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A method for single pair mating in an obligate parasitic nematode

Neil D. Sargison[a,1,2], Elizabeth Redman[b,1], Alison A. Morrison[c], David J. Bartley[c], Frank Jackson[c], Hardeep Naghra-van Gijzel[d,3], Nancy Holroyd[d], Matthew Berriman[d], James A. Cotton[d] and John S. Gilleard[b]

a University of Edinburgh, Royal (Dick) School of Veterinary Studies, Easter Bush Veterinary Centre, Roslin, Midlothian, EH25 9RG, United Kingdom

b Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, T2N 4N1 Canada

c Moredun Research Institute, Pentlands Science Park, Midlothian EH26 0PZ, United Kingdom;

d The Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SA, United Kingdom.

1 These authors contributed equally to the work.

2 To whom correspondence may be addressed. Email: neil.sargison@ed.ac.uk or jsgillea@ucalgary.ca

3 Current address: Computational Biology, Glaxo Smith Kline, Stevenage, United Kingdom.

Email addresses: neil.sargison@ed.ac.uk; libbyredman@hotmail.co.uk; Alison.Morrison@moredun.ac.uk; Dave.Bartley@moredun.ac.uk; frank.jackson1947@gmail.com; hardeep587@gmail.com; neh@sanger.ac.uk; mb4@sanger.ac.uk; jc17@sanger.ac.uk; jsgillea@ucalgary.ca

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26 ABSTRACT

27 Parasitic nematodes species have extremely high levels of genetic diversity, presenting a number of
28 experimental challenges for genomic and genetic work. Consequently, there is a need to develop
29 inbred laboratory strains with reduced levels of polymorphism. The most efficient approach to
30 inbred line development is single pair mating, but this is challenging for obligate parasites where the
31 adult sexual reproductive stages are inside the host, and so difficult to experimentally manipulate.
32 This paper describes a successful approach to single pair mating in a parasitic nematode,
33 *Haemonchus contortus*. The method allows for polyandrous mating behaviour and involves the
34 surgical transplantation of a single adult male worm with multiple immature adult females directly
35 into the sheep abomasum. We used a panel of microsatellite markers to monitor and validate the
36 single pair mating crosses and to ensure that the genotypes of progeny and subsequent filial
37 generations were consistent with those expected from a mating between a single female parent of
38 known genotype and a single male parent of unknown genotype. We have established two inbred
39 lines, that both show a significant overall reduction in genetic diversity based on microsatellite
40 genotyping and genome-wide single nucleotide polymorphism (SNP). There was an approximately
41 50% reduction in heterozygous SNP sites across the genome in MHco3.N1 line compared to the
42 MoHco3(ISE) parental strain. The MHco3.N1 inbred line has subsequently been used to provide DNA
43 template for whole genome sequencing of *H. contortus*. This work provides proof of concept and
44 methodologies for forward genetic analysis of obligate parasitic nematodes.

45

46

47 **1. Introduction**

48 Parasitic nematodes are amongst the most important pathogen groups causing quality of life
49 threatening disease in humans worldwide (Prichard et al., 2012). Approximately 2.0 billion people,
50 mostly living in impoverished regions where sanitation is poor, are affected by soil-transmitted
51 helminthiases. These diseases result in an array of clinical effects, ranging from gastrointestinal
52 disorders to anaemia, reduced physical fitness, decreased cognitive function and poor growth (De
53 Silva et al., 2003). The control of human soil-transmitted helminthiases is underpinned globally by
54 the use of anthelmintics in mass drug administration (MDA) programmes (McCarty et al., 2014;
55 Supali et al., 2013; Harris et al., 2015). Nematode parasites are also important causes of production
56 limiting diseases in ruminant livestock (Nieuwhof and Bishop, 2005), being particularly relevant in
57 already impoverished subtropical regions (Besier et al., 2016). The use of anthelmintic drugs had led
58 to the selection of drug resistance, which is now widespread in many parasites grazing livestock.
59 Concerns are now emerging regarding similar problems for the MDA programs being used to control
60 human helminths.

61 There is heritable variation in traits such as virulence, host-specificity, environmental
62 adaption and drug resistance, which are important constraints to sustainable helminth control in
63 both humans and animals (Criscoine et al., 2009). There is a huge potential to use genetic crossing
64 and mapping approaches in parasitic helminths to identify both single and quantitative trait loci
65 underlying phenotypes of interest (Chevalier et al., 2014). For example, genetic crossing experiments
66 are more powerful in identifying loci associated with anthelmintic resistance than laboratory
67 selection, or the study of specific candidate genes chosen largely on the basis experimental work
68 implicating them as encoding drug targets, or molecules involved in drug efflux (Rezansoff et al.,
69 2016). However, although genomic resources are rapidly advancing with genome projects being
70 undertaken for an increasing number of species, we still lack the basic tools and techniques to
71 undertake genetic crossing and mapping in parasitic nematodes. Undertaking genetic crosses
72 requires genetically and phenotypically divergent, and preferably near-isogenic parental lines.
73 Developing these for nematode parasites is challenging since they cannot be maintained *in vitro*
74 throughout their life cycle, hence experimental models depend upon the infection of the parasites'
75 mammalian hosts. High levels of host specificity make these models intractable for human parasites
76 and necessitate the development of animal models. The ruminant nematode parasite, *H. contortus* is
77 currently the most important model system for the study of anthelmintic drug resistance as well
78 being a key tool for anthelmintic drug and vaccine discovery research (Gilleard, 2013). A draft
79 genome sequence has recently been published (Laing et al., 2013) and assembly and annotation
80 improvements are on-going (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 larvae of the ISE 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 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 (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 four 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 post-infection 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

113 hours of recovery from the donor lamb, into the abomasum of a 4 month-old recipient lamb (lamb
114 A, Fig. 1). In addition, a single sexually-immature male and 20 sexually-immature females were
115 surgically transferred to two other lambs (lambs B and C, Fig. 1). The faecal trichostrongyle egg
116 counts (FEC) of the three recipient lambs were monitored daily from days 14 to 21 (1 to 7 days post
117 transplantation) using a standard salt floatation method with a minimum detection threshold of 1
118 egg per gram (epg) (Christie and Jackson, 1982). All three lambs had positive egg counts by day 18
119 that increased to 20 epg on day 21.

120

121 *2.3. Collection of progeny from single female adult nematodes following mating with a single male*

122 The three recipient lambs were euthanased 7 days after surgical transfer (day 21). The
123 single, transplanted male worms could not be recovered from any of the three recipient lambs at
124 autopsy but 12, 6 and 12 of the transplanted female worms were recovered from the abomasa of
125 recipient lambs A, B and C, respectively. All recovered female worms were immediately picked into
126 sterile phosphate buffered saline (PBS) and transferred individually into separate wells of 24 well
127 plates each containing 1 ml of warm RPMI 1640 cell culture media (Gibco) and incubated in 5% CO_2
128 at 37°C for 8 hours to promote egg shedding. They were then transferred to a 24°C incubator for 36
129 hours to permit hatching of any fertilised eggs. Although all of the recovered female worms shed
130 several hundred eggs, the development and hatching rate was extremely low. Consequently, only 4
131 female MHco3 *H. contortus* produced L_1 broods of sufficient size (minimum n = 100) to allow their
132 molecular and phenotypic characterisation and subsequent propagation of another generation.
133 These four females were arbitrarily named N1 (recovered from recipient lamb A), N2, N3 and N4
134 (recovered from recipient lamb B) respectively. DNA lysates were prepared from approximately half
135 of the L_1 stage larvae in each brood (F_1 progeny of the single parent mating) and from the head of
136 each adult female parent. The remaining L_1 were retained for coproculture development to the
137 infective L_3 larval stage, to allow infection of more animals to produce the next filial generation.

138

139 *2.4. Larval coproculture*

140 L_1 were either transferred onto a disc of cotton filter paper placed in a petri dish containing
141 5 ml of an OP50 *Escherichia coli* culture in Luria broth/streptomycin, or inoculated into 10 g of faeces
142 collected from a known 'worm-free' donor lamb that had been sequentially treated with 5 mg/kg of
143 fenbendazole and 7.5 mg/kg of levamisole 5 days previously. The filter paper/*E. coli* and larval
144 coprocultures were then placed individually in perforated plastic bags and incubated in a closed
145 laboratory incubator at 24°C for 7 days. L_3 were then recovered from the larval cultures by

146 Baermannisation (MAFF, 1986) and transferred in tapwater into tissue culture flasks and stored at
147 $8^{o}C$ for 3 weeks before they were used to infect donor lambs. 15, 20, 3 and zero L_3 were recovered
148 from the coprocultures of the broods of the adult female MHco3 individuals, N1, N2, N3 and N4.

149

150 *2.5. Propagation of inbred lines resulting from single pair matings*

151 In order to propagate filial lines, two 6 to 7 month-old 'worm-free' lambs were orally
152 infected with 15 MHco3.N1.F_1 L_3 larvae (lamb D, Fig. 1) and 20 MHco3.N2.F_1 L_3 (lamb E, Fig. 1). The
153 FECs of these two lambs were monitored from 14 days post-infection. Mean daily FECs were 1.2 (SD,
154 1.0) epg (lamb D), and 22 (SD, 16) epg (lamb E) between 21 and 60 days post-infection (Fig. 1). In
155 order to propagate the next filial generation, this process was repeated using L_3 larvae derived from
156 eggs (F_2 progeny) recovered from lambs D and E (Fig. 1). Two different 8 to 9 month-old 'worm-free'
157 lambs were infected separately with 7,500 MHco3.N1.F_2 (lamb F, Fig. 1) and MHco3.N2.F_2 L_3 (lamb
158 G, Fig. 1). The mean daily FECs between 20 and 60 days post-infection of lamb F and lamb G were
159 547 (SD, 265) epg and 420 (SD, 200) epg, respectively. An additional round of passage was
160 undertaken for the MHco3.N1 line only. 7,500 and 5,000 MHco3.N1.F_3 L_3 derived from eggs
161 recovered from lamb F were used to infect a 10 month-old lamb to produce a MHco3.N1.F_4 (lamb H,
162 Fig. 1) lines. The mean daily FECs of this lambs between 20 and 60 days post-infection was 33 (SD,
163 29) (Fig. 1).

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

169

170 *2.6. DNA Lysate preparation*

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

177 rDNA PCR assay, to confirm species identity as *H. contortus* (Redman et al., 2008b; Wimmer et al.,
178 2004).

179

180 *2.7. Single strand conformation polymorphism*

181 The genetic diversity of the GABA Cl subunit HG1 locus (Blackhall et al., 2003), as well as that
182 of GluCl α and β subunit loci (Blackhall et al., 1998) (Supplementary Fig. S2A) was examined by single
183 strand conformation polymorphism (SSCP) using previously described PCR primers (Blackhall et al.,
184 2003). The thermal cycler conditions used were: 95°C for 4 minutes; followed by 40 cycles of 95°C
185 for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds; a final extension stage of 72°C for 5
186 minutes. Amplicons were first visualised on a 1.2% agarose gels and then run on non-denaturing
187 polyacrylamide gels as previously described (Skuce et al., 2010).

188 To confirm the allelic assignments of the GABA Cl subunit HG1 SSCP genotyping, the PCR
189 products produced from two individual MHco3.N1.F_2 L_3 DNA heterozygotes (6F and 9G) were cloned
190 and sequenced. Briefly, amplicons were run on a 1% agarose electrophoretic gel to enable the
191 excision of 305 bp bands from which DNA was then isolated (QIAquik Gel Extraction kit, Qiagen).
192 DNA was ligated into pGEM®-T (Promega) plasmid vectors to allow transformation into JM109
193 competent *E. coli* cells (Stratagene). Plasmid DNA from the cultured transformed cells was then
194 purified using a Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced using
195 SP6 and T7 universal primers in both orientations.

196

197 *2.8. Microsatellite genotyping*

198 Microsatellite genotyping of 'bulk' DNA lysates, made from approximately 500 larvae, was
199 performed on MHco3(ISE), MHco3.N1.F_2, MHco3.N1.F_3, MHco3.N1.F_4, MHco3.N2.F_2 and
200 MHco3.N2.F_3 *H. contortus* lines. Ten microsatellite markers, previously shown to be polymorphic in
201 the MHco3(ISE) strain were used: Hcms25, Hcms33 and Hcms36 (Otsen et al., 2001), Hcms8a20,
202 Hcms22co3 (Redman et al., 2008b), HcmsX142, HcmsX256, HcmsX337 (Redman et al., 2008a),
203 Hcms3561 and Hcms18210 (Redman et al., 2012). Individual worm genotyping was also performed
204 for four of these loci, Hcms8a20, Hcms36, Hcms3561 and Hcms25 plus two additional loci,
205 HcmsX182 and HcmsX240 (Redman et al., 2008a), on the N1 and N2 adult female parent heads and
206 30 individual larvae for each of the following populations: MHco3.N1.F_1 and MHco3.N2.F_1 (L_1);
207 MHco3.N1.F_2, MHco3.N2.F_2, MHco3.N1.F_3, MHco3.N2.F_3 (L_3) and MHco3(ISE). All microsatellite
208 genotyping, on both 'bulk' and single worm DNA lysates, was performed using the same PCR

209 amplification methods and parameters as previously described (Redman et al., 2008b). Capillary
210 electrophoresis was performed using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster
211 City, CA) for the accurate sizing of microsatellite PCR products. The forward primer of each
212 microsatellite primer pair was 5'-end labelled with FAM, HEX, or NED fluorescent dyes (MWG) and
213 electrophoresed with a GeneScan ROX 400 (Applied Biosystems) internal size standard. Individual
214 chromatograms were analysed using Genemapper Software Version 4.0 (Applied Biosystems).

215

216 *2.9. Genetic analysis*

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

231

232 *2.10. Genome-wide SNP analysis*

233 Genomic libraries were prepared from 400 MHco3(ISE) and 400 MHco3.N1.F_3 adult worms
234 for Illumina sequencing using previously described methods (Laing et al., 2013; Kozarewa et al.,
235 2009) (Supplementary Table S3). Preliminary analysis and base-calling for data from the Illumina
236 HiSeq sequencing machines used the RTA1.8 analysis pipelines. Whole-genome shotgun sequence
237 data was generated from these libraries on two different Illumina platforms, producing different
238 numbers of reads and reads of different lengths (Supplementary Table S3). To produce comparable
239 data between the two biological samples, read pairs were randomly sampled from the larger (inbred
240 material) sequencing data by keeping each pair of reads in the subsampled file with a probability of
241 0.39, and by clipping 12 bp from each end of every read. Reads were mapped against the released

242 370Mb v1.0 genome assembly of *H. contortus* (Laing et al., 2013), available at the GenBank database
243 under project ID PRJEB506, using the mapper SMALT v0.7.0.1
244 (http://www.sanger.ac.uk/resources/software/smalt) in paired-end mode, with an indexing k-mer
245 size of 13 and step size of 1, mapping non-repetitively (-r -1), with a minimum identity of 0.8 to
246 report a mapping (-y 0.8) and exhaustively searching for alignments of each read independently of
247 its mate pair (-x), and only reporting reads as properly paired if they were mapped than 1000bp
248 apart on the reference genome. Single nucleotide polymorphism (SNP) variants were called jointly
249 from the three mapping output files using samtools v0.1.19-44428cd (Li et al., 2009) using the
250 *mpileup* command, skipping alignments with either mapping or base quality scores less than 13.
251 Density, distribution and types of variant calls were tallied using vcftools v0.1.11
252 (https://sourceforge.net/projects/vcftools/). Estimates of the nucleotide diversity (π) for the pools of
253 worms sequenced in each library were calculated independently from the variant calling approach
254 outlined above using PoPoolation2 v1.013 (Kofler et al., 2011).

255

256 *2.11. Ethics statement*

257 All experimental procedures described in this manuscript were examined and approved by
258 the Moredun Research Institute Experiments and Ethics Committee and were conducted under
259 approved UK Home Office licenses in accordance with the Animals (Scientific Procedures) Act of
260 1986. The Home Office license numbers are PPL 60/03223 and PPL 60/03899 and experimental IDs
261 for these studies were E06/58. E06/75 and E09/36.

262

263 **3. Results**

264 *3.1. The establishment of two independent inbred lines by single pair mating of H. contortus.*

265 Our preliminary experiments to replicate anecdotal reports suggesting that it might be
266 possible that a single male and a single female *H. contortus*, when transferred directly into the
267 abomasum of a 'worm-free' recipient sheep could survive for long enough to find each other, mate
268 and shed eggs were unsuccessful, highlighting the severe biological limitations to this approach.
269 Consequently, we developed a method in which a single immature male worm was transplanted
270 with a number of immature female worms, then following mating, the female worms were
271 recovered on autopsy, placed in individual wells of a 24 well plate and allowed to lay eggs (F_1
272 progeny) *in vitro*. Two of the recovered female worms, designated N1 and N2, produced broods of
273 sufficient size and viability to enable the propagation of the next filial generations. 15 and 20 L_3 from

274 the N1 and N2 founding female parents, respectively, were used to orally infect two separate lambs
275 to establish the MHco3.N1 and MHco3.N2 inbred lines (Fig. 1).

276

277 *3.2. Validation of single pair mating and assessment of polymorphism of inbred lines by*
278 *microsatellite genotyping*

279 Bulk DNA genotyping with 10 microsatellite markers, on DNA prepared from pools of
280 approximately 500 L_3 per population, was used to provide an initial assessment of the genetic
281 diversity of the MHco3.N1 and MHco3.N2 inbred lines and indicate of the success of the single pair
282 matings. The total number of alleles detected was reduced in both of the inbred lines relative to the
283 founding MHco3(ISE) population with a greater loss of overall diversity in MHco3.N1. Across the 10
284 markers, a total of 28 alleles in the MHco3(ISE) population was reduced to 15 and 20 alleles in the
285 derived MHco3.N1 and MHco3.N2 lines respectively. There was a loss of alleles at 7 out of the 10 loci
286 in both cases (Supplementary Table S1).

287 The MHco3(ISE) strain, the individual N1 and N2 founder female parents and populations of
288 the inbred MHco3.N1 and MHco3.N2 lines were analysed in more detail by genotyping individual
289 worms at six of the most discriminatory loci. The level of polymorphism of the parental MHco3(ISE)
290 strain was consistent with that previously observed with other panels of microsatellite loci (Redman
291 et al., 2008b) with a mean of 3.17 alleles per locus and an expected heterozygosity (H_E) of 0.572.
292 There was a clear reduction in polymorphism in F_1 and F_2 populations of both inbred lines with the
293 MHco3.N1 again showing the greatest reduction (Supplementary Table S2).

294 Pairwise F_{ST} estimates based on the multi-locus genotype data revealed a high degree of
295 genetic differentiation between the two inbred strains as well as between both lines and the
296 parental MHco3(ISE) strain. No statistically significant genetic differentiation was observed between
297 any of the filial populations within the same inbred line demonstrating that the genetic integrity of
298 both the inbred lines was maintained despite passage (Supplementary Fig. S1A). Further evidence
299 for reduction in genetic diversity by the single parent mating procedure was provided by principal
300 component analysis of individual worm multi-locus genotypes (Supplementary Fig. S1B).

301

302 *3.3. Examination of the HG1 GABA Cl locus using SSCP*

303 The HG1 gene which encodes a GABA-gated chloride channel (Blackhall et al., 2003) was
304 selected and used as an additional marker to monitor the single pair meeting and inbreeding
305 process, since this had been shown to have a high level of genetic diversity in the MHco3(ISE) strain

306 (Supplementary Fig. S2). SSCP profiles were obtained for 84 MHco3(ISE) L_3 and at least 15 distinct
307 profiles were discernable (Supplementary Fig. S3A). However, only three different SSCP profiles were
308 discernable from 57 F_1 and 65 F_2 progeny of the MHco3.N1 inbred line (Supplementary Fig. S3B).

309

310 *3.4. Comparison of MHco3(ISE) and MHco3.N1 genome-wide SNP polymorpshism*

311 Whole genome Illumina sequencing was performed on MHco3(ISE) and MHco3.N1
312 populations (Supplementary Table S3). The number of sites classified as heterozygous within the
313 MHco3.N1 population in the variant calls from *mpileup* was almost 50% fewer than those called for
314 the MHco3(ISE) populations. Using the number of reads supporting each allele at a site as a rough
315 estimate of the allele frequency in the pool of adult worms sequenced, there is a clear pattern of a
316 greater proportion of sites having minor alleles segregating at intermediate frequencies (between
317 0.15 and 0.35) within the MHco3.N1 population (Supplementary Fig. S4A). The MHco3.N1
318 population is particularly reduced in rare alleles, as expected from a recent, extreme population
319 bottleneck. This pattern is consistent with these nematodes being the offspring a single-pair mating,
320 where we would expect minor alleles to be present on just one of the four parental haplotypes. The
321 same pattern is clear in the subset of sites that are polymorphic in both populations, where the
322 nucleotide diversity (π) is lower in MHco3.N1 than in MHco3(ISE) at almost two-thirds of sites
323 (332/537) on the longest assembly scaffold (Supplementary Fig. S4B).

324

325 **4. Discussion**

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

336 This paper presents the development of a novel method to achieve a single pair parasitic
337 nematode mating involving the surgical transfer of multiple female and one male day 14 parasitic-

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

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

367 An overall loss of genetic polymorphism in both the MHco3.N1 and MHco3.N2 lines
368 compared with the parental MHco3 (ISE) strain was revealed by the microsatellite markers, the
369 GABA Cl SSCP profiles and the whole genome sequencing analysis. The Hco3.N1 line showed the
370 greatest loss of polymorphism of the two inbred lines based on the microsatellite genotyping with a
371 loss of 13 out of 28 alleles (46%) across the 10 microsatellite markers genotyped. Consequently, the

372 inbred MHco3.N1 population was propagated further and used as the reference strain for the *H.*
373 *contortus*, genome project (Laing et al., 2013). Subsequent, genome-wide SNP analysis was
374 consistent with the microsatellite analysis showing that MHco3.N1 line had an almost 40% reduction
375 in SNP positions called as heterozygous across the genome, and reduced nucleotide diversity at
376 shared heterozygous sites, compared to MHco3(ISE).

377 The two inbred lines retained largely different alleles from the MHco3(ISE) populations, as
378 demonstrated in the multilocus genotyping PCA plots and pairwise F_{ST} analyses. The production of
379 genetically divergent inbred *H. contortus* lines using this method could be exploited in a number of
380 ways. It could be used to develop genetically divergent strains with which to undertake genetic
381 crosses for the production of a genetic map, or to identify the position of genetic loci of interest,
382 such as those underlying anthelmintic resistance (Le Jambre et al., 1999). A prerequisite for the
383 creation of a genetic map is that the parent populations have minimal within-strain polymorphism,
384 but high levels of between-strain polymorphism, in order to allow the alternative parental alleles to
385 be identified in F_2 progeny resulting from the genetic cross. With the exception of the Chiswick
386 avermectin resistant (CAVR) strain, which arose as a serendipitous, extraneous, ivermectin resistant
387 contaminant of an Australian laboratory passaged *Trichostrongylus colubriformis* strain (Le Jambre,
388 1993), the currently available laboratory strains of *H. contortus* are too polymorphic to use in
389 conventional mapping studies. Hence generation of experimentally inbred, near-isogenic, genetically
390 divergent strains is useful. Cases of multigenic resistance could be investigated by segregating
391 different genetic loci contributing to an anthelmintic resistance phenotype into separate inbred
392 lines.

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

397

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411

412 **Contributions**

413 NDS, JSG and FJ conceived the method. NDS, AAM, DJB and FJ undertook the animal work and gross
414 parasitology. NDS, EM, HNv-G, NH, MB and JAC undertook the molecular studies and analysis of the
415 data. NDS, ER, JAC and JSG wrote the paper.

416

417 **Figure legend**

418 **Fig. 1. Genetic crossing and passaging approach to inbreed the MHco3(ISE) standard genome strain**
419 **of *H. contortus*.**

420 Schematic representation of experimental aim and summary of nomenclature.

421 *gDNA extracted from MHco3.N1.F3 adults used for genome sequencing (Laing et al., 2013)

422

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530

531 **Supplementary data**

532

533 **Supplementary Fig. S1. Genetic differentiation between populations of inbred worms using a**
534 **panel of four microsatellite markers.**

535 (A) Population pairwise FSTs comparing the F_1 and F_2 populations of the MHco3.N1 and MHco3.N2
536 inbred lines and the parent MHco3(ISE) population. Significant genetic divergence is highlighted by
537 underlining and bolding.

538 The data show a high degree of genetic differentiation between the two inbred strains
539 (pairwise F_{ST} = 0.300-0.512) as well as between both lines and the parental MHco3(ISE) strain. The
540 MHco3.N1 line appears to be the slightly more divergent from the parental MHco3(ISE) strain than
541 the MHco3.N2 line (eg. MHco3.N1.F_1 F_{ST} = 0.262 compared with MHco3.N2.F_1 F_{ST} = 0.197). The F_{ST}
542 values remain statistically significant between the MHco3(ISE) population and both inbred lines at
543 each filial generation. In contrast, no statistically significant genetic differentiation was observed
544 between any of the filial populations within the same inbred line.

545 (B) Individual multi-locus genotypes of worms from Hco3(ISE), MHco3.N1.F_1 and MHco3.N2.F_1
546 populations shown on a PCA plot as black triangles, green diamonds and red squares respectively.

547 The individual founding N1 and N2 female parental multi locus genotypes are shown as a larger
548 green diamond, or red square, respectively, and indicated by an arrow.

549 The individual worm multilocus genotypes of worms from the two inbred populations
550 formed much tighter clusters than those from the founding MHco3(ISE) population. The clusters of
551 for each inbred line were completely separate to each other and centred around the position of the
552 multilocus genotype their respective N1 and N2 individual founder female parents on the PCA plot.

553

554 **Supplementary Fig. S2. SSCP polyacrylamide gels.**

555 (A) Gel comparing polymorphisms in GluClα (lanes 1 – 4), GluClβ (lanes 6 – 9) and GABA Cl (lanes 11
556 – 14) subunits of four individual adult MHco3(ISE) *H. contortus* (1.5 mM $MgSO_4$ and 5 µl of PCR
557 product run on gel). Each of the MHco3(ISE) *H. contortus* has a different GABA Cl SSCP genotype.

558 (B) A polyacrylamide gel showing the GABA Cl SSCP genotypes of a MHco3(ISE) female head (lane 17)
559 and L_3 progeny (lanes 1, 2, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16 and 19) from eggs hatched to L_1 in RPMI
560 in the well of a 24 well plate, and then grown to L_3 in the same well on *E. coli* and filter paper.
561 Amplified PCR product was not seen on an agarose gel corresponding with lanes 3, 4, 8 and 9, while
562 lane 18 is a negative control. Different SSCP genotypic profiles are labelled A – E.

563

564 **Supplementary Fig. S3. GABA Cl subunit HG1 SSCP profiles.**

565 (A) Polyacrylamide gels showing a high level of polymorphism in MHco3(ISE) *H. contortus*. Each lane
566 shows the SSCP GABA Cl subunit HG1 genotype of an individual L_3.

567 (B) Polyacrylamide gels showing the GABA Cl SSCP genotypes of the N1 female (lanes 5, 15, 25 and
568 35) and of individual MHco3.N1.F_1 L_1 (lanes 1, 2, 4. 6 – 14, 16 – 19, 21, 23, 24, 26 – 32, 34, and 36 –
569 40).

570 (C) Polyacrylamide gels showing the GABA Cl SSCP genotypes of MHco3.N1.F_2 L_3.

571 Although the assignment of alleles from SSCP profiles is often ambiguous, the SSCP profile of
572 the founding N1 female parent was entirely consistent with those of the F_1 and F_2 progeny (data not
573 shown).

574

575 **Supplementary Fig. S4. Genomic analysis of allele frequency and nucleotide diversity.**

576 (A) Histogram of minor allele frequencies for MHco3(ISE) and MHco3.N1 inbred line based on
577 genome sequencing data, based on counts of reads supporting each of two alleles at all biallelic sites
578 in each sequencing library.

579 (B) Nucleotide diversity estimates for 537 sites on scaffold 1 of the *H. contortus* v1.0 assembly
580 polymorphic in both MHco3 (ISE) and MHco3.N1. The dashed line represents equal diversity in the
581 pools of worms from each population, the solid line is the best-fit linear regression through the
582 origin, with the 95% confidence interval for this fit shaded.
583
584