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Deep amplicon sequencing as a powerful new tool to screen for sequence polymorphisms associated with anthelmintic resistance in parasitic nematode populations ☆

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ABSTRACT

Parasitic gastrointestinal nematodes contribute to significant human morbidity and cause billions of dollars per year in lost agricultural production. Control is dependent on the use of anthelmintic drugs which, in the case of livestock parasites, is severely compromised by the widespread development of drug resistance. There are now concerns regarding the emergence of anthelmintic resistance in parasitic nematodes of humans in response to the selection pressure resulting from mass drug administration programs. Consequently, there is an urgent need for sensitive, scalable and accurate diagnostic tools to detect the emergence of anthelmintic resistance. Detecting and measuring the frequency of resistance-associated mutations in parasite populations has the potential to provide sensitive and quantitative assessment of resistance emergence from an early stage. Here, we describe the development and validation of deep amplicon sequencing as a powerful new approach to detect and quantify the frequency of single nucleotide polymorphisms associated with benzimidazole resistance. We have used parasite communities in sheep to undertake a proof-of-concept study of this approach. Sheep provide an excellent host system, as there are multiple co-infecting trichostrongylid nematode species, each likely with a varying prevalence of benzimidazole resistance. We demonstrate that the approach provides an accurate measure of resistance allele frequencies, and can reliably detect resistance alleles down to a frequency of 0.1%, making it particularly valuable for screening mutations in the early stages of resistance. We illustrate the power of the technique by screening UK sheep flocks for benzimidazole resistance-associated single nucleotide polymorphisms at three different codons of the β-tubulin gene in seven different parasite species from 164 populations (95 from ewes and 69 from lambs) in a single MiSeq sequencing run. This approach provides a powerful new tool to screen for the emergence of anthelmintic resistance mutations in parasitic nematode populations of both animals and humans.

1. Introduction

Gastrointestinal nematodes have a major impact on both human and animal health (Parkins and Holmes, 1989; Stromberg and Gasbarre, 2006; Hotez, 2011). The significant economic losses and health and welfare problems caused by parasitic nematodes of livestock have led to the routine use of broad spectrum anthelmintic drugs in agriculture over several decades (Stromberg and Gasbarre, 2006). As a result of frequent and sometimes indiscriminate use of anthelmintic drugs, resistance has become widespread in multiple livestock parasites, compromising the sustainability of control (Bartley et al., 2004; McKellar and Jackson, 2004; Wrigley et al., 2006; Sargison et al., 2010; Kaplan and Vidyashankar, 2012; Roeber et al., 2012; Voigt et al., 2012; Hodgson and Mulvaney, 2017). In the case of human parasitic nematodes, mass drug administration (MDA) programs in the developing world have substantially increased in coverage over the last 10–15 years, most recently in response to the WHO, 2012 Goals (World Health Organization, 2012). As a result, there are now increasing concerns regarding the emergence of benzimidazole resistance in human

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soil transmitted helminths (STHs) (Humphries et al., 2013; Ásbjörnsdóttir et al., 2017; Krücken et al., 2017). Consequently, there is an urgent need for more sensitive diagnostic tools to detect and monitor anthelmintic resistance in both human and animal parasites.

The standard method for detecting anthelmintic resistance in livestock parasites is the fecal egg count reduction test (FECRT). This technique involves conducting a fecal egg count (FEC) before and after drug treatment, typically on groups of at least 15 animals. A percentage reduction in the mean fecal egg count following treatment of <95%, with a lower 95% confidence level of <90%, is considered to indicate resistance (Coles et al., 1992). In order to be reliable, FECRTs need to be carefully conducted using validated protocols and statistical criteria. Consequently, those are time-consuming and expensive to use on a routine basis, and adapting those for use in humans, typically in poorer regions, presents significant practical and logistical challenges. Further, FECRTs are insensitive and unable to detect resistance at an early stage. A number of in vitro laboratory assays have been developed to assess drug resistance phenotypes for a number of livestock parasites, and include the egg hatch, larval motility and larval development assays (Martin and Le Jambre, 1979; Coles et al., 2006; Demeler et al., 2012; Storey et al., 2014). However, these assays require fresh fecal samples, are labor-intensive, insensitive, and difficult to standardize. Some of the limitations of current in vivo and in vitro anthelmintic resistance diagnostic tests could be potentially overcome through the use of molecular tests that detect specific mutations associated with the resistance phenotype. In particular, such tests are potentially more sensitive, allowing the early detection of resistance. In the case of the benzimidazole drug class, the most important drug resistance conferring mutations are known for a number of livestock parasites, which provides an opportunity to develop molecular tests and provide a proof-of-concept. There is a considerable body of research showing that polymorphisms in three different codons of the isotype-1 β-tubulin gene of trichostrongylid nematodes of domestic ruminants are associated with benzimidazole resistance (Roos et al., 1990; Kwa et al., 1993, 1994, 1995; Njue and Prichard, 2003; Gilleard, 2006). The F200Y (TTC>T**A**C) single nucleotide polymorphism (SNP) is the most intensively studied and the most prevalent in sheep parasites worldwide. This SNP was originally shown to confer resistance to benomyl in a variety of fungi (von Samson-Himmelstjerna et al., 2007) and was subsequently shown to confer benzimidazole resistance in the sheep parasite *Haemonchus contortus* by heterologous expression experiments in *Caenorhabditis elegans* (see Kwa et al., 1994, 1995) This SNP has been consistently shown to be associated with benzimidazole resistance in many *H. contortus* populations across the world as well as in several other trichostrongylid species such as *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (see Silvestre and Humbert, 2002; Ghisi et al., 2007; Niciura et al., 2012; Chagas et al., 2016; Zhang et al., 2016). F167Y (TTC>T**A**C), E198A (GAA>G**C**A) and E198L (GAA>**TT**A) isotype-1 β-tubulin polymorphisms, all known to confer benzimidazole resistance in fungi, have also been identified in benzimidazole-resistant *H. contortus* and/or *Te. circumcincta* populations in some geographic regions with evidence of selection in the field (Orbach et al., 1986; Nowak and Kück, 1994; Prichard, 2001; Silvestre and Cabaret, 2002; Lila et al., 2003; Ghisi et al., 2007; Liu et al., 2014; Redman et al., 2015).

The understanding of the molecular basis of benzimidazole resistance in ovine parasitic nematodes makes it possible to develop molecular techniques for diagnosis and surveillance. Until now, approaches have included allele-specific PCR, real-time PCR and pyrosequencing genotyping assays in species such as *H. contortus*, *Te. circumcincta* and *Trichostrongylus* spp. (Álvarez-Sánchez et al., 2005; von Samson-Himmelstjerna et al., 2007; Walsh et al., 2007; Demeler et al., 2013; Chaudhry et al., 2014; Knapp-Lawitzke et al., 2015; Redman et al., 2015; Ramünke et al., 2016). However, for all these approaches, individual assays need to be separately developed and optimized for each polymorphism in each species, and there are practical limitations regarding the reliable detection of resistance alleles when present at low frequencies. In this paper, we describe the first known use of deep amplicon sequencing to determine the frequency of benzimidazole resistance alleles in parasitic nematodes communities. We demonstrate the ability of the approach to determine the frequency of multiple resistance polymorphisms, even when present at low frequencies, in multiple parasite species and in multiple populations in a single assay. This represents a new and versatile approach, which has the potential to achieve more sensitive and larger scale screening of resistance-associated mutations, in a range of parasitic nematodes simultaneously obtained from either animals or humans.

2. Materials and methods

2.1. Parasite materials

2.1.1. L1s from sheep field samples

Aliquots of 1000 trichostrongylid L1s, harvested in a previous study from pooled fecal samples collected from farms around the United Kingdom (UK) were used to prepare DNA lysates; 20 individual fecal samples were collected from ewes from 95 farms, and from lambs from 69 farms (Burgess et al., 2012; Redman et al., 2015). In total, 54 farms had samples from both ewes and lambs, with the remaining 56 farms having samples from either ewes or lambs. A pooled composite containing equal amounts of each individual fecal sample was generated from the collected ewe and lamb samples from each farm, before eggs were harvested and allowed to hatch to L1s. Further details of the sample collection and preparation is provided in the original study (Burgess et al., 2012).

2.1.2. Single species populations of infective L3s

Pure populations of L3s of various nematode species were obtained from experimentally passaged and previously characterized monospecific isolates: *Cooperia oncophora*, *Cooperia punctata*, *Ostertagia ostertagi*, *H. contortus*, *Haemonchus placei*, *Trichostrongylus axei*, *Tr. colubriformis* and *Nematodirus helvetianus* from Merial Ltd, USA (Jeffrey Shyrock) and *Te. circumcincta*, *Cooperia curticei*, *Nematodirus battus* and *Trichostrongylus vitrinus* from the Moredun Research Institute (Dr David Bartley, UK). Approximately 1000 larvae from each pure strain were fixed in ethanol and were used to generate reference sequences for each of the respective parasite species.

2.1.3. Morphologically identified adult male nematodes

Three morphologically identified adult male nematodes of both *Cooperia pectinata* and *Haemonchus similis* were obtained. The morphologically identified *C. pectinata* were provided by Dr. Murilo A. Bichuette (Universidade Estadual Paulista (UNESP) Brazil), while the *H. similis* were provided by Dr. Cesar C. Bassetto (UNESP, Brazil).

2.1.4. DNA preparation

DNA lysates were prepared from the pools of L1s or L3s using a previously described method (Avramenko et al., 2015). DNA lysates from adult nematodes were prepared by cutting off the heads and 1/3 of the body, and lysing in a final volume of 100 μL following the same lysis protocol described in Avramenko et al.

(2015). DNA was then purified from the crude DNA lysates using the QIAGEN QIAmp DNA mini kit (Cat# 51306), following the manufacturer's recommended protocol.

2.1.5. Primer design

Multiple forward and reverse oligonucleotide primers were designed to PCR-amplify from exons 4 and 5 (spanning intron 4) of the isotype-1 β-tubulin gene of multiple trichostrongylid nematode species. The forward primers are anchored in codons 136–142, and the reverse primers anchored in codons 211–219, which were chosen on the basis of their sequence conservation between species and specificity for the isotype-1 β-tubulin gene. The amplified fragment spans codons 167, 198 and 200. The amplicon also includes codons 153 and 165, which are discriminatory between isotype-1 and isotype-2 β-tubulin (Njue and Prichard, 2003), allowing a quality assurance check on the sequence data generated for isotype-1 β-tubulin specificity. The primers were designed to have similar annealing conditions, to allow their use in a single multiplex PCR. In total, four primer sets (forward and reverse) were designed to amplify from multiple Clade V nematode species as mentioned below. For species for which there are no isotype-1 β-tubulin sequences available in public databases, it was assumed that the primer sites would be sufficiently conserved to anneal primers designed for another species within the same genus with an available reference sequence. These primers have been validated to amplify the isotype-1 β-tubulin target sequence from the following species using the multiplex PCR conditions described in Section 2.2.1.: *C. oncophora, C. punctata, C. pectinata, C. curticei, H. contortus, H. placei, Te. circumcincta, O. ostertagi, Tr. axei, Tr. colubriformis* and *Tr. vitrinus.* Primer sequences are provided in Supplementary Table S1. Primer names include acronyms identifying species the primer variant was designed against: Oo = *O. ostertagi*, Co = *C. oncophora,* Hc = *H. contortus*, Tc = *Te. circumcincta*, Tcol = *Tr. colubriformis.*

2.2. Deep amplicon sequencing

2.2.1. PCR amplification of the isotype-1 β-tubulin fragment

Samples were prepared for sequencing using a protocol adapted from our previously published method (Avramenko et al., 2015). Further information is also available at https://www.nemabiome.ca. Using the primers described previously, we created adapted primers suitable for Illumina next-generation sequencing as described previously (Avramenko et al., 2015). Briefly, the primer design is as follows: 5′-Illumina adapter sequence – 0-3N′s – Primer binding regions -3′. The forward Illumina adapter sequence used is: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′, while the reverse Illumina adapter sequence is: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′. Primers were staggered, with 0-3 N′s included between the adapter and the primer-binding region, to increase the diversity of generated amplicons to prevent oversaturation of the sequencing channels. The Illumina adapter oligonucleotide sequences were obtained from the Illumina Adapter Sequences document (February 2016; Oligonucleotide sequences © 2016 Illumina, Inc. All rights reserved). A complete list of all adapted primers used can be found in Supplementary Table S1. All primers used for Illumina sequencing were ordered from Eurofins Operon (Luxembourg) with QuickLC purification.

All forward and all reverse primers were mixed in equal concentrations to create a single forward and a single reverse primer mixture with a final concentration of 10 μM. These mixtures are subsequently referred to as the "Forward primer mix" and "Reverse primer mix". The following PCR conditions were used: 5 μL of 5× NEB Q5 Reaction Buffer (New England Biolabs Ltd, USA) 0.5 μL of 10 mM dNTPs, 1.25 μL of 10 μM Forward primer mixture, 1.25 μL of 10 μM Reverse primer mixture, 0.25 μL of NEB Q5 polymerase, 14.75 μL of molecular grade water and 2 μL of DNA lysate. The thermocycling parameters were 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, 65 °C for 15 s, and 72 °C for 25 s, followed by 72 °C for 2 min. All PCR steps were carried out with best practices to reduce aerosol formation, including the use of filter pipette tips, working in a PCR cabinet, and the use of the easy release Microseal 'A' Film (Bio-Rad, USA MSA5001) PCR plate cover.

2.2.2. Purification of first round PCR products

PCR products were purified with AMPure XP Magnetic Beads (1X) (Beckman Coulter, Inc USA), following the manufacturer's recommended protocol. All samples were eluted in 32.5 μL of molecular-grade water.

2.2.3. Addition of indices and P5/P7 sequencing regions

Illumina barcode indices, as well as the P5/P7 sequencing regions, were added to the isotype-1 β-tubulin amplicons by limited cycle PCR amplification, using primers contained in the Nextera XT Index Kit v2 set (Oligonucleotide sequences © 2016 Illumina, Inc. USA). These primer sequences are provided in Supplementary Table S2. Primers were combined to make up to 384 unique forward/reverse sample barcode combinations per run. The following PCR conditions were used: 5 μL of 5X KAPA HiFi HotStart Fidelity Buffer (KAPA Biosystems, USA), 1.25 μL of Forward Primer (S502-S522) (10 μM), 1.25 μL of Reverse Primer (N701-N729) (10 μM), 0.75 μL of dNTPs (10 mM), 0.5 μL KAPA HiFi HotStart Polymerase (0.5 U), 13.25 μL of molecular-grade H_2O, and 3 μL of first-round clean PCR product as template. The thermocycling parameters were: 98 °C for 45 s, followed by nine cycles of 98 °C for 20 s, 63 °C for 20 s, 72 °C for 2 min. Amplicons were purified with AMPure XP magnetic beads (1X), following the same steps as described previously.

2.2.4. Pooling and sequencing of libraries

The concentration of each sample was determined using the NanoVue plus Spectrophotometer (General Electric, USA). A master sequencing mix was created by pooling ~50 ng of purified PCR product from each sample. The final concentration of the pooled library was assessed with the KAPA qPCR Library Quantification Kit (KAPA Biosystems, USA), following the manufacturer's recommended protocol. The library was diluted to 4 nM based upon the quantitative PCR results. The prepared pooled library was run on an Illumina Desktop Sequencer using a 500-cycle paired-end reagent kit (MiSeq Reagent Kit, v2, MS-103–2003) at a final concentration of 15 pM, with the addition of 25% PhiX control v3 (Illumina, FC-110-3001). The MiSeq was set to generate only FASTQ files, with no post-run analysis. Samples were automatically demultiplexed with the MiSeq, based on the supplied index combinations. All protocols were carried out using Illumina's standard MiSeq operating protocol (Illumina Inc.). All sequencing data generated during this study are available from the EMBL Nucleotide Sequence Database (ENA) repository, study accession **PRJEB27413**.

2.3. Sequence analysis

2.3.1. Sequence processing and classification

Overlapping paired-end reads from each sample were joined to form contigs using PEAR v. 0.9.6 (Paired-end read merger), using default parameters (Zhang et al., 2014). This was done to generate contiguous FASTQ sequences to be used in subsequent analysis steps. The Mothur software package (Schloss et al., 2009) was used to assign specific sequences to their respective nematode species of origin. Overlapping paired-end reads were joined in Mothur v.1.36.1 to create single contigs for analysis (Schloss et al., 2009). Sequences were removed if they were <200 bp or >450 bp, or if

there were ambiguities in the overlapping region. Sequences were then aligned in Mothur, using the default Needleman-wunsch algorithm, to a database of trichostrongylid nematode isotype-1 β-tubulin sequences generated from a collection of nematode species (database can be viewed in Supplementary Data S1; details can be viewed in Section 3.1). Sequences were removed if they did not align to at least 10% of any sequence with at least 60% similarity, in order to remove any non-isotype-1 β-tubulin sequences. The remaining sequences were compared with the isotype-1 β-tubulin database, and taxonomically classified using the k-nearest-neighbor method with $k = 3$. Sequences for which the three nearest matches were not from a single species, were then assessed at the next taxonomic level, until a consensus could be reached. Once all sequences were classified to the species level, a list of sequences belonging to each species, from each sample, was created. This list was used to divide the FASTQ sequences from each sample generated by PEAR, into individual FASTQ files from each species, from each sample. Sequences were divided into the following classifications if they were present in the sample: *C. oncophora, C. punctata, C. pectinata, C. curticei, H. contortus, H. placei, Te. circumcincta, O. ostertagi, Tr. axei, Tr. colubriformis* and *Tr. vitrinus*. Sequences that did not match any species (or were classified at a higher taxonomic level) were not considered further.

2.3.2. Identification of non-synonymous mutations in codons 167, 198 and 200

Consensus isotype-1 β-tubulin reference sequences with known susceptible haplotypes at codons 167, 198 and 200 were created for each species previously mentioned, and can be found in Supplementary Data S2. The FASTQ files for each species from each sample, were aligned to the respective susceptible reference isotype-1 β-tubulin sequence with the Burrows-Wheeler Aligner (BWA) v. 0,7.12-r1039, using default parameters (Li and Durbin, 2009). The resulting SAM file was converted to a BAM file and sorted with SAMtools version 0.1.19 (Li et al., 2009). Picard Tools version 1.139 (Broad Institute: https://broadinstitute.github.io/picard/) was used to add header and sample information back to the BAM file, which was lost in previous steps, and to create a reference sequence directory used for variant calling.

Variants for each species were called using VarDict version 1.5 (Lai et al., 2016), with "amplicon aware variant calling" activated, using a minimum frequency of 0.1% and a maximum allowed mismatches of 40 compared with the reference sequence. The number of mismatches was kept high, due to the large amount of sequence variation found within the intron. Identified variants were translated and annotated using the SnpEff bioinformatic tool (Cingolani et al., 2012). Variants were only assessed if at least 200 reads were present for the respective species. SnpSift was used to sort variants and keep only those with moderate to high effects (Cingolani et al., 2012). This step removes all variants resulting in synonymous changes and keeps only those with significant changes such as non-synonymous and frame-shift mutations. Only variants resulting in changes at codons 167, 198 and 200 were analyzed for the purposes of identifying the benzimidazole resistance-associated mutations described in this paper.

2.4. Isotype-1 β-tubulin sequence database and phylogenetic analyses

Consensus sequences were generated by aligning all sequences from each species contained within the isotype-1 β-tubulin sequence database (Supplementary Data S1) with Geneious v.10.1.3 (Biomatters Ltd, New Zealand) with the MUSCLE alignment tool, using default parameters. Details of the generation of the sequence database is presented in Section 3.1. Consensus sequences for each species were generated with a 75% threshold, which can be seen in Supplementary Data S2. These were then aligned with MUSCLE, and were used to produce sequence identities between consensus sequences for each species.

All sequences from the isotype-1 β-tubulin sequence database (Supplementary Data S1) were aligned with MUSCLE and a neighbor-joining tree was produced using the Geneious Tree Builder, using the Jukes-Cantor distance model, with the trimmed *C. elegans ben-1* gene (GenBank Accession number NM_065327.3) as an outgroup and 2000 bootstrap replicates.

2.5. Statistics

All statistics were calculated in IBM SPSS Statistics version 24. Lin's Concordance Correlation Coefficient was calculated in SPSS with the syntax provided by https://gjyp.nl/marta/Lin.sps. r_c values >0.8 are classified as a near perfect agreement ($r_c = 1$, would represent perfect agreement).

3. Results

3.1. Production of an isotype-1 β-tubulin reference sequence database for trichostrongylid nematodes from ruminants

Isotype-1 β-tubulin amplicons were generated, using the forward and reverse PCR primer mix, from DNA templates derived from ~1000 L3s for each single species population (including: *C. oncophora*, *C. punctata*, *C. curticei*, *O. ostertagi*, *H. contortus*, *H. placei*, *Tr. axei*, *Tr. colubriformis* and *N. helvetianus, N. battus*, *Te. circumcincta*, and *Tr. vitrinus*). In the case of *C. pectinata* and *H. similis*, sequences were derived from DNA from three morphologically identified adult male worms each. Several thousand sequences were generated for each species and 30 reference sequences were chosen to represent the spectrum of sequence diversity from each species. An *Oesophagostomum columbianum* sequence (GenBank Accession number KP792296.1) was also added to the database, being the only available reference sequence for this genus. This database can be viewed in Supplementary Data S1.

3.2. Isotype-1 β-tubulin intra- and inter-species sequence diversity

All sequences in the isotype-1 β-tubulin reference database were aligned by species using the MUSCLE alignment tool, and intra-species variation assessed. Pairwise identities of sequences within a species ranged between 84.3 and 100%. The lowest pairwise identity between any two sequences within each species can be observed in Table 1. As sequences were derived from exper-

Table 1
Lowest pairwise sequence identity observed within nematode species.

Species	Lowest pairwise sequence identity (%)	Species	Lowest pairwise sequence identity (%)
Cooperia curticei	95.1	*Nematodirus battus*	93.0
Cooperia pectinata	97.0	*Ostertagia ostertagi*	91.7
Cooperia punctata	92.1	*Teladorsagia circumcincta*	84.3
Cooperia oncophora	91.7	*Trichostrongylus axei*	88.2
Haemonchus contortus	92.5	*Trichostrongylus vitrinus*	93.5
Haemonchus placei	95.7	*Trichostrongylus colubriformis*	92.1
Haemonchus similis	97.4		
Nematodirus helvetianus	87.0		

imentally passaged isolates, or morphologically identified adults from a single isolate, the global intra-species diversity may be higher than estimated in this study.

Inter-species sequence identities ranged from 46.0 to 92.6% (Supplementary Table S3), with the intron, and 81.1–98.2% (Supplementary Table S4) without the intron. Percent identities of consensus sequences among species were generally lower than within species, however, occasionally sequences within a species had a lower pairwise sequence identity than those seen between the consensus sequences of different species within a genus. A neighbor-joining phylogenetic tree was generated for all sequences in the isotype-1 β-tubulin database (Fig. 1). All sequences form monophyletic clusters for each species, demonstrating that the isotype-1 β-tubulin sequences in the database can be taxonomically assigned to the correct species despite occasional overlap in the overall intra and inter-species percentage sequence identity.

3.3. Validation of deep amplicon sequencing for determining F167Y (TTC>TAC), E198L (GAA>TTA) and F200Y (TTC>TAC) isotype-1 β-tubulin polymorphism frequencies for H. contortus and Te. circumcincta using pyrosequence genotyping data

Previously, we used characterized populations of *H. contortus* and *Te. circumcincta* from seven UK sheep farms to assess the accuracy of the deep amplicon sequencing approach for determining the frequency of F167Y (TTC>T**A**C), E198L (GAA>**TT**A) and F200Y (TTC>T**A**C) isotype-1 β-tubulin polymorphisms (Redman et al., 2015). We had determined the F167Y (TTC>T**A**C), E198L (GAA>**TT**A) and F200Y (TTC>T**A**C) isotype-1 β-tubulin polymorphism frequencies in these populations using pyrosequence genotyping of 32 individual worms for each species on each of the seven farms (Redman et al., 2015). We deep-sequenced isotype-1

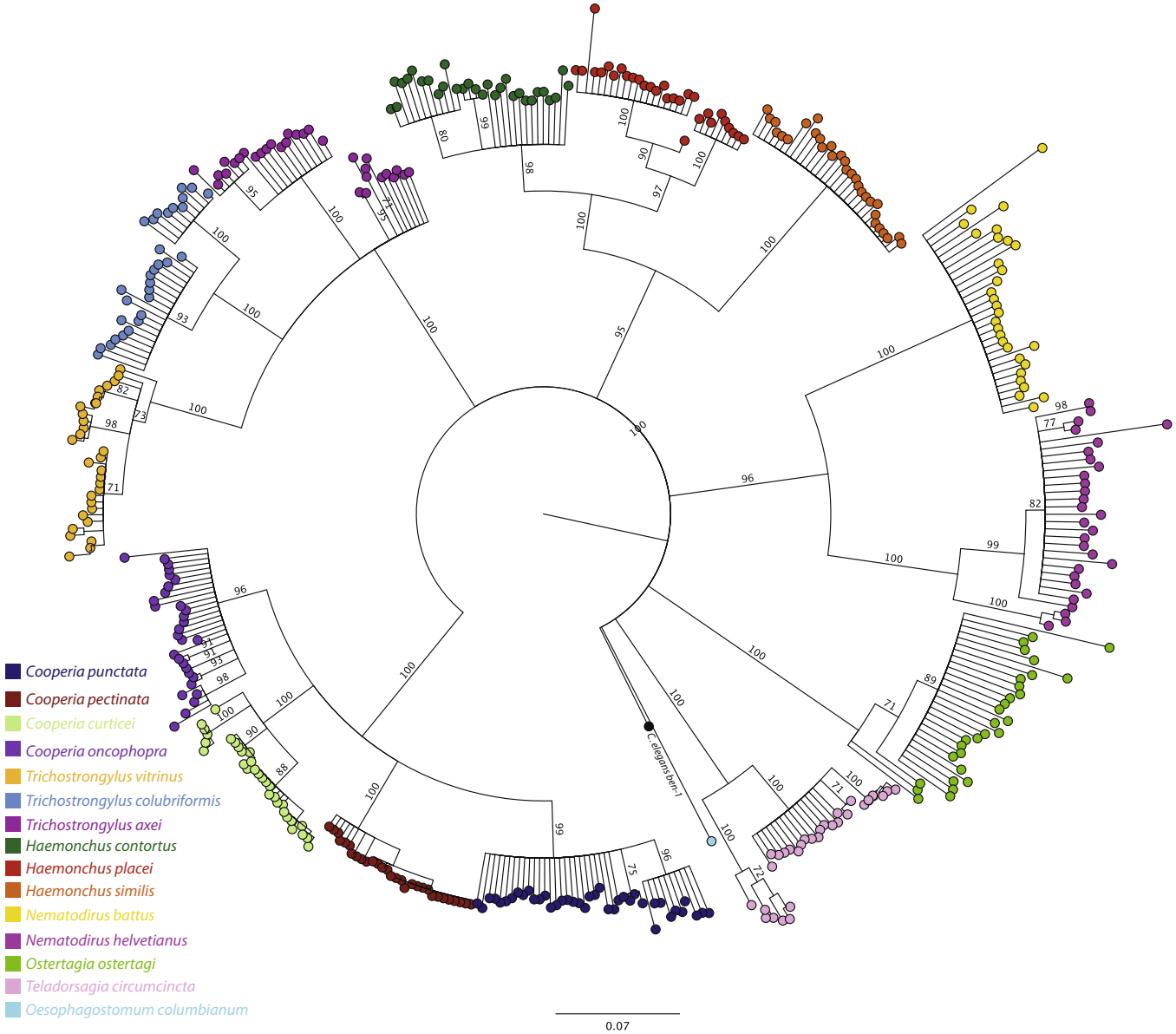

Fig. 1. Phylogenetic neighbor-joining tree of isotype-1 β-tubulin sequences from parasitic nematodes of ruminants. A neighbor-joining tree using all sequences in the isotype-1 β-tubulin database is shown. Alignments were performed using the Geneious MUSCLE Alignment tool and the neighbor-joining tree produced using the Geneious Tree Builder, using the Jukes-Cantor distance model, rooted on *Caenorhabditis elegans ben-1* GenBank Accession number: NM_065327) and 2000 bootstrap replicates. Bootstrap values >70 are shown. Each coloured node represents a single isotype-1 β-tubulin sequence. Species are colour coded and labeled; they are listed in the key in the order in which they appear in a clockwise direction.

β-tubulin amplicons generated from DNA templates prepared from a total of ~1000 L1s for each of the seven parasite populations. Between ~3000 and ~14,000 Illumina sequence reads for each of the two parasite species, from each sample, were used to determine the F167Y (TTC>T**A**C), E198L (GAA>**TT**A) and F200Y (TTC>T**A**C) isotype-1 β-tubulin polymorphism frequencies in each of the seven populations. These polymorphism frequencies were then compared with those previously determined by pyrosequence genotyping of individual worms (Fig. 2A and B). The frequencies of each polymorphism determined by the deep amplicon sequencing assay were very similar to those determined by individual worm pyrosequence genotyping (Fig. 2A and B), with the exception of *Te. circumcincta* from sample 95 for which there was a notable discrepancy. Consequently, pyrosequence genotyping of an additional 49 individual larvae that had been archived from this population was undertaken. This produced allele frequencies that were much more similar to those determined by the deep amplicon sequencing; 45% F200Y (TTC>T**A**C) SNP and 29% E198L (GAA>**TT**A) frequencies based on individual pyrosequence genotyping compare with 57.2% (F200Y (TTC>T**A**C)) and 38.8% E198L (GAA>**TT**A) for the deep amplicon sequencing (Fig. 2B). A scatterplot comparing the polymorphism frequencies determined by the two methods for both species combined is shown in Fig. 2C. Lin's Concordance Correlation Coefficient was calculated to assess the overall level of agreement between the two methods; $r_c = 0.983$; 95% Confidence Interval (CI): 0.968–0.991 (Kuei Lin, (1989)). This represents a very high level of agreement between the polymorphism frequencies determined by the deep-sequencing and individual larvae pyrosequence genotyping results.

3.4. Application of deep amplicon sequencing to determine the frequency of isotype-1 β-tubulin resistance associated polymorphisms for seven different nematode species on UK sheep farms

Populations of ~1000 trichostrongylid L1s derived from pooled fecal samples from ewes on 95 UK sheep farms, and from lambs on 69 UK sheep farms, were screened for isotype-1 β-tubulin polymorphisms at codons 167, 198 and 200 using the deep amplicon sequencing assay. Samples were collected from both ewes and lambs on 54 of the farms assessed, with the remainder having either ewe or lamb samples assessed. Between 1,118 and 37,553 (mean = 16,866) sequence reads were generated from each parasite population. Sequences mapped to *Te. circumcincta*, *H. contortus, Tr. axei, Tr. colubriformis*, *Tr. vitrinus, C. oncophora and C. curticei* isotype-1 β-tubulin reference sequences. *Teladorsagia circumcincta* isotype-1 β-tubulin sequences were identified in samples from ewes and lambs on all farms, consistent with its ubiquitous presence on UK sheep farms (Burgess et al., 2012) (Fig. 3A). There was a high frequency of the F200Y (TTC>T**A**C) polymorphism in the *Te. circumcincta* populations on nearly all farms surveyed (Fig. 3A). The mean frequency of this polymorphism was 67.53% for worms from ewes and 65.52% for those from lambs (Table 2). The E198L (GAA>**TT**A) polymorphism was detected at a low frequency on most farms (overall mean of 6.41% and 7.80% for ewes

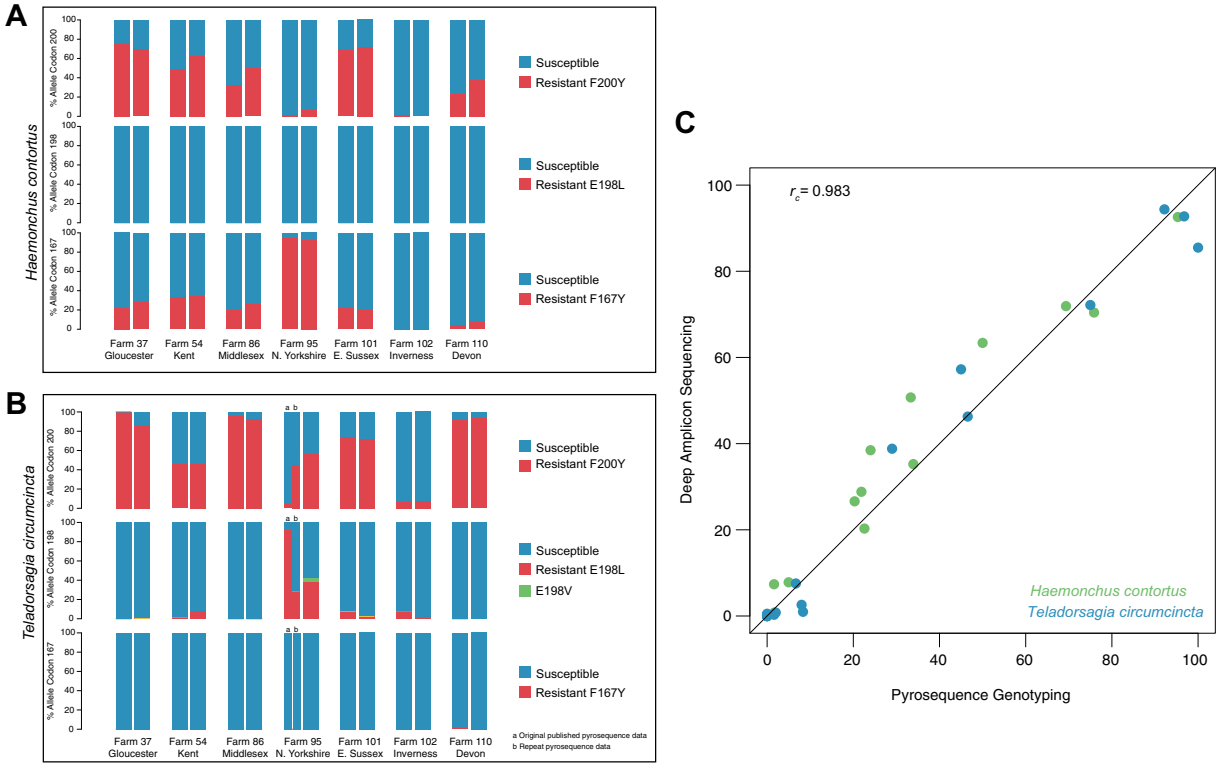

Fig. 2. Comparison of F167Y, E198L and F200Y isotype-1 β-tubulin polymorphism frequencies in *Haemonchus contortus* and *Teladorsagia circumcincta*, determined by deep amplicon sequencing with individual worm pyrosequence genotyping. (A) and (B) show the single nucleotide polymorphism (SNP) frequencies at codons 167, 198 and 200 of the isotype-1 β-tubulin determined by pyrosequence genotyping and deep amplicon sequencing of *H. contortus* and *Te. Circumcincta,* respectively, in each of seven UK farms. The left bar of each pair shows the results of pyrosequence genotyping of 32 individual worms in each population for the F167Y (TTC>T**A**C), E198L (GAA>**TT**A) and F200Y (TTC>T**A**C) SNPs. The right bar of each pair show results of the deep amplicon sequencing assay performed on pools of 1000 L1s. Given the discrepancy between the deep amplicon sequencing and the original pyrosequence genotyping data for *Te. circumcincta* from farm 95, the latter was repeated with an additional 49 larvae. The original published pyrosequence results are denoted with an 'a', while the repeat results are denoted with a 'b'. (C) A scatterplot of the estimated resistance allele frequency of the pyrosequence genotyping assay versus the deep amplicon sequencing assay. The newly generated results from farm 95 (*Te. circumcincta*) were included in this figure. A reference line has been drawn, indicating perfect agreement between the two assays. Lin's Concordance Correlation Coefficient was calculated, assessing the level of agreement between the two assays (0.983), which indicates a near perfect agreement between the two methods. N. Yorkshire, North Yorkshire, UK; E. Sussex, East Sussex, UK.

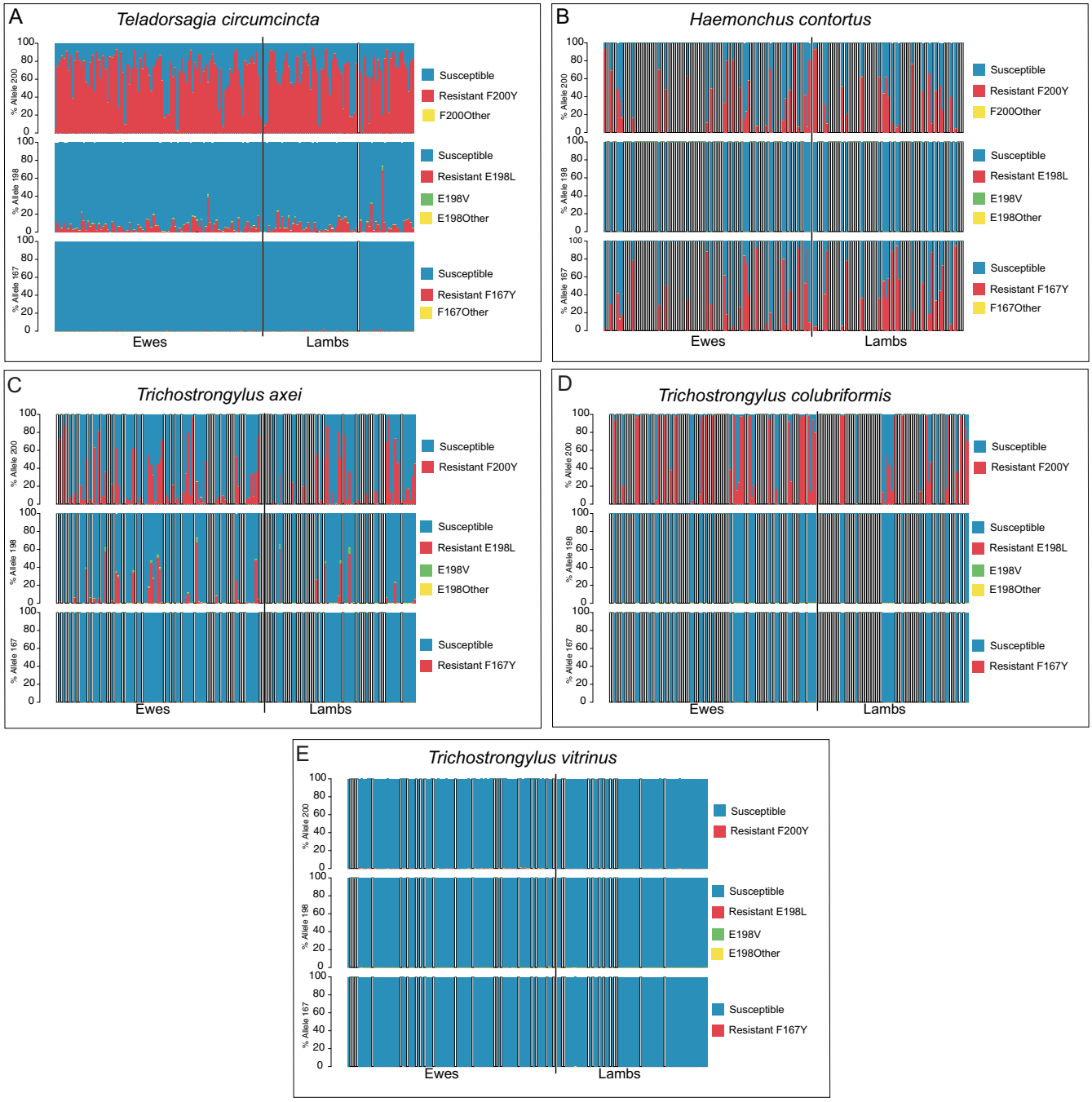

Fig. 3. The relative proportions of isotype-1 β-tubulin resistance allele frequencies on UK sheep farms for five different trichostrongylid nematode species. (A) *Teladorsagia circumcincta*; (B) *Haemonchus contortus*; (C) *Trichostrongylus axei*; (D) *Trichostrongylus colubriformis*; (E) *Trichostrongylus vitrinus*. Susceptible alleles (F167, E198 and F200) are shown in blue, while the previously described resistance-associated polymorphisms (F167Y (TTC>T**A**C), E198L (GAA>**TT**A) and F200Y (TTC>T**A**C)) are displayed in red. An E198V (GAA>G**T**A) polymorphism at codon 198 is displayed in green. All other identified non-synonymous polymorphisms were grouped together and are displayed in yellow. Populations for which either no isotype-1 β-tubulin sequences were found for that species, or there were fewer than 200 sequences identified, are indicated by a white bar on each chart.

and lambs, respectively) as was the E198V (GAA>G**T**A) polymorphism (0.52% and 0.60% in ewes and lambs, respectively (Fig. 3A; Table 2). The F167Y (TTC>T**A**C) mutation was only detected in *Te. circumcincta* at a very low level (overall mean of 0.17% and 0.25% in ewes and lambs, respectively) (Fig. 3A; Table 2).

Haemonchus contortus isotype-1 β-tubulin sequences were identified in 30/95 ewe and 23/69 lamb samples, consistent with the more sporadic prevalence of this parasite species on UK sheep farms (Burgess et al., 2012) (Fig. 3B). There was a high frequency of the F200Y (TTC>T**A**C) polymorphism in *H. contortus* on most of the farms on which this parasite was detected with an overall mean of 43.12% and 42.08% for ewes and lambs, respectively (Fig. 2B; Table 2). The E198A (GAA>G**C**A), E198L (GAA>**TT**A) and E198V (GAA>G**T**A) polymorphisms were not detected in any *H. contortus* population, but there was a high frequency of the F167Y (TTC>T**A**C) polymorphism on many farms with an overall mean of 41.08% and 48.99% for ewes and lambs, respectively (Fig. 2B and Table 2).

Trichostrongylus axei isotype-1 β-tubulin sequences were detected on most farms (59/95 for ewes and 39/69 for lambs)

Table 2
Mean resistance allele frequency by nematode species.

	Species	F200Y (%)	F200 Other (%)	E198L (%)	E198V (%)	E198 Other (%)	F167Y (%)	F167 Other (%)
Ewe	*Teladorsagia circumcincta*	67.53	0.00	6.41	0.52	0.01	0.17	0.01
	Haemonchus contortus	43.12	0.00	0.00	0.00	0.00	41.08	0.05
	Trichostrongylus axei	27.01	0.01	9.90	0.61	0.01	0.01	0.03
	Trichostrongylus colubriformis	61.67	0.01	0.00	0.00	0.00	0.01	0.00
	Trichostrongylus vitrinus	0.47	0.00	0.00	0.00	0.03	0.01	0.01
	Cooperia oncophora	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Cooperia curticei	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Lamb	*Teladorsagia circumcincta*	65.52	0.00	7.80	0.60	0.02	0.25	0.00
	Haemonchus contortus	42.08	0.00	0.00	0.00	0.00	48.99	0.00
	Trichostrongylus axei	26.03	0.00	7.09	0.56	0.02	0.01	0.02
	Trichostrongylus colubriformis	53.37	0.00	0.00	0.00	0.00	0.00	0.01
	Trichostrongylus vitrinus	0.14	0.01	0.00	0.00	0.01	0.01	0.01
	Cooperia oncophora	0.08	0.00	0.00	0.00	0.00	0.00	0.00
	Cooperia curticei	0.00	0.00	0.00	0.00	0.00	0.00	0.00

and the F200Y (TTC>T**A**C) polymorphism was detected at a variable frequency, which was high in some cases (overall mean of 27.01% and 26.03% for ewes and lambs, respectively) (Fig. 4A; Table 2). The E198L (GAA>**TT**A) polymorphism was also detected in *Tr. axei* at relatively high frequencies on some farms with an overall mean of 9.90% and 7.09% for ewes and lambs, respectively. The E198V (GAA>G**T**A) mutation was detected at a much lower frequency in *Tr. axei* (0.61% and 0.56% for ewes and lambs, respectively) and the F167Y (TTC>T**A**C) mutation was only detected at a very low frequency in a few *Tr. axei* populations (overall mean of 0.01% in both ewes and lambs) (Fig. 3C; Table 2).

Trichostrongylus colubriformis isotype-1 β-tubulin sequences were detected on many farms (37/95 for ewes and 21/69 for lambs) and the F200Y (TTC>T**A**C) polymorphism was detected at a high frequency in many cases with an overall mean of 61.67% and 53.37% for ewes and lambs, respectively (Fig. 3D; Table 2). No non-synonymous polymorphisms were detected at codon 198 in *Tr. colubriformis*, and the F167Y (TTC>T**A**C) polymorphism was only detected at a very low frequency (overall mean of 0.01% in ewes) (Fig. 3D; Table 2). *Trichostrongylus vitrinus* isotype-1 β-tubulin sequences were detected on most farms, but in contrast to the other parasite species, had only very low frequencies of the F200Y (TTC>T**A**C) polymorphism (overall mean frequency of 0.47% and 0.14% in ewes and lambs, respectively). The E198A (GAA>G**C**A), E198L (GAA>**TT**A) and E198V (GAA>G**T**A) polymorphisms were not identified in *Tr. vitrinus* on any farm (Fig. 3E and Table 2). However, the F167Y (TTC>T**A**C) polymorphism was detected at an extremely low frequency (overall mean of 0.01% in both ewes and lambs).

Cooperia oncophora was not identified on any of the ewe farms, but was identified on 5/69 lamb farms. The F200Y (TTC>T**A**C) was detected on only one farm at 0.38%, no non-synonymous polymorphisms were identified at position 167 or 198 on any farm (Table 2). Finally, *Cooperia curticei* sequences were detected on numerous farms (27/95 ewes and 17/69 lambs). However, the F167Y (TTC>T**A**C), polymorphism was only identified on a single ewe farm at 0.13%. The F200Y (TTC>T**A**C), E198V (GAA>G**T**A) and E198L (GAA>**TT**A) were not identified on any ewe or lamb farms (Table 2).

3.5. Comparison of benzimidazole resistance associated polymorphism frequencies in parasite populations from ewes and lambs from the same farms

We compared the frequency of each polymorphism for each species between ewes and lambs from the same farms. In total, there were 54 farms from which both ewe and lamb samples were collected and analyzed. On several of these farms, certain species were either not present, or there were too few reads (<200) for a species to obtain accurate polymorphism frequencies. In these cases, the samples were removed from the analysis, leaving only farms with an accurate percentage for both the ewes and lambs from a single farm for each species. Dot plots comparing the observed frequency for each polymorphism between the ewe and lamb samples are shown in Fig. 4. Lin's Concordance Correlation Coefficient was calculated, assessing the level of agreement between the ewe and lamb samples for each polymorphism for each species (Fig. 4). Overall, there was a very high level of agreement between the ewe and lamb samples from the same farm for all polymorphisms assessed. This provides further support for the accuracy and repeatability of the approach since the frequencies of resistance alleles for each parasite species are expected to be similar in animals co-grazing the same pastures.

4. Discussion

4.1. Development of a deep amplicon next-generation sequencing assay to scan for anthelmintic resistance mutations in parasitic nematode populations

In this paper, we describe a powerful new approach for screening parasitic nematode populations for the presence of polymorphisms associated with benzimidazole resistance. The only previous attempt to apply deep amplicon next-generation sequencing to detect anthelmintic resistance mutations was in a study of *Parascaris equorum* populations derived from equine fecal samples from 11 farms (Tydén et al., 2013). In that case, deep amplicon sequencing of the isotype-1 and isotype-2 β-tubulin genes did not reveal any of the known SNPs associated with benzimidazole resistance, so its utility in determining the frequency of resistance mutations could not be assessed (Tydén et al., 2013). Here, we have applied the approach on a much wider scale for gastrointestinal nematode parasites of sheep. This is an excellent system in which to test the proof-of-concept of deep amplicon sequencing for anthelmintic resistance polymorphism detection for a number of reasons. There are multiple species of the nematode superfamily Trichostrongyloidea that commonly occur as co-infections in sheep, and benzimidazole resistance is widespread for a number of these species in many regions of the world. Furthermore, polymorphisms in the isotype-1 β-tubulin gene in codons 167, 198 and 200, which are known to be associated with benzimidazole resistance, have been reported in one or more of these trichostrongylid nematode species of sheep (Redman et al., 2015). We have developed a set of variant primers that can be used in a single multiplex PCR to amplify a 300–350 bp fragment,

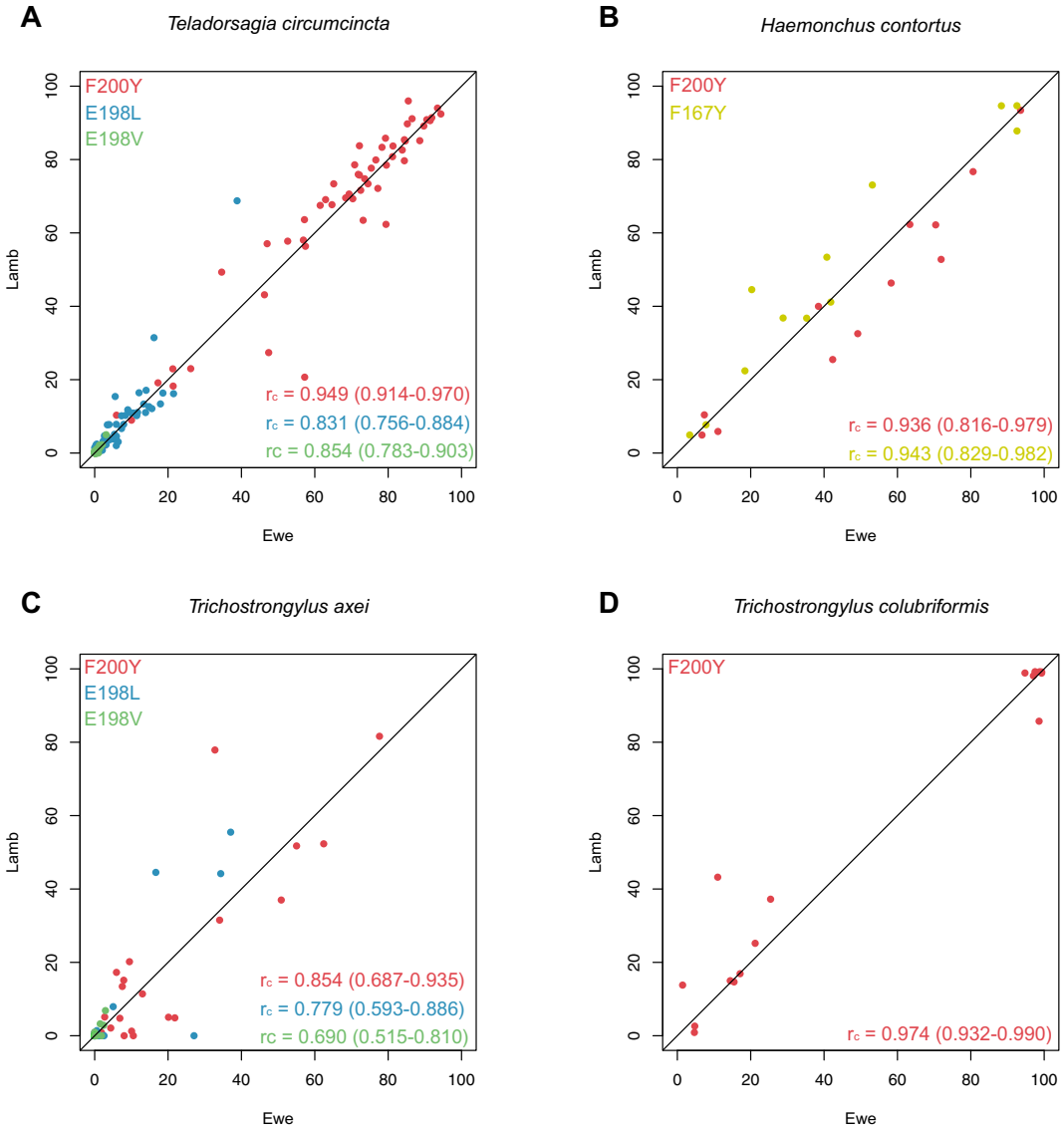

Fig. 4. Comparison of resistance allele frequencies in nematode parasite populations obtained from ewes and lambs from each of 54 UK sheep farms. Resistance allele frequency was compared between ewes and lambs on farms in which both ewes and lambs were sampled (54 farms total) for *Teladorsagia circumcincta* (A), *Haemonchus contortus* (B), *Trichostrongylus axei* (C), and *Trichostrongylus colubriformis* (D). On several of these farms, certain species were either not present, or there were too few reads (<200) for a species to obtain accurate single nucleotide polymorphism (SNP) frequencies. In these cases, the samples were removed from analysis, leaving only farms with an accurate percentage for both the ewes and lambs from a single farm for each species. Dot plots are shown comparing the observed frequencies of each polymorphism for each species between ewes and lambs. A reference line has been drawn, indicating perfect agreement between the ewe and lamb samples. The F200Y (TTC>T**A**C) polymorphism is displayed in red; E198L (GAA>**TT**A) in blue; E198V (GAA>G**T**A) in green; F167Y (TTC>T**A**C) in yellow. Lin's Concordance Correlation Coefficient was calculated, assessing the level of agreement between the ewe and lamb samples and is displayed with the same colour as the respective polymorphism, and 95% confidence intervals are in parentheses.

encompassing codons 167, 198 and 200 of the isotype-1 β-tubulin gene, from all of the trichostrongylid nematode species that commonly occur in domestic sheep. We then used this primer set in a deep amplicon sequencing assay to determine the frequency of non-synonymous polymorphisms in codons 167, 198 and 200 in parasite populations. In this assay, amplicons were generated from mixed species populations of L1s harvested from fecal samples and then sequenced at depth using the Illumina MiSeq platform. The resultant sequences were then bioinformatically assigned to their corresponding parasite species on the basis of sequence identity when compared against an isotype-1 β-tubulin reference sequence database. The frequencies of the polymorphisms of interest were then determined from the data for each separate species using a bioinformatic pipeline that we had developed.

There is a wide array of different variant calling bioinformatic tools that are available to use, and the choice of VarDict as the variant caller used for this application is a critical aspect of our bioinformatic pipeline. We also tested the use the Genome Analysis Tool Kit (GATK) UnifiedGenotyper, GATK HaplotypeCaller and Freebayes. However, these had significant drawbacks for this particular application. These variant callers were originally designed to call variants in whole genome datasets, which have a significant reduction in depth compared with amplicon data. Additionally, they use the assumption that the data is diploid in nature, meaning that variants should occur either at a frequency of 0%, 50%, or 100% in the samples, and, as such, try to fit the observations to this assumption. Given that these data have frequencies in the range of fractions of a percent, workarounds must be used to overcome

these assumptions. These workarounds tend to greatly inflate computational times of these other variant callers. Additionally, both of the GATK variant callers lacked the ability to call multiple nucleotide polymorphisms (MNPs), which as evidenced in this data set is crucial to calling some of the resistance variants observed in these samples (i.e. The E198L (GAA>**TT**A) polymorphism). Freebayes has the ability to call MNPs, but took orders of magnitude longer to run on these samples compared with VarDict and was no more accurate. Overall, VarDict was the most useful, as it had the ability to be run with "amplicon aware variant calling", which greatly reduces the time to analyze the data, as it does not try to fit the data to a set ploidy/frequency.

4.2. The deep amplicon next-generation sequencing assay provides accurate estimates of polymorphism frequencies in parasite populations

In the case of *H. contortus* and *Te. circumcincta*, we have shown that this deep amplicon sequencing assay provides an accurate estimate of the true polymorphism frequency in the parasite populations. We compared the polymorphism frequencies determined by the deep amplicon sequencing assay with those previously determined by pyrosequence-genotyping of 32 individual worms from the same parasite populations for seven of the farms. There was a very strong agreement between the two assays with $r_c = 0.983$ (a value of 1 indicates that the values are identical is every instance). The one sample for which there was a discrepancy appears to have been due to an inaccuracy in the original pyrosequence genotyping data.

Another indication of the accuracy of the data, overall, comes from the comparison of polymorphism frequencies between the parasite populations in ewes and lambs. One would predict that the resistance-associated polymorphism frequencies should be similar in ewe and lamb populations on the same farm as these two groups generally co-graze the same pastures and thus share the same parasite population. Although the species distributions could vary between ewes and lambs due to differences in host immunity, there is no obvious biological reason to expect major differences in isotype-1 β-tubulin polymorphism frequencies within a parasite species between co-grazing ewes and lambs. We found a high level of agreement between the polymorphism frequencies of ewes and lambs on the same farm for *H. contortus*, *Te. circumcincta*, *Tr. axei* and *Tr. colubriformis* (Fig. 4). This information supports the pyrosequence-genotyping validation experiments in suggesting that the deep amplicon sequencing assay provides a reasonably accurate estimate of polymorphism frequencies in the overall parasite population.

4.3. Benefits of deep amplicon sequencing over current methods of anthelmintic resistance polymorphism detection

This approach is a significant advance in the detection and monitoring of polymorphisms associated with anthelmintic resistance compared with current methods in terms of versatility, sensitivity and the scale of application. At present, the most commonly used approaches to detect polymorphisms associated with anthelmintic resistance are real-time PCR and pyrosequence genotyping but these methods have a number of limitations (Álvarez-Sánchez et al., 2005; Walsh et al., 2007). Real-time PCR assays need to be carefully optimized and validated for each individual polymorphism, making the development of assays that can screen for multiple polymorphisms in multiple species a challenging task. Similarly, a real-time PCR assay optimized in one laboratory can take significant time and effort to optimize and validate in a new laboratory, with each individual assay again needing separate validation. In addition, although real-time PCR is in principal a very sensitive technique, it has limited sensitivity in practice. Interpreting results when the target polymorphism frequency is very low and, consequently, the Ct values high, can be difficult and this ultimately limits the sensitivity of the assay. Pyrosequence-genotyping also requires considerable effort to develop a separate assay for each individual polymorphism of interest, in each parasite species, and there are significant limits to the sensitivity and scale of application of this approach. Pyrosequence-genotyping can be used in two ways. Firstly, it can be used to genotype individual parasites within a population (typically 30 or more individuals). This approach can provide accurate information regarding the frequency of the targeted mutation in the parasite population. However, the cost and labor involved precludes the use of individual worm genotyping to screen large numbers of populations for multiple mutations in multiple parasite species. Secondly, it can be applied to DNA from pools of parasites, allowing more populations to be screened. However, in this case, the sensitivity of detection is limited. We, and others, have previously shown that the detection of target mutations is only reliable when they are present at frequencies of higher than 5–10% in parasite populations (Chaudhry et al., 2015; Ramünke et al., 2016). Additionally, it is difficult or impossible to design primers specific enough to each species, resulting in genus-, rather than species-specific primers. These limitations are illustrated by the only previous large-scale study of benzimidazole resistance associated mutations at codons 167, 198 and 200 of the isotype-1 β-tubulin gene in ruminant parasites (Ramünke et al., 2016). This study was undertaken using a pyrosequence-genotyping assay using DNA from pooled L3s from fecal cultures (Ramünke et al., 2016). This study used a cut-off of 10% sensitivity for identification of the presence of resistance-associated polymorphisms due to the limits of sensitivity of the assay when used on pooled material (Ramünke et al., 2016).

The data in this paper illustrate some of the major benefits of deep amplicon sequencing for the screening of anthelmintic resistance mutations in parasitic nematode populations. A single multiplex PCR, followed by library preparation and a single Illumina MiSeq run simultaneously, screened for and determined the frequency of, three different benzimidazole resistance-associated polymorphisms in seven different nematode species in 164 parasite populations (95 from ewes and 69 from lambs). It would take 21 different, independently optimized and validated, real-time PCR polymorphism assays (and a total of 3,444 total real-time PCR assay reactions) to screen the same number of populations. The deep amplicon sequencing approach also allows the identification of individual species, while pyrosequencing assays are typically limited to a specific genus (Ramünke et al., 2016). The deep sequencing approach is also very sensitive, allowing the detection of mutations present at very low frequencies. In theory, the sensitivity of the technique is limited only by the number of worms used to make the DNA and the depth of sequence reads generated per sample. In this case, we used pools of 1000 L1s per farm and a read depth of ~16,000 reads per sample. In principal, this allows resistance mutations to be detected down to a frequency of 0.05% in a population. We applied a cut-off of 0.1% based on considerations of contamination such as cross-well contamination and barcode misidentification, which occur when using these technologies, in addition to PCR and sequencing induced errors (Avramenko et al., 2015). Additionally, the deep amplicon assay was able to distinguish the E198L (GAA>**TT**A) and E198V (GAA>G**T**A) mutations present. These were possibly missed in previous pyrosequence genotyping studies, as they occur at low frequencies in the population. The ability to reliably detect resistance mutations at low frequencies in populations will be an important tool to detect the emergence of resistance to a drug at

an early stage in its development. For example, developing equivalent assays for the human soil transmitted helminths has the potential to allow wide-scale monitoring for the emergence of benzimidazole resistance at the earliest stages.

Another advantage worth noting is that the deep amplicon sequencing approach has the potential to detect species and polymorphisms that are unanticipated in a sample, whereas the other current assays are limited to the detection of the particular species and polymorphisms for which they have been optimized. Additionally, any variant contained in the amplicon can be identified and assessed.

4.4. Application of the deep amplicon sequencing assay provides a wealth of new information on benzimidazole resistance-associated polymorphism frequencies in parasite populations of UK sheep

The deep sequencing assay was used to screen parasite populations in ewes from 95 farms and from lambs on 69 farms across the UK (with 54 ewe and lamb samples originating from the same farms). The pooling strategy was designed to give a comprehensive overview of the polymorphism frequencies in the parasite populations. Twenty fecal samples from either ewes or lambs were pooled and 1000 L1s, harvested from each pool, were used to prepare DNA templates. In all seven trichostrongylid species examined in the UK populations, the F200Y (TTC>T**A**C) mutation was at the highest frequency. This finding is consistent with previous studies in the UK (Skuce et al., 2010; Morrison et al., 2014; Redman et al., 2015) and other parts of the world that have been examined (Chaudhry et al., 2015; Ramünke et al., 2016; Zhang et al., 2016). However, there are major differences between the different parasite species in both the frequency of the F200Y (TTC>T**A**C) mutation and the relative frequency of mutations at the 167 and 198 codons. These are illustrated in Fig. 5.

In the case of *Te. circumcincta,* the F200Y (TTC>T**A**C) SNP was present at high frequency in the majority of parasite populations. Indeed, it was the highest of all seven species examined, suggesting that benzimidazole resistance is most advanced in this parasite species in sheep across the UK. In spite of this, the F167Y (TTC>T**A**C) SNP, which is the second most common benzimidazole resistance SNP reported in previous studies, was very rare in UK *Te. circumcincta* populations overall (0.17% ewes and 0.25% lambs), with the highest frequency on a single farm being 2.92%. We detected two SNPs in codon 198 - E198L (GAA>**TT**A) – in *Te. circumcincta* on the majority of UK sheep farms. Although at a relatively low frequency overall (6.41% in ewes and 7.80% in lambs), it was present at high frequency on a number of farms (68.76% in the highest case). This finding is consistent with that of a previous, smaller study of just seven UK sheep farms (Redman et al., 2015), where we reported this polymorphism at a high frequency (91.66%) in *Te. circumcincta* on one of the farms. This polymorphism has only been reported twice before in the literature (other than our previous UK study) (Choi et al., 2017; Keegan et al., 2017). In the former study, it was identified on one farm at 0.17% (Keegan et al., 2017). Although no functional studies have yet been performed on the E198L (GAA>**TT**A) genotype in nematodes, this substitution has been associated with a high level of benzimidazole resistance (carbendazim) in the fungus *Gibberella zeae* (see Liu et al., 2014). A fungal isolate with the E198L (GAA>**TT**A) polymorphism had a resistance factor of 52.16 compared with wild type (compared with resistance factors of between 10.58 and 27.61 for strains harboring the F200Y (TTC>T**A**C) polymorphism) (Liu et al., 2014). Previously, we also reported evidence of selection of this polymorphism based on neutrality tests of sequence data of the resistance haplotypes, suggesting a functional role in resistance (Redman et al., 2015). The deep amplicon sequencing also answered a question regarding this polymorphism that was raised in our previous study (Redman et al., 2015). The presence of an E to L substitution was surprising because it involved two SNPs compared with the susceptible allele at this codon (GAA>**TT**A). The deep amplicon sequencing, for which we have scanned for polymorphisms at much lower frequency and on a much wider scale, identified an E198V (GAA>G**T**A) genotype present in almost all the populations, in which the E198L (GAA>**TT**A) occurred. This V (G**T**A) codon is an intermediate step between the susceptible E (G**A**A) and the putative resistance associated L(**TT**A) codon, providing a potential explanation for the latter's occurrence. One hypothesis would be that the E198V (GAA>G**T**A) polymorphism provides a

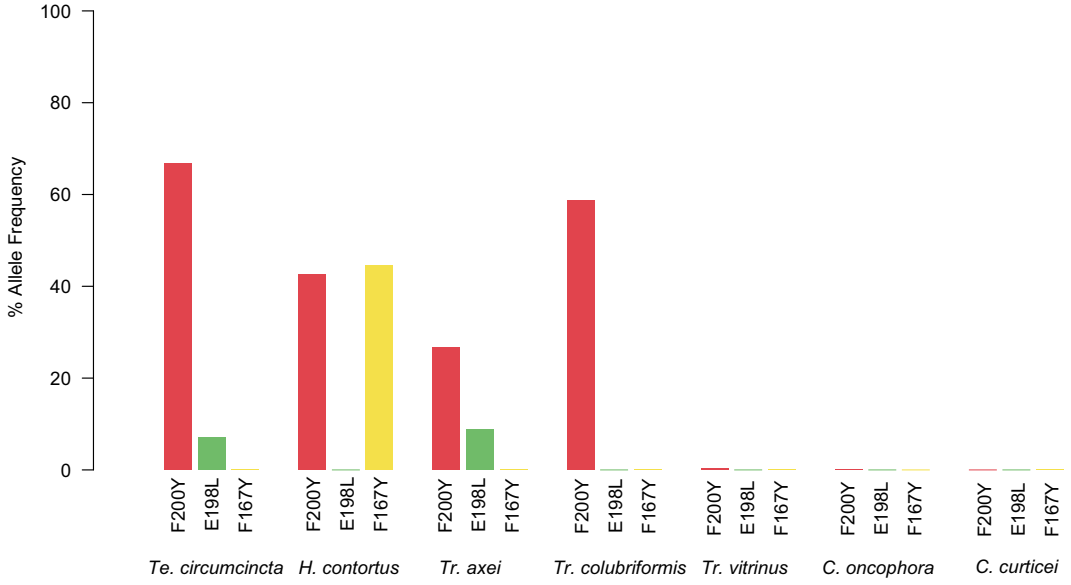

Fig. 5. Summary of the mean polymorphism frequencies at codons 167,198 and 200 for each nematode species across all UK sheep farms sampled. Mean polymorphism frequencies for F200Y (TTC>T**A**C) (Red), E198L (GAA>**TT**A) (Green) and F167Y (TTC>T**A**C) (Yellow) from both ewes and lambs from UK sheep farms are shown. Means for each nematode species are shown.

low level of resistance and the E198L (GAA>**TT**A) a higher level of resistance, allowing selection to occur and thus increase in frequency under drug selection pressure. The E198V (GAA>G**T**A) polymorphism has been identified as conferring levels of benzimidazole resistance in various fungal species (Albertini et al., 1999; Banno et al., 2008; Yang et al., 2015).

The results for *H. contortus* were notably different from those of *Te. circumcincta*. Although the F200Y (TTC>T**A**C) SNP was at the highest frequency overall (43.12% in ewes and 42.08% in lambs), F167Y (TTC>T**A**C) was present at almost as high a frequency (41.08% in ewes and 48.99% in lambs overall), which contrasts with the *Te. circumcincta* results on the same farms, where the F167Y (TTC>T**A**C) is very rare (Redman et al., 2015). Further, in the case of *H. contortus*, the reported polymorphisms at position 198 (E198A (GAA>G**C**A), E198V (GAA>G**T**A) or E198L (GAA>**TT**A)) were not found on any farm for *H. contortus*.

The three *Trichostrongylus* spp., although sampled from the same farms, again show striking differences from each other, in terms of the relative frequencies of polymorphisms at the three benzimidazole resistance-associated codons. The resistance polymorphism profile for *Tr. axei* was quite similar to *Te. circumcincta*, albeit less advanced. The F200Y (TTC>T**A**C) polymorphism was present at the highest frequency, with the F167Y (TTC>T**A**C) being present at extremely low frequencies overall (0.03 % ewes and 0.01% lambs). The E198L (GAA>**TT**A) was present at high frequencies on many farms with the E198V (GAA>G**T**A) almost always being present at a much lower frequency on those farms where the E198L (GAA>**TT**A) was present. In contrast, for *Tr. colubriformis*, although the F200Y (TTC>T**A**C) was present at high frequency on many farms (indeed at fixation on a number of farms) (61.67% ewes, 53.37% lambs), no polymorphisms were detected at codon 198 on any farm, and the F167Y (TTC>T**A**C) was only present at an extremely low frequency overall in ewes (0.01%). Strikingly, in the case of *Tr. vitrinus*, the F200Y (TTC>T**A**C) SNP was only present at a low frequency (0.47% ewes, 0.14% lambs), with the 198 polymorphisms being completely absent and F167Y (TTC>T**A**C) SNP being present at an extremely low frequency (0.01% ewes, 0.01% lambs), suggesting that benzimidazole resistance is yet to emerge in this parasite species.

A detailed discussion of the hypothesis behind the origins and spread of benzimidazole resistance mutations in parasite populations are beyond the scope of this paper and have been discussed elsewhere (Gilleard and Beech, 2007; Redman et al., 2015; Gilleard and Redman, 2016). However, it is worth highlighting a number of general points regarding the patterns of benzimidazole resistance-associated polymorphisms uncovered by this work. Although F200Y (TTC>T**A**C) is the most common benzimidazole resistance-associated SNP for all seven trichostrongylid species, its frequency varies greatly between each species (Table 2, Fig. 5). The benzimidazole resistance-associated polymorphisms at codons 198 and 167 show an even higher degree of variability between different species, being present at high frequencies in some species and being absent from others. This is interesting, given the seven parasite species were obtained from the same animals on the same farms and thus were subject to the same drug selection pressures and other epidemiological and management factors. Consequently, the differences in benzimidazole resistance between these parasite species are most likely due to differences in the biology of the specific parasites. Alternatively, predominance of parasites at particular times throughout the year might influence their exposure to anthelmintics, as a result of dosing schedules. As an example, *Te. circumcincta* emerges earlier in the season compared with *Trichostrongylus* spp. and is therefore likely to have greater exposure to drug, resulting in higher selection pressure. It is intriguing why benzimidazole resistance-associated polymorphisms appear to have not yet emerged in *Tr. vitrinus*, whilst at least one of them is present at high frequency in the other very closely related species isolated from the same hosts. One possible hypothesis is that the polymorphisms carry more of a fitness cost in *Tr. vitrinus* than the other species. Further work on both the molecular epidemiology of anthelmintic resistance and the functional biology of the relevant polymorphisms will be required to test this hypothesis.

We have developed a novel approach for scanning for benzimidazole resistance polymorphisms in parasitic nematode populations that is based on deep amplicon next–generation sequencing. This has many potential applications in both veterinary and human parasitology. The scalability of the approach, combined with its high sensitivity, make it powerful approach to screen for resistance-associated polymorphisms at the earliest stages of resistance emergence. Methods of early monitoring are urgently needed in order to direct the implementation of parasite control strategies that minimize the development and spread of resistance. One example of this critical need is in the screening of human soil transmitted helminth populations to investigate the suspected emergence of benzimidazole resistance due to mass drug administration programs. The approach also generates a huge amount of sequence data that can subsequently be used in molecular epidemiological investigations of the emergence and spread of resistance, as well as to assess and screen for other polymorphisms within the DNA fragment that might contribute to resistance.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.10.005.

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