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Short communication

A panel of microsatellite markers to discriminate and study interactions between *Haemonchus contortus* and *Haemonchus placei*

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ABSTRACT

Haemonchus contortus and *Haemonchus placei* are two closely related economically important parasites of ruminants. Their close morphological similarity, common occurrence as co-infections and ability to hybridize makes definitive diagnosis and epidemiological studies in field populations challenging. In this paper, we describe the development of a panel of microsatellite markers that can be used to discriminate and study the genetics of these two parasite species in co-infections and mixed field populations. We have identified two additional microsatellites (Hp52 and Hp53), in addition to three previously reported microsatellites (Hcms3561, Hcms53265 and Hcms36) that have a discrete set of alleles between the two species. Multilocus genotyping of worms with this 5 marker panel from 3 geographically diverse *H. placei* isolates and 4 geographically diverse *H. contortus* populations allows unambiguous species assignment of individual worms. This panel of markers should provide a valuable resource in studying the biology and epidemiology of these important ruminant parasite species in the field.

1. Introduction

Haemonchus contortus and *Haemonchus placei* species are economically important gastrointestinal nematodes of ruminants which cause serious negative impacts on animal welfare and productivity (O'Connor et al., 2006; van Dijk et al., 2010). These two species are extremely closely related and can hybridize to produce viable F_1 progeny which are fertile upon mating with parental species (Le Jambre, 1979, 1981). Although sheep and cattle are the respective preferred hosts, these parasites often co-infect the alternate host species and co-infections are common, particularly when cattle and sheep share the same pastures (Amarante et al., 1997; Chaudhry et al., 2015).

Attempts to use morphology and morphometrics to distinguish *Haemonchus* species, have encountered considerable issues with overlapping measurement ranges (Santos et al., 2014). Molecular techniques offer advantages over morphology for the identification of parasites, their genetic characterization, the isolation and characterization of expressed genes, and the detection of drug resistance and mutations (Gasser, 1999; Gasser et al., 2016). Species-specific microsatellite genotyping of populations of gastro-intestinal worms is well-established in the literature and represents a convenient technique to characterize strains of parasitic nematodes such as *H. contortus* and *Teladorsagia circumcincta* (Hoekstra et al., 1997; Redman et al., 2008a). The use of microsatellites to discriminate between *H. contortus* and *H. placei* individual worms and also identify hybrid worms was first described using a panel of three markers on parasite populations from Pakistan and India (Chaudhry et al., 2015). In this study, we build on this previous work by characterising two additional markers and validating the discriminatory power of the expanded marker panel on *H. placei* and *H. contortus* isolates from more diverse geographical regions.

2. Materials and methods

Our initial screening and characterization of the previous and new markers was performed on a *H. contortus* and a *H. placei* isolate from Sao Paulo, Brazil. The *H. contortus* isolate (SpHco2) was isolated from larvae from two male lambs raised on a farm in Sao Paulo state, Brazil naturally infected with gastrointestinal nematodes (GIN) in 2006, while the *H. placei* isolate (SpHpl1) larvae were isolated from a bovine naturally infected with GIN in Sao Paulo state in 2005. 25,000 infective larvae (L3s) of each isolate were used to infect a donor sheep (*H. contortus*, SpHco2) and a calf (*H. placei*, SpHpl1) to generate adult worms for this study. These donor animals (sheep and calf) were maintained indoors and, before being infected with the isolates, received monepantel

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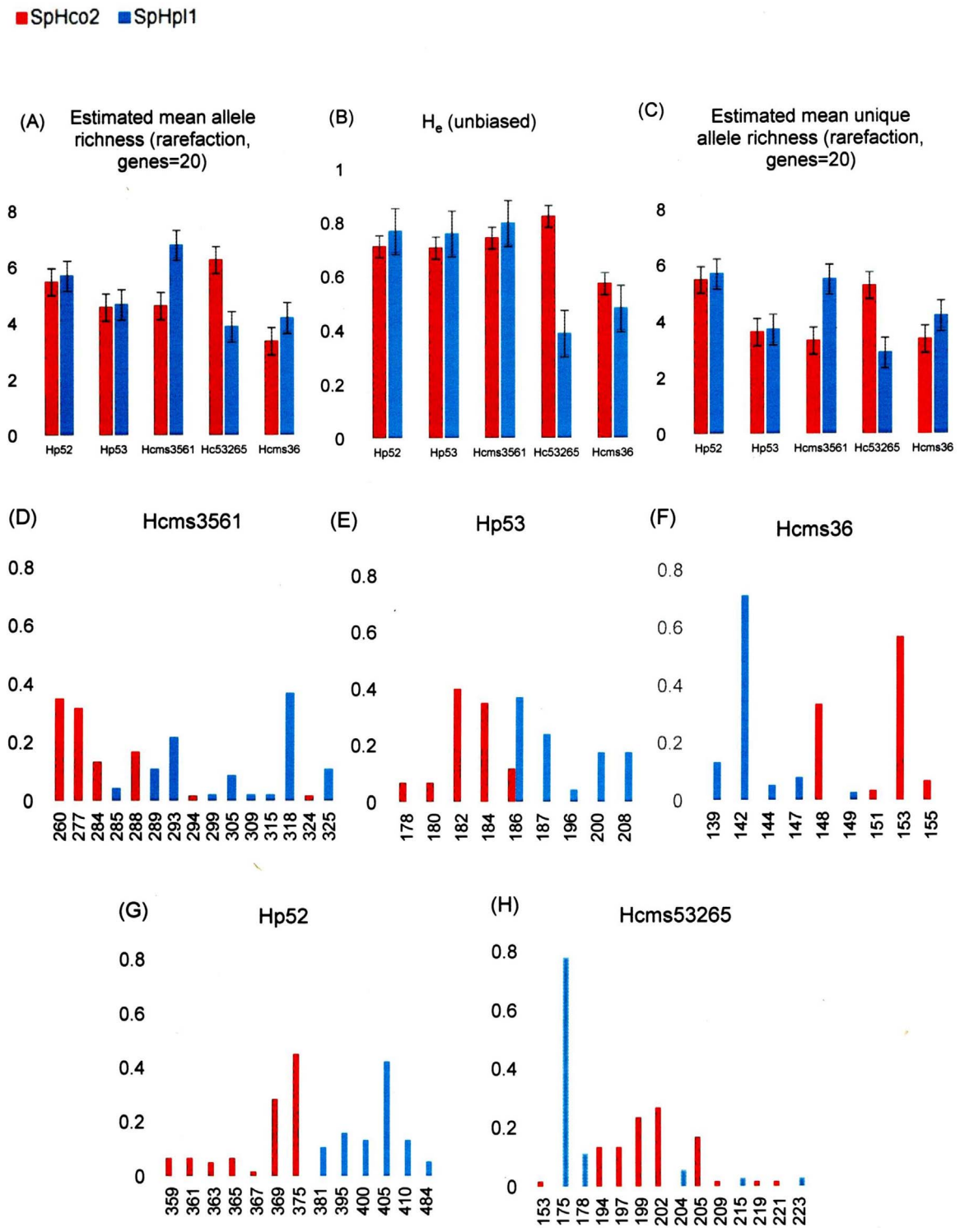

Fig. 1. Genetic diversity of *Haemonchus contortus* (SpHco2) and *Haemonchus placei* (SpHpl1) populations from Brazil based on microsatellite marker genotyping. In each chart Red = SpHco2 and Blue = SpHpl1. Panel A: The estimated mean allelic richness. Panel B: The unbiased expected heterozygosity ($H_{Eunbiased}$). Panel C: The estimated mean unique allele richness (C) are shown for each loci in each species. For Panels A-C: Error bars indicate the standard error of the mean. $H_{Eunbiased}$ was calculated with Arlequin ver 3.5.2 and both allelic richness and unique allele richness were estimated with rarefaction (genes = 20) using HP-Rare 1.1 to correct for unequal sample sizes. There were no statistical differences between species for any of these measures of genetic diversity when tested with the Wilcoxon rank sum test ($p = 0.05$). Panels D-H: Allele frequencies and ranges for each of the five microsatellite loci (Hcms3561, Hp53, Hcms36, Hp52 and Hcms53265 respectively on populations SpHco2(red) and SpHpl1 (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(2.5 mg/kg, Zolvix®, Novartis Animal Health) in a single oral dose to eliminate any natural nematode infection.

Their worm-free status was confirmed before the infection by a series of faecal examinations using a modified McMaster technique (Ueno and Gonçalves, 1998) and if no eggs were detected a more sensitive flotation method (modified Willis) was performed (Ueno and Gonçalves, 1998). Calf and sheep were kept in separate pens to avoid cross-contamination and, during the trial, they had free access to tap

Table 1
Summary statistics and AMOVA analysis for genotype data for the five microsatellites (Hcms3561, Hp53, Hp52, Hcms36 and Hcms53265) of *Haemonchus contortus* (SpHco2) and *Haemonchus placei* (SpHpl1) populations.

Loci	Hcms3561	Hp53	Hp52	Hcms36	Hcms53265
Haemonchus contortus					
Genotypes	30	30	30	30	30
Na	6	5	7	4	9
H_o	0.76667	0.66667	0.63333	0.53333	0.73333
H_e	0.74350	0.70678	0.71299	0.57175	0.82373
p-value	0.14048	0.84859	0.16947	0.43332	0.31930
s.d.	0.00025	0.00036	0.00037	0.00045	0.00047
Haemonchus placei					
Genotypes	23	23	19	19	18
Na	9	5	6	5	5
H_o	0.47826	0.56522	0.63158	0.52632	0.27778
H_e	0.79903	0.76039	0.76956	0.48080	0.38889
p-value	0.00003*	0.06515	0.22626	0.47369	0.02102*
s.d.	0.00001	0.00024	0.00045	0.00052	0.00011
AMOVA:					
% Variation among populations	19.65	24.42	25.83	46.85	36.01
% Variation among individuals within populations	13.41	11.30	10.50	0.62	9.78
% Variation within individuals	66.94	64.28	63.67	52.54	54.21
Fixation indices:					
F_{IS}	0.17	0.15	0.14	0.01	0.15
p-value	0.01271	0.05181	0.05376	0.51320	0.03226
F_{ST}	0.20	0.24	0.26	0.47	0.36
p-value	0	0	0	0	0
F_{IT}	0.33	0.36	0.36	0.47	0.46
p-value	0	0	0	0	0

Na: number of alleles, H_o: observed heterozygosity and H_e: expected heterozygosity.
* *p*-value < 0.05 indicate a significant deviation from Hardy–Weinberg equilibrium.
Global AMOVA results: average F-Statistics over all loci for fixation indices: inbreeding coefficient (F_{IS}): 0.12080, fixation index (F_{ST}): 0.30763 and overall fixation index (F_{IT}): 0.39819.

water and grass hay (*Cynodon dactylon* cv. Tifton 85 *ad libitum*) purchased from a farm with no ruminants, avoiding risks of food contamination by nematode L3. Faecal cultures were performed separately for the production of L3. The species identity were confirmed based on the length of the sheath tail extension using an ocular micrometer (Zeiss®), *H. placei* larvae usually have a sheath tail longer than 85 µm and *H. contortus* a sheath tail shorter than 85 µm (Santos et al., 2014). The donor animals were euthanized 106 and 161 days post-infection (DPI), for the sheep and calf respectively and adult worms recovered and immediately fixed in 70% ethanol. Adult male worms were morphologically identified, in accordance to the Manual of Veterinary Parasitology Laboratory Techniques using male tail and spicule morphology (MAFF, 1986), prior to DNA preparation. Individual adult males (n = 30 for SpHco2 and n = 23 for SpHpl1) were randomly chosen for detailed molecular analysis and following sharp dissection DNA lysates were prepared from the anterior half using previously described methods (Redman et al., 2008a). The rDNA ITS-2 sequence of SpHpl1 and SpHco2 adult worms were generated to confirm their identity, based on SNPs differences between the *Haemonchus* species (Chaudhry et al., 2015).

In addition to these Brazilian strains, another three *H. contortus* and two *H. placei* strains previously archived in the laboratory were used in this study. They originated from a wide range of geographical regions to explore the ability of the markers to discriminate species in different global genetic populations of these species. The *H. contortus* strains used were MHco3(ISE) (Scotland), MHco4(WRS) (S. Africa), MHco10(CAVR) (Australia). The *H. placei* strains were RAHpl2 (Argentina) and MHpl1 (Australia).

3. Microsatellites

To date only three microsatellite loci (Hcms36, Hcms3561 and Hcms53265) have been identified as population genetic loci for both *Haemonchus contortus* and *Haemonchus placei* (Chaudhry et al., 2015). These loci can be consistently amplified from both species, are polymorphic and yield distinct allele profiles. To expand this panel of microsatellite loci two different strategies were attempted. The first was to screen microsatellite loci already identified for *H. contortus,* for their ability to amplify *H. placei*. Seven *Haemonchus contortus* microsatellites located on the X-chromosome (Redman et al., 2008b) were tested for their ability to amplify *Haemonchus placei* individuals. Two (HcmsX142 and HcmsX182) of the seven loci, showed potential and were selected for further investigation. The second approach was to bioinformatically identify potential microsatellites that could, theoretically be amplified from both *H. placei* and *H. contortus,* based on their sequence alignments. Of the twelve loci that were identified in this manner, five loci (Hp102, Hp43, Hp35, Hp52 and Hp53) produced distinct allele ranges for the different species and were consequently chosen for further study (data not shown).

These seven microsatellite markers (HcmsX142, HcmsX182, Hp102, Hp43, Hp35, Hp52 and Hp53), that had shown potential for amplifying both species, were used to genotype the 23 and 30 individual adult worms from *H. contortus* (SpHco2) and *H. placei* (SpHpl1) respectively. Of these two loci, Hp52 and Hp53, consistently produced distinct, easily binned amplicons and were therefore chosen to add to the previously described markers (Hcms3561, Hcms36, Hcms53265) (data not shown).

The characteristics and primers pairs for Hcms3561, Hcms36, Hcms53265 have been previously described (Chaudhry et al., 2015). Details of the two new markers are as follows: Hp52 has a repeat sequence of $[AAAGA]_5$ AAAGG AAAGN $[AAAGA]_9$ and was amplified using primers 5′-GAGTGAGACATCGTAATCCA-3′ and 5′-GGTATCAAACTACTACTTGTC-3′ an annealing temperature of 54 °C and allele size range (340–470 bp). Hp53 has repeat sequence of $[ACAT]_7$ NACAT $[ACAT]_7$ and was amplified using primers 5′-TCGACTCTCACCCGAATACA-3′ and 5′-TTTGTGGCAGTTGGCGTT-3′at an annealing temperature of 54 °C and allele size range of 170–220 bp. PCR was performed in 20 µL using Qiagen® multiplex PCR kit (QIAGEN, Hilden, Germany) and 0.1 µM forward and reverse primers and 2 µL of lysate DNA. Thermocycling conditions evaluated were 95 °C for 15 min followed by 40

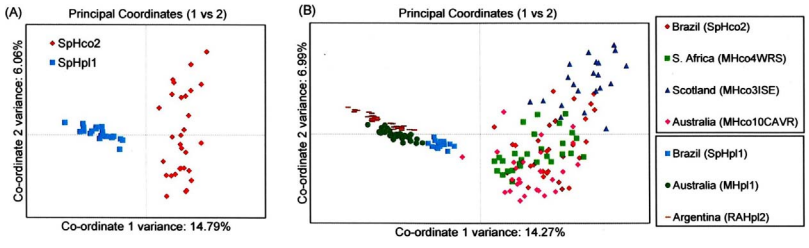

Fig. 2. Principle Component Analysis (PCA) of individual adult worm multi-locus genotypes based on five loci (Hcms53265, Hcms3561, Hp52, Hp53 and Hcms36). Panel A compares the SpHco1 (*Haemonchus contortus*) and SpHpl2 (*Haemonchus placei*) strains from Sao Paulo Satate, Brazil. Panel B compares geographically diverse isolates *H. contorus* and *H. placei* strains (4 isolates of *H. contortus* and 3 isolates of *H. placei*) including the two strains from Brazil (SpHco2 and SpHpl1). The country of origin for the strains is indicated in the legend. PCA was performed in GenAlEx version 6.5 with individual genotypes of the markers preserved; therefore each data point represents an individual worm. The percentage of variation explained by the first two coordinates is shown in the X- and Y-axes of the graph.

cycles of 94 °C for 30 s, 54 °C for 90 s and 72 °C for 30 s with a single final extension cycle of 72 °C for 15 min. The forward primer of each microsatellite primer pair was 5'end labelled with 6-FAM and HEX fluorescent dyes (MWG) and electrophoresed. Microsatellite PCR products by capillary electrophoresis were performed using GeneScan ROX 400 internal size standard was used on the ABI Prism 3100 genetic analyser (Applied Biosystems, USA®). Individual chromatograms were analysed using Peak Scanner™ software to determine the genotypes of each sample and each of these five molecular markers produced distinct genotypes with a single or double peak (homozygous or heterozygous) as predictable in a diploid organism.

The unbiased expected and observed heterozygosity (H_E and H_o) were calculated with Arlequin 3.5.2. (Excoffier and Lischer, 2010). Allele richness (A_c) and unique allele richness (Au_c) for each locus were estimated with rarefaction (genes = 20) using HP-Rare 1.1 (Kalinowski, 2005) to correct for unequal sample sizes. Differences in these measures of genetic diversity between populations were tested for statistical significance using a Wilcoxon rank sum test (Wilcoxon, 1945). Correction for variation in sample sizes was achieved by using the unbiased expected heterozygosity ($H_{Eunbias}$) and by estimating corrected allele richness values A_c. In Arlequin 3.5.2 (Excoffier and Lischer, 2010) Guo and Thompson (1992) exact test was used to statistically evaluate deviations from Hardy-Weinberg equilibrium using a Markov chain (10000 steps, $p = 0.05$) for all loci/population combinations. Partition of microsatellite diversity between and within populations was estimated through an analysis of molecular variance AMOVA with the data defined as 'standard' rather than 'microsatellite', as loci did not necessarily adhere to the stepwise mutation model. Pairwise F_{ST} values were calculated and significance testing was undertaken by random permutation in Arlequin 3.5.2. (Excoffier and Lischer, 2010). PCA was performed in GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012) with individual genotypes of the markers preserved; therefore each data point represents an individual worm.

4. Results

The mean allele richness was 5.05 for SpHpl1 and 4.86 for SpHco2 (Fig. 1A) and the mean expected heterozygosity was 0.6397 (± SD 0.19038) for SpHpl1 and 0.71175 (± SD 0.09107) for SpHco2 (Fig. 1B). There were no statistically significant differences in the overall genetic diversity, between the two species.

For the SpHpl1 strain, there were significant differences between observed (H_o) and expected (H_e) heterozygosity in two of the five loci; Hcms3561 (H_o/H_e 0.47826/0.79903,) and Hcms53265 (H_o/H_e 0.27778/0.38889). For SpHco2, there was no statistical differences ($p > 0.05$) in the other five loci tested (Table 1).

Both strains had a high number of unique alleles (Fig. 1C, mean unique allele richness, A_u). The allele ranges generated for the two species at these five loci were distinct (Fig. 1D–H), except the allele 186 bp (loci Hp53) that amplified from both species. This was reflected in a high pairwise F_{ST} estimate of 0.2922. Multilocus genotypes of the Brazilian *H. contortus* (SpHco2) n = 30 and *H. placei* (SpHpl1) n = 23 was showed in separately clusters on PCA plots (Fig. 2A). In addition, between 20 and 35 worms from additional *H. placei* isolates (RAHpl2 and MHpl1) and *H. contortus* isolates (MHco3(ISE), MHco4(WRS) and MHco10 (CAVR)) were genotyped (Fig. 2B). All three *H. placei* isolates clustered together and separately from all four *H. contortus* isolates on the PCA plots of the multilocus genotypes (Fig. 2, Panel B).

5. Discussion

Both strains had a high number of unique alleles indicating the potential power of these loci to discriminate between these two species. In fact there was only one allele out of all five loci that was amplified from both species; Hp53 allele 186 bp. Consequently the allele ranges generated for the two species at these five loci were distinct.

Multilocus genotypes of the SpHco2 (*H. contortus*) and SpHpl1 (*H. placei*) isolates formed completely separate clusters on PCA plots. In order to further test the ability of this five marker panel to unambiguously discriminate between *H. contortus* and *H. placei* two additional *H. placei* isolates; RAHpl2 and MHpl1 and three additional *H. contortus* isolates; MHco3(ISE), MHco4(WRS) and MHco10(CAVR) were genotyped. The *H. contortus* strains have been morphologically characterized and passaged for many years and the origins of these strains are geographically diverse; MHco3(ISE) Scotland, MHco4(WRS) South Africa, MHco10(CAVR) Australia (Redman et al., 2008a). The *H. placei* isolates are also geographically diverse; RAHpl2 is a field isolate derived from a calf on a farm in Rosario, Argentina and morphologically identified as *H. placei* (Dr. J. Cabaret, personal communication), MHpl1 is a morphologically characterized *H. placei* isolate originally derived from Australia (Le Jambre, 1979) and which has now been passaged by experimental infection at the Moredun Institute (Edinbugh, Scotland) for many years.

In conclusion, we describe a panel of five microsatellite markers (Hcms36, Hcms3561, Hp53, Hp52 and Hcms53265) that are able to unambiguously discriminate between individual worms of *H. contortus* and *H. placei* across very geographically diverse isolates.

Conflict of interest statement

The authors declare no conflict of interest.

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