to confirm species identity as *H. contortus* (Wimmer et al., 2004; Redman et al., 2008b).

2.7. Single strand conformation polymorphism

The genetic diversity of the GABA Cl subunit HG1 locus (Blackhall et al., 2003), as well as that of GluCl α and β subunit loci (Blackhall et al., 1998) (Supplementary Fig. S1A) was examined by single strand conformation polymorphism (SSCP) using previously described PCR primers (Blackhall et al., 2003). The thermal cycler conditions used were: 95 °C for 4 min; followed by 40 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s; and a final extension stage of 72 °C for 5 min. Amplicons were first visualised on a 1.2% agarose gels and then run on non-denaturing polyacrylamide gels as previously described (Skuce et al., 2010).

To confirm the allelic assignments of the GABA Cl subunit HG1 SSCP genotyping, the PCR products produced from two individual MHco3.N1.F_2 L_3 DNA heterozygotes (6F and 9G) were cloned and sequenced. Briefly, amplicons were run on a 1% agarose electrophoretic gel to enable the excision of 305 bp bands from which DNA was then isolated (QIAquik Gel Extraction kit, Qiagen, UK). DNA was ligated into pGEM®-T (Promega, UK) plasmid vectors to allow transformation into JM109 competent *E. coli* cells (Strata-

gene, UK). Plasmid DNA from the cultured transformed cells was then purified using a Wizard® Plus SV Minipreps DNA Purification System (Promega, UK) and sequenced using SP6 and T7 universal primers in both orientations.

2.8. Microsatellite genotyping

Microsatellite genotyping of 'bulk' DNA lysates, made from approximately 500 larvae, was performed on MHco3(ISE), MHco3.N1.F_2, MHco3.N1.F_3, MHco3.N1.F_4, MHco3.N2.F_2 and MHco3.N2.F_3 *H. contortus* lines. Ten microsatellite markers, previously shown to be polymorphic in the MHco3(ISE) strain, were used: Hcms25, Hcms33 and Hcms36 (Otsen et al., 2001), Hcms8a20, Hcms22co3 (Redman et al., 2008b), HcmsX142, HcmsX256, HcmsX337 (Redman et al., 2008a), Hcms3561 and Hcms18210 (Redman et al., 2012). Individual worm genotyping was also performed for four of these loci (Hcms8a20, Hcms36, Hcms3561 and Hcms25) plus two additional loci (HcmsX182 and HcmsX240) (Redman et al., 2008a) on the N1 and N2 adult female parent heads and 30 individual larvae for each of the following populations: MHco3.N1.F_1 and MHco3.N2.F_1 (L_1); MHco3.N1.F_2, MHco3.N2.F_2, MHco3.N1.F_3, MHco3.N2.F_3 (L_3) and MHco3(ISE). All microsatellite genotyping, on both 'bulk' and single worm DNA lysates, was performed using the same PCR amplification methods and parameters as previously described (Redman et al., 2008b). Capillary electrophoresis was performed using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) for the accurate sizing of microsatellite PCR products. The forward primer of each microsatellite primer pair was 5′-end labelled with FAM, HEX, or NED fluorescent dyes (MWG-Biotech AG, UK) and electrophoresed with a GeneScan ROX 400 (Applied Biosystems) internal size standard. Individual chromatograms were analysed using Genemapper Software Version 4.0 (Applied Biosystems).

2.9. Genetic analysis

Multilocus genotype principal coordinates analysis was conducted using GenAlEx version 6.1 add-in software (Peakall and Smouse, 2006) for Microsoft Excel to provide a schematic indication of the degree of inbreeding. The average number of alleles per locus, observed heterozygosities (H_o), and unbiased estimates of expected heterozygosity (H_e) were calculated using Arlequin version 3.11 software (Nei, 1978; Excoffier et al., 2005). Data were defined as 'standard' rather than 'microsatellite' because the loci did not adhere to the stepwise mutation model. Exact tests for Hardy–Weinberg equilibrium were tested per locus using Fisher's exact probability test based on contingency tables (Raymond and Rousset, 1995), where P values <0.05 were taken as evidence of significant deviation. Significance levels were estimated using 100,000 Markov chain steps. Pairwise linkage disequilibrium was tested for using a likelihood-ratio test (Slatkin and Excoffier, 1996). For each locus, estimates of inbreeding (F_{is}) were calculated using an algorithm based on the formula $(H_e - H_o)/H_e$. Pairwise Fst values were calculated using Arlequin version 3.11 software. Analysis of Molecular Variance (AMOVA) was performed to test for population differentiation of samples at various levels, locus by locus, using the Arlequin version 3.11 software.

2.10. Genome-wide single nucleotide polymorphism (SNP) analysis

Genomic libraries were prepared from 400 MHco3(ISE) and 400 MHco3.N1.F_3 adult worms for Illumina sequencing using previously described methods (Laing et al., 2013; Kozarewa et al., 2009) (Supplementary Table S1). Preliminary analysis and base calling for data from the Illumina HiSeq sequencing machines used the RTA1.8 analysis pipelines. Whole genome shotgun sequence data was generated from these libraries on two different Illumina platforms, producing different numbers of reads and reads of different lengths (Supplementary Table S1). To produce comparable data between the two biological samples, read pairs were randomly sampled from the larger (inbred material) sequencing data by keeping each pair of reads in the subsampled file with a probability of 0.39, and by clipping 12 bp from each end of every read. Reads were mapped against the released 370 Mb v1.0 genome assembly of *H. contortus* (Laing et al., 2013), available at the GenBank database under project ID PRJEB506, using the mapper SMALT v0.7.0.1 (http://www.sanger.ac.uk/resources/software/smalt) in paired-end mode, with an indexing k-mer size of 13 and step size of 1, mapping non-repetitively (−r −1), with a minimum identity of 0.8 to report a mapping (−y 0.8) and exhaustively searching for alignments of each read independently of its mate pair (−x), and only reporting reads as properly paired if they were mapped less than 1,000 base pairs apart on the reference genome. SNP variants were called jointly from the three mapping output files using samtools v0.1.19-44428cd (Li et al., 2009) using the *mpileup* command, skipping alignments with either mapping or base quality scores less than 13. Density, distribution and types of variant calls were tallied using vcftools v0.1.11 (https://sourceforge.net/projects/vcftools/). Estimates of the nucleotide diversity (π) for the pools of worms sequenced in each library were calculated independently from the variant calling approach outlined above using PoPoolation2 v1.013 (Kofler et al., 2011).

2.11. Ethics statement

All experimental procedures described in this manuscript were examined and approved by the Moredun Research Institute (UK) Experiments and Ethics Committee and were conducted under approved UK Home Office licenses in accordance with the Animals (Scientific Procedures) Act of 1986. The Home Office license numbers are PPL 60/03223 and PPL 60/03899 and experimental IDs for these studies were E06/58. E06/75 and E09/36.

3. Results

3.1. The establishment of two independent inbred lines by single pair mating of H. contortus

Our preliminary experiments to replicate anecdotal reports suggesting that it might be possible that a single male and a single female *H. contortus*, when transferred directly into the abomasum of a 'worm-free' recipient sheep could survive for long enough to find each other, mate and shed eggs were unsuccessful, highlighting the severe biological limitations to this approach. Consequently, we developed a method in which a single immature male worm was transplanted with a number of immature female worms, then following mating, the female worms were recovered on autopsy, placed in individual wells of a 24 well plate and allowed to lay eggs (F_1 progeny) in vitro. Two of the recovered female worms, designated N1 and N2, produced broods of sufficient size and viability to enable the propagation of the next filial generations. Fifteen and 20 L_3 from the N1 and N2 founding female parents, respectively, were used to orally infect two separate lambs to establish the MHco3.N1 and MHco3.N2 inbred lines (Fig. 1).

3.2. Validation of single pair mating and assessment of polymorphism of inbred lines by microsatellite genotyping

Bulk DNA genotyping with 10 microsatellite markers, on DNA prepared from pools of approximately 500 L_3 per population, was used to provide an initial assessment of the genetic diversity of

the MHco3.N1 and MHco3.N2 inbred lines and indicate the success of the single pair matings. The total number of alleles detected was reduced in both of the inbred lines relative to the founding MHco3 (ISE) population with a greater loss of overall diversity in MHco3. N1. Across the 10 markers, a total of 28 alleles in the MHco3(ISE) population was reduced to 15 and 20 alleles in the derived MHco3.N1 and MHco3.N2 lines, respectively. There was a loss of alleles at seven out of the 10 loci in both cases (Supplementary Tables S2, S3).

The MHco3(ISE) strain, the individual N1 and N2 founder female parents and populations of the inbred MHco3.N1 and MHco3.N2 lines were analysed in more detail by genotyping individual worms at six of the most discriminatory loci. The level of polymorphism of the parental MHco3(ISE) strain was consistent with that previously observed with other panels of microsatellite loci (Redman et al., 2008b) with a mean of 3.17 alleles per locus and an expected heterozygosity (H_E) of 0.572. There was a clear reduction in polymorphism in F_1 and F_2 populations of both inbred lines with MHco3.N1 again showing the greatest reduction (Supplementary Table S4).

Pairwise F_{ST} estimates based on the multilocus genotype data revealed a high degree of genetic differentiation between the two inbred strains as well as between both lines and the parental MHco3(ISE) strain. No statistically significant genetic differentiation was observed between any of the filial populations within the same inbred line, demonstrating that the genetic integrity of both the inbred lines was maintained despite passage (Supplementary Fig. S2A). Further evidence for reduction in genetic diversity by the single parent mating procedure was provided by principal component analysis of individual worm multilocus genotypes (Supplementary Fig. S2B).

3.3. Examination of the HG1 GABA Cl locus using SSCP

The HG1 gene which encodes a GABA-gated chloride channel (Blackhall et al., 2003) was selected and used as an additional marker to monitor the single pair mating and inbreeding process, since this had been shown to have a high level of genetic diversity in the MHco3(ISE) strain (Supplementary Fig. S1). SSCP profiles were obtained for 84 MHco3(ISE) L_3 and at least 15 distinct profiles were discernable (Supplementary Fig. S3A). However, only three different SSCP profiles were discernable from 57 F_1 and 65 F_2 progeny of the MHco3.N1 inbred line (Supplementary Fig. S3B).

3.4. Comparison of MHco3(ISE) and MHco3.N1 genome-wide SNP

Whole genome Illumina sequencing was performed on MHco3 (ISE) and MHco3.N1 populations (Supplementary Table S1). The number of sites classified as heterozygous within the MHco3.N1 population in the variant calls from *mpileup* was almost 50% fewer than those called for the MHco3(ISE) populations. Using the number of reads supporting each allele at a site as a rough estimate of the allele frequency in the pool of adult worms sequenced, there is a clear pattern of a greater proportion of sites having minor alleles segregating at intermediate frequencies (between 0.15 and 0.35) within the MHco3.N1 population (Supplementary Fig. S4A). The MHco3.N1 population is particularly reduced in rare alleles, as expected from a recent, extreme population bottleneck. This pattern is consistent with these nematodes being the offspring a single pair mating, where we would expect minor alleles to be present on just one of the four parental haplotypes. The same pattern is clear in the subset of sites that are polymorphic in both populations, where π is lower in MHco3.N1 than in MHco3(ISE) at almost two-thirds of sites (332/537) on the longest assembly scaffold (Supplementary Fig. S4B).

4. Discussion

The original ISE strain of *H. contortus* had been previously inbred from the outbred SE population (Otsen et al., 2000, 2001). This was achieved by dissecting the eggs from an adult female SE strain of *H. contortus*, culturing these eggs for 7 days in 'worm-free' faeces, and then injecting recovered L_3 into the forestomachs of recipient sheep. The recipient sheep were euthanased 1 week after they had started shedding trichostrongyle eggs. A single benzimidazole susceptible adult female *H. contortus* had then been selected on the basis of its β-tubulin isotype 1 genotype (Kwa et al., 1994), and the process repeated through 15 generations (Roos et al., 2004), to yield what was considered to be an inbred benzimidazole-susceptible isolate. However, genetic analysis with microsatellite markers subsequently revealed high levels of genetic polymorphism in the MHco3(ISE) strain of *H. contortus* (Redman et al., 2008b).

This paper presents the development of a novel method to achieve a single pair parasitic nematode mating involving the surgical transfer of multiple female and one male day 14 parasitic stage *H. contortus* to the abomasa of recipient lambs. The experimental protocol took into account the known polyandrous mating behaviour of *H. contortus* (Redman et al., 2008a) by using a single male transplanted with multiple females. The method exploited the ability to differentiate between male and female nematodes before they reach sexual maturity, which is a prerequisite for genetic crosses (Chevalier et al., 2016). The transplanted male worm successfully fertilised multiple female worms in each case and this experimental design ensured each female brood was from a single pair mating event. The experimental design also prevented any risk of extraneous parasitic nematode infection of recipient sheep by euthanasing them and recovering egg laying female *H. contortus* well within the minimum prepatent period of contaminant parasitic nematodes. Potential issues caused by fly-borne parasitic nematode contamination of coprocultures were addressed by their incubation in an isolated closed environment, primarily on filter paper in a live *E. coli* system. The methods used to prevent parasitic nematode contamination were apparently effective for the development of the MHco3.N1 and MHoc3.N2 lines, since the genetic analyses presented in this paper are consistent with those expected from a single pair mating event. Failure to recover the male parent *H. contortus* from any of the three recipient lambs, while between 30% and 60% of the females were recovered, was disappointing. This could be due to chance, or might suggest that the behaviour of male parasitic nematodes in seeking out females predisposes to their loss from the abomasum. Determination of both parental genotypes founding the inbred lines would have aided further genetic validation based on the male parental genotype.

The microsatellite individual genotyping data was entirely consistent with that expected if the two MHco3.N1 and MHco3.N2 inbred lines were founded by single pair matings of the N1 and N2 female parents. Although the lack of knowledge of the male parental genotypes precluded definitive Mendelian genetic analysis of the crosses, the data overall provided strong support for the success of the single pair matings. The appropriate maternal alleles for each microsatellite marker were present in the filial generations of each cross and the total number of alleles present was entirely consistent with single pair mating. The multilocus genotype analysis of MHco3.N1.F_1 and MHco3.N2.F_1 worms was also strongly supportive of successful single pair mating, with the F_1 multilocus genotypes forming tight clusters around the respective maternal parental genotypes on principal component analysis (PCA) plots.

An overall loss of genetic polymorphism in both the MHco3.N1 and MHco3.N2 lines, compared with the parental MHco3 (ISE) strain, was revealed by the microsatellite markers, the GABA Cl SSCP profiles and the whole genome sequencing analysis. The

Hco3.N1 line showed the greatest loss of polymorphism of the two inbred lines based on the microsatellite genotyping with a loss of 13 out of 28 alleles (46%) across the 10 microsatellite markers genotyped. Consequently, the inbred MHco3.N1 population was propagated further and used as the reference strain for the *H. contortus* genome project (Laing et al., 2013). Subsequent, genome-wide SNP analysis was consistent with the microsatellite analysis, showing that MHco3.N1 line had an almost 40% reduction in SNP positions called as heterozygous across the genome, and reduced nucleotide diversity at shared heterozygous sites compared with MHco3(ISE).

The two inbred lines retained largely different alleles from the MHco3(ISE) populations, as demonstrated in the multilocus genotyping PCA plots and pairwise F_{ST} analyses. The production of genetically divergent inbred *H. contortus* lines using this method could be exploited in a number of ways. It could be used to develop genetically divergent strains with which to undertake genetic crosses for the production of a genetic map, or to identify the positions of genetic loci of interest such as those underlying anthelmintic resistance (Le Jambre et al., 1999). A prerequisite for the creation of a genetic map is that the parent populations have minimal within-strain polymorphism, but high levels of between-strain polymorphism, in order to allow the alternative parental alleles to be identified in F_2 progeny resulting from the genetic cross. With the exception of the Chiswick avermectin-resistant (CAVR) strain, which arose as a serendipitous, extraneous, ivermectin-resistant contaminant of an Australian laboratory passaged *Trichostrongylus colubriformis* strain (Le Jambre, 1993), the currently available laboratory strains of *H. contortus* are too polymorphic to use in conventional mapping studies. Hence generation of experimentally inbred, near-isogenic, genetically divergent strains is useful. Cases of multigenic resistance could be investigated by segregating different genetic loci contributing to an anthelmintic resistance phenotype into separate inbred lines.

In conclusion, the proof of concept of molecular and genetic validation of a single parent mating method to inbreed *H. contortus* will provide a potentially useful tool in the further development of genomic resources that are needed to inform sustainable nematode parasite control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpara.2017.08.010.

References

Besier, R.B., Kahn, L.P., Sargison, N.D., Van Wyk, J.A., 2016. The pathophysiology, ecology and epidemiology of *Haemonchus contortus* infection in small ruminants. In: Gasser, R.B., vonSamson-Himmelstjerna, G. (Eds.), *Haemonchus contortus* and Haemonchosis – Past, Present and Future Trends. Adv. Parasitol. 93, 95–143.

Blackhall, W.J., Pouliot, J.F., Prichard, R.K., Beech, R.N., 1998. *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. Exp. Parasitol. 90, 42–48.

Blackhall, W.J., Prichard, R.K., Beech, R.N., 2003. Selection at a [gamma]-aminobutyric acid receptor gene in *Haemonchus contortus* resistant to avermectins/milbemycins. Mol. Biochem. Parasitol. 131, 137–145.

Chevalier, F.D., Valentim, C.L.L., LoVerde, P.T., Anderson, T.J.C., 2014. Efficient linkage mapping using exome capture and extreme QTL in schistosome parasites. BMC Genomics 15, 617 http://www.biomedcentral.com/1471-2164/15/617.

Chevalier, F.D., Le Clec'h, W., Carolina Alvesde, A., Mattos, LoVerde, P.T., Anderson, T.J.C., 2016. Real-time PCR for sexing *Schistosoma mansoni* cercariae. Mol. Biochem. Parasitol. 205, 35–38.

Christie, M., Jackson, F., 1982. Specific identification of strongyle eggs in small samples of sheep faeces. Res. Vet. Sci. 32, 113–117.

Criscoine, C.D., Valentim, C.L.L., Hirai, H., LoVerde, P.T., Anderson, T.J.C., 2009. Genomic linkage map of the human blood fluke *Schistosoma mansoni*. Genome Biol. 10, R71. https://doi.org/10.1186/gb-2009-10-6-r71.

De Silva, N.R., Brooker, S., Hotez, P.J., Montresor, A., Engels, D., Savioli, L., 2003. Soil-transmitted helminth infections: updating the global picture. Trends Parasitol. 19, 547–551.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol. Bioinform. Online 1, 47–50.

Gilleard, J.S., 2013. *Haemonchus contortus* as a paradigm and model to study anthelmintic drug resistance. Parasitology 140, 1506–1522.

Gilleard, J.S., Redman, E., 2016. Genetic diversity and population structure of *Haemonchus contortus*. Adv. Parasitol. 93, 31–68. https://doi.org/10.1016/bs.apar.2016.02.009.

Harris, J.R., Worrell, C.M., Davis, S.M., Odero, K., Mogeni, O.D., Deming, M.S., Mohammed, A., Montgomery, J.M., Njenga, S.M., Fox, L.M., Addiss, D.G., 2015. Unprogrammed deworming in the Kibera Slum, Nairobi: implications for control of soil-transmitted helminthiases. PLoS Negl. Trop. Dis. 9, e0003590. https://doi.org/10.1371/journal.pntd.0003590.

Kofler, R., Pandey, R.V., Schlötterer, C., 2011. PoPoolation2: identifying differentiation between populations using sequencing of pooled DNA samples (Pool-Seq). Bioinformatics 27, 3435–3436.

Kozarewa, I., Ning, Z., Quail, M.A., Sanders, M.J., Berriman, M., Turner, D.J., 2009. Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. Nat. Methods 6, 291–295. https://doi.org/10.1038/nmeth.1311.

Kwa, M.S.G., Veenstra, J.G., Roos, M.H., 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in [beta]-tubulin isotype 1. Mol. Biochem. Parasitol. 63, 299–303.

Laing, R., Kikuchi, T., Martinelli, A., Tsai, I.J., Beech, R., Redman, E., Holroyd, R., Bartley, D.J., Beasley, H., Britton, C., Curran, D., Devaney, E., Gilabert, A., Hunt, M., Johnston, S., Kryukov, I., Li, K., Morrison, A., Reid, A., Sargison, N., Saunders, G., Wasmuth, J., Wolstenholme, A., Berriman, M., Gilleard, J.S., Cotton, J.A., 2013. The genome and transcriptome of *Haemonchus contortus*, a key model parasite for anthelmintic drug and vaccine discovery. Genome Biol. 14, R88. https://doi.org/10.1186/gb-2013-14-8-r88.

Laing, R., Martinelli, A., Tracey, A., Holroyd, N., Gilleard, J.S., Cotton, J.A., 2016. *Haemonchus contortus*: genome structure, organization and comparative genomics. Adv. Parasitol. 93, 569–598. https://doi.org/10.1016/bs.apar.2016.02.016.

Le Jambre, L.F., 1993. Ivermectin resistant *Haemonchus contortus* in Australia. Aust. Vet. J. 70, 357.

Le Jambre, L.F., Lenane, I.J., Wardrop, A.J., 1999. A hybridization technique to identify anthelmintic resistance genes in *Haemonchus*. Int. J. Parasitol. 29, 1979–1985.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R.1000 Genome Project Data Processing Subgroup, 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079.

MAFF (Ministry of Agriculture Fisheries and Food), 1986. Part 1 Helminthology. In: Manual of Veterinary Parasitological Laboratory Techniques, Reference Book 418. third ed. Her Majesty's Stationary Office, London, UK, pp. 3–67.

McCarty, T.R., Turkeltaub, J.A., Hotez, P.J., 2014. Global progress towards eliminating gastrointestinal helminth infections. Curr. Opin. Gastroenterol. 30, 18–24.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583–590.

Nieuwhof, G.J., Bishop, S.C., 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. Anim. Sci. 81, 23–29.

Otsen, M., Plas, M.E., Lenstra, J.A., Roos, M.H., Hoekstra, R., 2000. Microsatellite diversity of isolates of the parasitic nematode *Haemonchus contortus*. Mol. Biochem. Parasitol. 110, 69–77.

Otsen, M., Hoekstra, R., Plas, M.E., Buntjer, J.B., Lenstra, J.A., Roos, M.A., 2001. Amplified fragment length polymorphism analysis of genetic diversity of

Haemonchus contortus during selection for drug resistance. Int. J. Parasitol. 31, 1138–1143.

Palmer, D.G., McCombe, I.L., 1996. Lectin staining of trichostrongylid nematode eggs of sheep: rapid identification of *Haemonchus contortus* eggs with peanut agglutinin. Int. J. Parasitol. 26, 447–450.

Peakall, R., Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6, 288–295.

Prichard, R.K., Básañez, M.G., Boatin, B.A., McCarthy, J.S., García, H.H., Yang, G.J., Sripa, B., Lustigman, S., 2012. A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl. Trop. Dis. 6, e1549. https://doi.org/10.1371/journal.pntd.0001549.

Raymond, M., Rousset, F., 1995. An exact test for population differentiation. Evolution 49, 1280–1283.

Redman, E., Packard, E., Grillo, V., Smith, J., Jackson, F., Gilleard, J.S., 2008a. Microsatellite analysis reveals marked genetic differentiation between *Haemonchus contortus* laboratory isolates and provides a rapid system of genetic fingerprinting. Int. J. Parasitol. 38, 111–122.

Redman, E., Grillo, V., Saunders, G., Packard, E., Jackson, F., Berriman, M., Gilleard, J. S., 2008b. Genetics of mating and sex determination in the parasitic nematode *Haemonchus contortus*. Genetics 180, 1877–1887.

Redman, L., Sargison, N.D., Donnan, A.A., Bartley, D.J., Whitelaw, F., Jackson, F., Gilleard, S.J., 2012. Introgression of ivermectin resistance genes into a susceptible *Haemonchus contortus* strain by multiple backcrossing. PLoS Pathog. 8, e1002534. https://doi.org/10.1371/journal.ppat.1002534.

Rezansoff, A.M., Laing, R., Gilleard, J.S., 2016. 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*. Int. J. Parasitol. 46, 653–661.

Roos, M.H., Otsen, M., Hoekstra, R., Veenstra, J.G., Lenstra, J.A., 2004. Genetic analysis of inbreeding of two strains of the parasitic nematode *Haemonchus contortus*. Int. J. Parasitol. 34, 109–115.

Skuce, P., Stenhouse, L., Jackson, F., Hypsa, V., Gilleard, J., 2010. Benzimidazole resistance allele haplotype diversity in United Kingdom isolates of *Teladorsagia circumcincta* supports a hypothesis of multiple origins of resistance by recurrent mutation. Int. J. Parasitol. 40, 1247–1255.

Slatkin, M., Excoffier, L., 1996. Testing for linkage disequilibrium in genotypic data using the Expectation-Maximization algorithm. Heredity (Edinb.) 76 (Pt 4), 377–383.

Supali, T., Djuardi, Y., Bradley, M., Noordin, R., Rückert, P., Fischer, P.U., 2013. Impact of six rounds of mass drug administration on brugian filariasis and soil transmitted helminth infections in Eastern Indonesia. PLoS Negl. Trop. Dis. 7, e2586. https://doi.org/10.1371/journal.pntd.0002586.

Van Wyk, J.A., Mayhew, E., 2013. Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: a practical lab guide. Onderstepoort J. Vet. Res. 80, 1–14.

Wimmer, B., Craig, B.H., Pilkington, J.G., Pemberton, J.M., 2004. Non-invasive assessment of parasitic nematode species diversity in wild Soay sheep using molecular markers. Int. J. Parasitol. 34, 625–631.