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ORIGINAL PAPER

Population genetics of benzimidazole-resistant *Haemonchus contortus* and *Haemonchus placei* from buffalo and cattle: implications for the emergence and spread of resistance mutations

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Abstract
The population genetics of nematode parasites are poorly understood with practical reference to the selection and spread of anthelmintic resistance mutations. *Haemonchus* species are important to study the nematode population genetics due to their clinical importance in ruminant livestock, and the availability of genomic resources. In the present study, it has been examined that *Haemonchus contortus* and *Haemonchus placei* populations from three buffalo and nine cattle hosts. Seventy-three individual adult worms of *H. contortus* and 148 of *H. placei* were analysed using a panel of seven microsatellite markers. The number of alleles per locus in *H. contortus* and *H. placei* indicated that all populations were polymorphic for the microsatellites used in the present study. Genetic diversity parameters included high levels of allelic richness and heterozygosity, indicating effective population sizes, high mutation rates and high transmission frequencies in the area. Genetic structure parameters revealed low genetic differentiation between and high levels of genetic variation within *H. contortus* and *H. placei* populations. Population dynamic analyses showed an absence of heterozygosity excess in both species, suggesting that there was no deviation from genetic drift equilibrium. Our results provide a proof of concept for better understanding of the consequences of specific control strategies, climatic change or management strategies on the population genetics of anthelmintic resistance alleles in *Haemonchus* spp. infecting co-managed buffalo and cattle.

Keywords Anthelmintic resistance · Population genetics · Genetic diversity · Genetic structure

Introduction

High levels of genetic diversity have been described in parasitic nematodes of the superfamily Trichostrongyloidea, attributed to large effective population sizes and high mutation rates (Blouin et al. 1995; Gilleard and Beech 2007; Prichard 2001). Studies in small ruminants have reaffirmed the contribution of high parasitic population size to genetic diversity in *Haemonchus contortus* (Chaudhry and Gilleard 2015; Prichard 2001; Redman et al. 2015). A number of studies have investigated how population genetic structure is partitioned between parasitic nematodes (Archie and Ezenwa 2011; Redman et al. 2008; Redman et al. 2015; Silvestre et al. 2009; Troell et al. 2003). A previous study of the global population genetic structure of *H. contortus* in small ruminants revealed a high level of genetic differentiation between continents, with populations from each essentially forming monophyletic groups (Troell et al. 2003). Similarly, high levels of genetic divergence were found between laboratory strains of *H. contortus* derived from different part of the world (Redman et al. 2008). These results are not surprising because of the limited potential for gene flow among geographically isolated populations. Within the countries, an early study of *H.*

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contortus field isolates in the USA suggested that there was essentially no genetic differentiation within North America due to very large effective population sizes combined with high gene flow by animal movement (Blouin et al. 1995). However, subsequent studies using more discriminatory markers suggest that relatively low but significant levels of regional genetic differentiation occur between *H. contortus* populations in Australia, UK, France and Sweden (Hunt et al. 2008; Redman et al. 2015; Silvestre et al. 2009; Troell et al. 2003). The results from the UK in particular show that genetic differentiation exists, even where high levels of animal movement are known to occur (Redman et al. 2015).

Few studies have investigated the population dynamics of parasitic nematodes due to their complex epidemiology involving free living or parasitic stages and effects of climate, animal management and host responses (Yin et al. 2016). For example, in case of *H. contortus* in small ruminants, the vast majority of environmental stages die during cold winters in Sweden and the UK, or dry and hot periods in Australia. Consequently, parasite survival at these particular times of year is confined to adult worms and inhibited early 4th stage larvae in their hosts. Subsequently, there is significant potential for population bottlenecks, which may allow sufficient genetic drift to account for genetic structure in these regions.

To date, most population genetic analyses have been performed in *H. contortus* from sheep and goats (Brasil et al. 2012; Chaudhry et al. 2015a; Chaudhry et al. 2016; Hunt et al. 2008; Hussain et al. 2014; Redman et al. 2015; Silvestre et al. 2009; Yin et al. 2016). Despite the economic importance of *H. contortus* and *H. placei* in buffalo and cattle in many subtropical regions, the only population genetic studies in these hosts are based on rDNA ITS-2 and ND4 mitochondrial markers (Ali et al. 2014; Brasil et al. 2012; Hussain et al. 2014). Although these markers are informative, they are not always sufficiently variable to show the population genetics of the parasites. Here for the first time, we use a panel of seven microsatellite markers to show the population genetic structure of *H. contortus* and *H. placei* from buffalo and cattle in six different locations of the Punjab province of Pakistan.

Materials and methods

Field parasite samples

In the present study, several different regions were chosen from the Punjab province of Pakistan, where it was anticipated the prevalence of *Haemonchus* to be high. Adult *Haemonchus* spp. worms were obtained from the abomasa of three buffalo and nine cattle, immediately following slaughter at six different abattoirs (Lahore, Faisalabad, Sargodha, Sahiwal, Okara and Gujranwala). The numbers of *H. contortus* and *H. placei* collected from the three buffalo (Pop3B, Pop11B and Pop12B) and nine cattle (Pop1C, Pop2C, Pop4C, Pop5C, Pop6C, Pop7C, Pop8C, Pop9C, Pop10C) populations are described in Supplementary Table S1.

Genomic DNA extraction

Adult worms were fixed in 80% ethanol immediately following removal from the host abomasa. The heads of individual worms were dissected and lysed in single 0.5-uL tube containing 40 μl lysis buffer and proteinase K (10 mg/ml, New England BioLabs). One microliter of 1:5 dilution of neat single worm lysate was used as PCR template and identical dilutions of lysate buffer, made in parallel, were used as negative controls. Pooled lysates of each population were prepared using 1μl aliquots of each individual neat adult worm lysate. 1 μL of a 1:20 dilution of pooled lysates was used as PCR template (Chaudhry et al. 2016).

Pyrosequence specie-specific genotyping for the position 24 SNP of ITS-2 rDNA

Genotyping of the SNP at position 24 (P24) of the rDNA ITS-2 region was used to confirm the identity of *Haemonchus* spp. in buffalo and cattle parasite populations as previously described by Chaudhry et al. (2015b). Briefly, the rDNA ITS-2 region was amplified from individual *Haemonchus* adult worm lysates using a "universal" forward primer complementary to 5.8S rDNA coding sequence and biotin labelled reverse primer complimentary to the 28S rDNA coding sequence. Final PCR conditions were 1× thermopol reaction buffer, 2 mM $MgSO_4$, 100 μM dNTPs, 0.1 μM forward and reverse primers and 1.25 U Taq DNA polymerase (New England Biolabs). Thermo-cycling parameters were 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min with a single final extension cycle of 72 °C for 5 min. Following PCR amplification of rDNA ITS-2, the single nucleotide polymorphism (SNP) at P24 was determined by pyrosequence genotyping using the PryoMark ID system (Biotage, Sweden). The sequencing primer used was Hsq24 (5′- CATATACTACAATGTGGCTA-3′) and the nucleotide dispensation order was CGAGTCACA. Peak heights were measured using the SNP mode in the PSQ 96 single nucleotide position software (Biotage, Sweden). Worms were designated as *H. contortus*, *H. placei* or putative hybrids based on being homozygous A, homozygous G or heterozygous A/G, respectively at P24 (Chaudhry et al. 2015a).

Microsatellite genotyping of *H. contortus* and *H. placei* populations

Seven previously published microsatellite markers (Hcms3561, Hcms53265, Hcms36, Hpms43, Hpms52, Hpms53, Hpms102) were selected based on known polymorphism in *H. contortus*

and *H. placei* worms (Chaudhry et al. 2015a; Chaudhry et al. 2016; Santos et al. 2017). A summary of primers sequences and allele ranges are given in Supplementary Table S1. PCR amplification was performed using a 25μl master mix containing final concentrations of 1X thermopol reaction buffer, 2 mM $MgSO_4$, 100uM of each dNTP, 0.1μM forward and reverse primers and 1.25 U Taq DNA polymerase (New England Biolabs). Thermo-cycling parameters were 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s, 54 °C for 30 s and 72 °C for 1 min 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 fluorescent dye (IDT,Canada) and the GeneScan ROX 400 internal size standard was used on the ABI Prism 3100 genetic analyser (Applied Biosystems, USA). Individual chromatograms were analysed using Gene Mapper software version 4.0 for the accurate size of the amplicons and determine genotypes (Applied Biosystems, USA) previously described by Chaudhry et al. (2016).

The multi-locus microsatellite genotype data analysis was previously described by Chaudhry and Gilleard (2015). A log likelihood ratio test statistic (*G* test) was used to estimate the linkage equilibrium, using Arlequin 3.11 (Excoffier et al. 2005). Expected and observed heterozygosities (H_e and H_o) and allele variation (A_C) for each locus were calculated using Arlequin 3.11. An exact test was used to statistically evaluate deviations from Hardy-Weinberg equilibrium for all populations (Guo and Thompson 1992). Significance levels were adjusted using the sequential method of Bonferroni correction for multiple comparisons in the same dataset (Rice 1989). Analysis of molecular variance (AMOVA) was estimated through partition of genetic variation between and within populations (Excoffier et al. 1992). Fixation index (pairwise F_{ST}) values were calculated from the multi-locus microsatellite genotype data, by random permutation in Arlequin 3.11. Principal coordinate analysis (PCoA) was performed using GenALEx software preserving individual worm genotypes to plot coordinates of individuals (Peakall and Smouse 2012). The bottleneck software was used to assess any possible recent reduction in effective population size (Luikart and Cornuet 1998).

Results

H. contortus and *H. placei* co-infections are common in buffalo and cattle

Up to 20 individual worms from each host were pyrosequence genotyped for the rDNA ITS-2 P24 SNP (228 worms genotyped in total) (Supplementary Table S1). All worms were identified as *H. contortus* (homozygous A at rDNA ITS-2 P24) in population number Pop1C of cattle and Pop3B of buffalo and *H. placei* (homozygous G at rDNA ITS-2 P24) in population number Pop4C, Pop5C, Pop6C and Pop7C of cattle and Pop11B of buffalo. The remainder five populations (Pop2C, Pop8C, Pop9C, Pop10C and Pop12B) of the buffalo and cattle examined contained a mixture of *H. contortus* (homozygous A at rDNA ITS-2 P24) and *H. placei* (homozygous G at rDNA ITS-2 P24) indicating co-infection with the two species (Supplementary Table S1). Three of the individual hosts (two cattle and one buffalo) also each contained a single worm with a heterozygous A/G genotype at the rDNA ITS-2 P24 position, suggesting that they were *H. contortus*/*H. placei* hybrids.

Genetic diversity in *H. contortus* and *H. placei* populations

We selected five *H. contortus* populations (total 73 individual worms) and nine *H. placei* populations (total 148 individual worms) based on their P24 SNPs in the rDNA ITS-2 region. Seventy-three individual worms from *H. contortus* and 148 worms from *H. placei* populations were then genotyped using a panel of seven microsatellite markers (Supplementary Table S1). To measure the level of allelic variation in the populations, diversity indices were estimated in *H. contortus* and *H. placei*. All populations were polymorphic at all loci, with the overall number of alleles per locus (A) ranging from 2 to 11 in *H. contortus* and 2 to 10 in *H. placei*. A number of unique alleles (A_U) was observed in each population. Furthermore, *H. contortus* and *H. placei* showed high levels of mean allele richness (A_C) [Hc: 4.629 ± 0.521, Hp: 5.444 ± 0.413] (Table 1). The dataset was then used to measure genetic characteristics such as heterozygosity. In *H. contortus*, the observed heterozygosity (H_o) ranged from 0.125 to 0.937 and expected heterozygosity (H_e) ranged from 0.125 to 0.892. In *H. placei*, the H_o ranged from 0.213 to 0.909 and H_e ranged from 0.118 to 0.947. Overall, there was a high level of mean heterozygosity in both *H. contortus* and *H. placei* [(Hc: H_o 0.636/ H_e 0.626) (Hp: H_o 0.629/H_e 0.670)] (Table 1). Departure from Hardy-Weinberg equilibrium was used to assess genetic variability. There was some significant departure from Hardy-Weinberg equilibrium, even after Bonferroni correction, in addition to relative *P* values for 3 out of 35 loci combinations for *H. contortus* and 7 out of the 63 loci combination for *H. placei* populations (Table 1). In addition, H_o was less than the H_e and inbreeding coefficient (F_{IS}) values were high (Table 1). The presence of null alleles (N_0) for microsatellite loci has been previously reported and is likely associated for these departures from Hardy-Weinberg equilibrium high level in both species (Chaudhry et al. 2015a; Chaudhry et al. 2016). There was no evidence to support linkage disequilibrium for any combination of loci across all populations, indicating that alleles at these loci were randomly associating and not genetically linked. Overall results indicated high level of genetic diversity was attributed to a large population

Table 1 Population genetic data for each microsatellite from five *H. contortus* and nine *H. placei* populations based on panel of seven markers

H. contortus	Hc36 (31 [b])	Hpms43 (17 [b])	Hpms52 (22 [b])	Hpms53 (16 [b])	Hpms102 (34 [b])	Hcms3561 (24 [b])	Hc53265 (62 [b])	All loci
Pop1C (20[a])								
N_o	0	0	1	2	1	2	0	
H_e	0.74487	0.56795	0.64865	0.34127	0.69701	0.68413	0.87051	0.65063
H_o	1.00000	0.70000	0.68421	0.27778	0.57895	0.77778	0.90000	0.70267
p value	0.14984	0.56519	0.46149	0.26899	0.00026	0.17937	0.26287	
F_{IS}	− 0.35472	− 0.24009	− 0.05643	0.19048	0.17328	− 0.14149	− 0.03480	
A_U	6 (0)	4 (1)	4 (0)	3 (0)	6 (1)	3 (0)	8 (0)	4.857 [C]
Pop2C (16[a])								
N_o	0	0	1	4	0	0	0	
H_e	0.75806	0.27218	0.65517	0.15942	0.81048	0.57863	0.83065	0.58066
H_o	0.81250	0.31250	0.60000	0.16667	0.68750	0.75000	0.93750	0.60952
p value	0.49250	1.00000	0.66101	1.00000	0.00270	0.00277	0.90764	
F_{IS}	− 0.07438	− 0.15385	0.08696	− 0.04762	0.15601	− 0.30909	− 0.13350	
A_U	5 (0)	2 (0)	4 (0)	2 (0)	11 (6)	4 (0)	8 (0)	5.143 [C]
Pop3B (20[a])								
N_o	0	0	8	0	0	2	6	
H_e	0.77051	0.66154	0.63406	0.34359	0.69487	0.71270	0.85979	0.66815
H_o	0.85000	0.55000	0.66667	0.40000	0.70000	0.77778	0.92857	0.69615
p value	0.18131	0.01338	0.07325	1.00000	0.32706	0.98828	0.33936	
F_{IS}	− 0.10616	0.17228	− 0.05389	− 0.16923	− 0.00758	− 0.09425	− 0.08333	
A_U	5 (0)	3 (0)	4 (0)	3 (1)	6 (2)	5 (0)	9 (2)	5.000 [C]
Pop9C (8 [a])								
N_o	0	0	1	0	0	1	1	
H_e	0.81667	0.56667	0.47253	0.12500	0.45000	0.73626	0.83516	0.57176
H_o	0.50000	0.50000	0.28571	0.12500	0.37500	1.00000	1.00000	0.54082
p value	0.09344	0.08564	0.44389	1.00000	0.39140	0.12120	0.97897	
F_{IS}	0.40426	0.12500	0.41463	0.00000	0.17647	− 0.40000	− 0.21739	
A_U	5 (0)	3 (0)	3 (0)	2 (0)	5 (2)	4 (0)	6 (0)	4.000 [C]
Pop12B (9[a])								
N_o	2	1	4	2	1	3	1	
H_e	0.80220	0.63333	0.46667	0.38462	0.69167	0.75758	0.89167	0.66110
H_o	0.57143	0.75000	0.60000	0.42857	0.50000	0.83333	0.75000	0.63333
p value	0.20927	0.32012	1.00000	1.00000	0.03486	1.00000	0.45168	
F_{IS}	0.30435	− 0.20000	− 0.33333	− 0.12500	0.29114	− 0.11111	0.16832	
A_U	5 (0)	3 (0)	2 (0)	3 (0)	4 (0)	4 (0)	8 (0)	4.143 [C]
H. placei	Hc36 (25 [b])	Hpms43 (53 [b])	Hpms52 (69 [b])	Hpms53 (58 [b])	Hpms102 (66 [b])	Hcms3561 (53 [b])	Hc53265 (31 [b])	All loci
Pop4C (20[a])								
N_o	0	5	1	0	8	5	3	
H_e	0.70000	0.86667	0.94737	0.90000	058333	0.80000	0.11765	0.63784
H_o	0.46667	0.78161	0.87767	0.68974	0.70652	0.72874	0.21390	0.70215
p value	0.04267	0.09711	0.95855	0.34029	0.58604	0.94411	0.17921	
F_{IS}	− 0.52000	− 0.11315	− 0.08180	− 0.31538	0.18085	− 0.10164	0.45763	
A_U	2 (0)	7 (1)	9 (0)	7 (1)	5 (0)	5 (0)	2 (0)	5.286 [C]
Pop5C (20[a])								
N_o	0	12	3	6	13	11	5	
H_e	0.46667	0.83333	0.78610	0.63228	0.80220	0.73856	0.23908	0.64260
H_o	0.60000	0.87500	0.76471	0.50000	1.00000	0.66667	0.26667	0.66758

Table 1 (continued)

H. contortus	Hc36 (31 [b])	Hpms43 (17 [b])	Hpms52 (22 [b])	Hpms53 (16 [b])	Hpms102 (34 [b])	Hcms3561 (24 [b])	Hc53265 (62 [b])	All loci
p value	0.32735	0.87860	0.74365	0.01722	1.00000	0.69878	1.00000	
F_{IS}	− 0.29545	− 0.05376	0.02804	0.21552	− 0.27273	0.10280	− 0.12000	
A_U	2 (1)	6 (1)	10 (1)	5 (0)	7 (0)	4 (0)	20 (0)	5.143 [C]
Pop6C (8[a])								
N_o	1	1	0	1	2	0	1	
H_e	0.57500	0.59477	0.89153	0.73333	0.87879	0.70588	0.23333	0.65895
H_o	0.50000	0.33333	0.57243	0.75000	0.83333	0.44444	0.25695	0.49036
p value	0.66434	0.03911	0.01269	0.65715	0.76947	0.22638	0.06665	
F_{IS}	0.13846	0.45455	0.36778	−0.02439	0.05660	0.38462	1.00000	
A_U	3 (0)	4 (0)	10 (0)	6 (0)	6 (0)	5 (0)	2 (0)	5.143 [C]
Pop7C (20[a])								
N_o	0	1	6	4	5	8	0	
H_e	0.52254	0.79801	0.78307	0.69355	0.78851	0.78261	0.45641	0.68767
H_o	0.85000	0.78947	0.35714	0.56250	0.66667	0.83333	0.60000	0.66559
p value	0.00533	0.67662	0.00043	0.20411	0.01393	0.41396	1.31847	
F_{IS}	− 0.69110	0.01099	0.55326	0.19403	0.15916	− 0.06796	− 0.32558	
A_U	2 (0)	5 (0)	6 (0)	6 (0)	8 (0)	6 (0)	3 (0)	5.143 [C]
Pop8C (18[a])								
N_o	0	1	7	7	8	5	0	
H_e	0.50952	0.77718	0.83550	0.67965	0.75789	0.79077	0.43968	0.68432
H_o	0.50000	0.82353	0.50912	0.63636	0.50000	0.92308	0.44444	0.55976
p value	0.55953	1.00000	0.00000	0.11726	0.02062	0.72083	1.00000	
F_{IS}	0.01923	− 0.06161	0.89583	0.06667	0.35252	− 0.17551	− 0.01115	
A_U	3 (0)	6 (1)	7 (2)	4 (0)	6 (0)	7 (0)	4 (1)	5.286 [C]
Pop9C (12[a])								
N_o	0	1	1	3	1	1	0	
H_e	0.48913	0.71861	0.89610	0.84314	0.83983	0.83117	0.51948	0.73392
H_o	0.66667	0.81818	0.45455	0.77778	0.45455	0.90909	0.63636	0.67388
p value	0.63773	1.00000	0.00162	0.50537	0.00032	0.01318	1.0000	
F_{IS}	− 0.38583	− 0.14650	0.50495	0.08197	0.47090	− 0.09890	− 0.23894	
A_U	3 (0)	6 (0)	10 (1)	7 (1)	7 (0)	7 (0)	4 (1)	6.286 [C]
Pop10C (19[a])								
N_o	0	0	7	3	2	1	6	
H_e	0.56899	0.70413	0.73188	0.67540	0.76292	0.77619	0.45290	0.66749
H_o	0.84211	0.94737	0.41667	0.75000	0.58824	0.88889	0.58333	0.71666
p value	0.02296	0.13148	0.00433	0.68492	0.05477	0.57634	0.63338	
F_{IS}	− 0.50000	− 0.35849	0.44162	− 0.11455	0.23445	− 0.15011	− 0.30508	
A_U	3 (0)	5 (0)	8 (0)	7 (0)	6 (0)	8 (0)	3 (0)	5.714 [C]
Pop11B (20[a])								
N_o	0	0	7	1	0	10	1	
H_e	0.52179	0.71282	0.83385	0.79232	0.78462	0.71053	0.24751	0.65763
H_o	0.70000	0.70000	0.30769	0.73684	0.60000	0.80000	0.26316	0.58681
p value	0.09606	0.67413	0.00001	0.35896	0.00120	0.94949	1.00000	
F_{IS}	− 0.35369	0.01845	0.64045	0.07182	0.24000	− 0.13386	− 0.06509	
A_U	3 (0)	6 (0)	6 (0)	7 (0)	9 (0)	5 (0)	5 (2)	5.857 [C]
Pop12B (11[a])								
N_o	0	0	0	0	1	7	0	
H_e	0.48485	0.70563	0.86147	0.84948	0.83158	0.71429	0.17749	0.66054

Table 1 (continued)

H. contortus	Hc36 (31 [b])	Hpms43 (17 [b])	Hpms52 (22 [b])	Hpms53 (16 [b])	Hpms102 (34 [b])	Hcms3561 (24 [b])	Hc53265 (62 [b])	All loci
H_o	0.54545	1.00000	0.72727	0.90909	0.90000	0.84574	0.18182	0.60909
p value	1.00000	0.12833	0.28568	0.79616	0.97725	0.02889	1.00000	
F_{IS}	− 0.13208	− 0.44737	0.16230	− 0.07527	− 0.08725	1.00000	− 0.02564	
A_U	2 (0)	4 (0)	10 (1)	7 (0)	7 (0)	3 (0)	3 (0)	5.143 [C]

N_o, apparent null homozygotes, i.e., number of worms in the population which failed to give an amplification product for a particular marker; H_e, expected heterozygosity; H_o, observed heterozygosity; F_{IS}, inbreeding coefficient; *P* values indicate a significant deviation from Hardy–Weinberg equilibrium following bonferroni correction; *A*, number of alleles; A_U, unique alleles

[a] Total number of individuals genotyped for each population is given in parenthesis under the population name

[b] Total number of alleles for each marker across all populations is given in parenthesis below each marker name

[c] Mean number of alleles in each population for eight markers

size, high population densities, high mutation rates and high transmission frequencies in the area.

Genetic structure of *H. controtus* and *H. placei* populations

To measure the level of genetic differentiation, pairwise F_{ST} values were estimated in both populations. In *H. contortus*, these values showed relatively low levels of genetic differentiation ranging from 0.0078 to 0.0753. However, population groups Pop1C/Pop9C, Pop2C/Pop9C, Pop12B/Pop9C and Pop3B/Pop9C showed high F_{ST} values, ranging from 0.1522 to 0.2364 (Table 2). In *H. placei*, overall F_{ST} value also indicated a low level of genetic differentiation range from 0.0056 to 0.0521 (Table 2). Genetic differentiation was showed when the populations from each region were comprised with each other in a pairwise manner. The low level of genetic differentiation in *H. contortus* and *H. placei* indicates a high level of gene flow. AMOVA was conducted to estimate the genetic variation within and among the populations. In *H. contortus*, AMOVA showed that genetic variation was distributed 94% within populations and 7.20% among the populations. In *H. placei*, AMOVA showed that genetic variation was distributed 92% within population and 4.65% among the population. These results suggest a high rate of cross-mating and recombination within the parasite populations as a whole. To measure the level of genetic distance, PCoA was performed in *H. contortus* and *H. placei* and shown as a two-dimensional plot to illustrate

Table 2 Pairwise F_{ST} values based on genotyping individual worms from five *H. contortus* and nine *H. placei* populations with seven microsatellite markers

H. placei	Pop 4C	Pop5C	Pop6C	Pop7C	Pop8C	Pop9C	Pop10B	Pop11B
Pop5C	− 0.0256							
Pop6C	− 0.0382	− 0.0382						
Pop7C	0.0056	0.0069	− 0.0291					
Pop8C	− C0.0245	− 0.0245	− 0.0444	− 0.0057				
Pop9C	− 0.0284	− 0.0284	− 0.0449	0.0212	− 0.0249			
Pop10C	− 0.0081	− 0.0090	− 0.0486	0.0106	− 0.0134	− 0.0231		
Pop11B	− 0.0164	− 0.0164	− 0.0466	− 0.0168	− 0.0228	− 0.0135	− 0.0134	
Pop12B	− 0.0361	− 0.0361	− 0.0521	− 0.0106	− 0.0365	− 0.0368	− 0.0208	− 0.03052
H. contortus	Pop1C		Pop2C		Pop3B		Pop9C	
Pop2C	0.0078							
Pop3B	0.0534		0.0753					
Pop9C	0.1926		0.2364		0.1522			
Pop12B	−0.0096		0.0376		0.0200		0.1624	

the extent to which populations are genetically distinct. In both *H. contortus* and *H. placei*, the two axes accounted for 51.64% (28.31 + 23.33) and 48.79% (29.29 + 19.50) of the variation and showed that populations from different regions of Punjab formed overlapping clusters, hence were not geographically sub-structured (Fig. 1).

Population dynamics of *H. controtus* and *H. placei*

The population bottlenecking analysis of both *H. contortus* and *H. placei* showed that there was no heterozygosity excess according to the Sign Test and Wilcoxon Test (Table 3). The mode shift analysis established that all populations had a normal L-shaped distribution. The overall results suggest that population studied did not appear to deviate from genetic drift equilibrium.

Discussion

Anthelmintic resistance in *Haemonchus* spp. of large ruminants now represents a serious challenge to the livestock industry worldwide (Kaplan and Vidyashankar 2012). Hence, it is important to understand the population genetics of *H. contortus* and *H. placei* of large ruminants and their implications for the emergence and the spread of resistance mutations. The data reported in the present study reveal a high level of genetic diversity in *H. contortus* and *H. placei* from buffalo and cattle hosts in Pakistan. Similar results were seen in previous Indian and Pakistani studies of sheep and goats (Chaudhry et al. 2015a; Chaudhry et al. 2016). Our results differ from those of Blouin et al. (1995), Brasil et al. (2012) and Jacquiet et al. (1995), which showed higher genetic diversity in *H. contortus* than in *H. placei* in North America, Brazil,

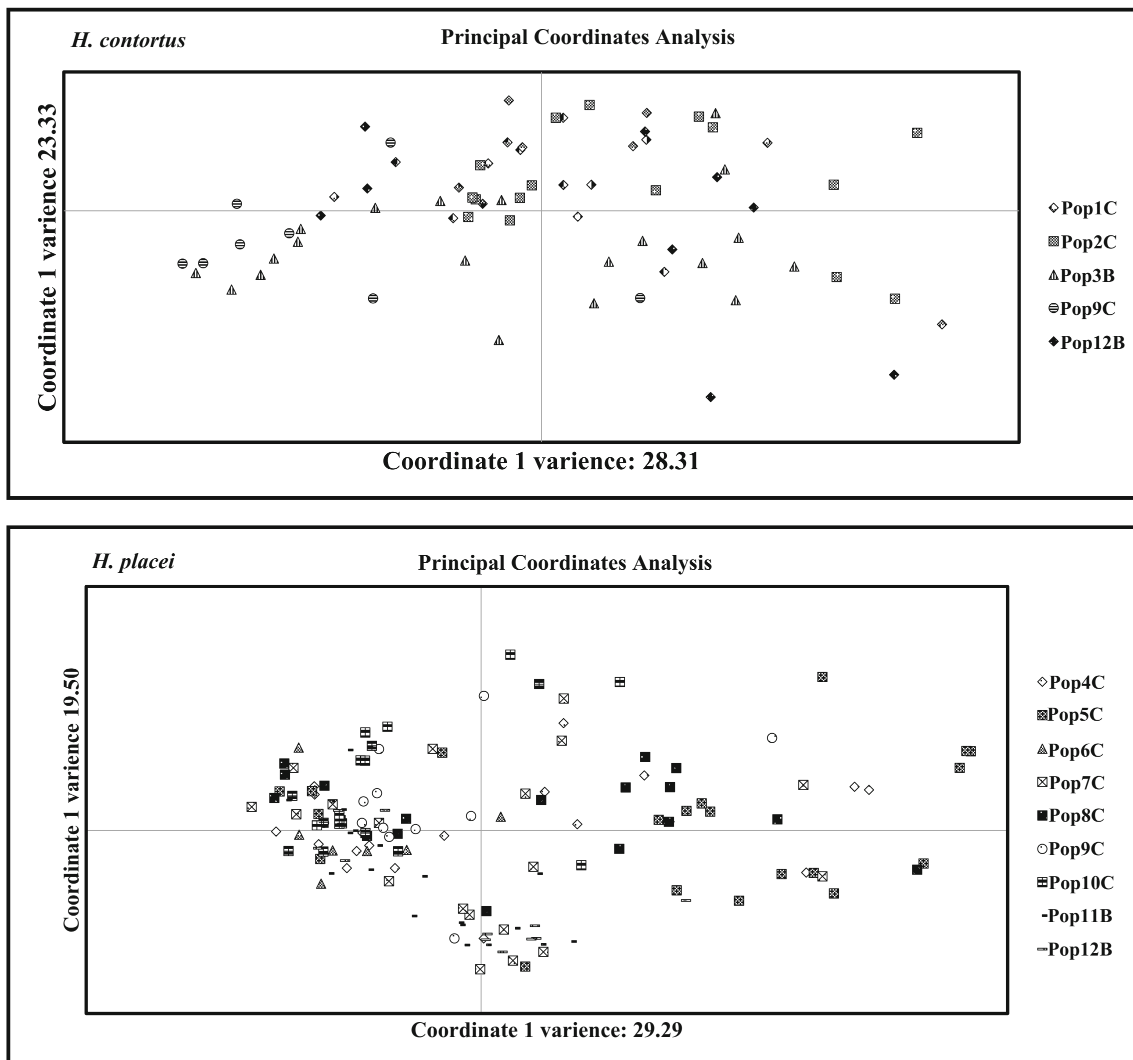

Fig. 1 Principal coordinate analysis represent five *H. contortus* and nine *H. placei* populations using seven microsatellite markers. Each data point represents an individual worm. Each population represents by different sketch

Table 3 Population bottleneck analysis of five *H. contortus* (*Hc*) and nine *H. placei* (*Hp*) populations with seven microsatellite markers

Populations	Sign test			Wilcoxon test			Mode shift
	IAM*	TPM*	SMM*	IAM*	TPM*	SMM*	
Pop1C (*Hc*)	0.124	0.131	0.301	0.015	0.039	0.937	Normal L-shape distribution
Pop2C (*Hc*)	0.307	0.329	0.142	0.468	0.687	0.375	Normal L-shape distribution
Pop3B (*Hc*)	0.132	0.133	0.277	0.015	0.023	0.812	Normal L-shape distribution
Pop9C (*Hc*)	0.638	0.607	0.626	0.937	1.000	0.687	Normal L-shape distribution
Pop12B (*Hc*)	0.147	0.146	0.133	0.054	0.109	0.296	Normal L-shape distribution
Pop1C (*Hp*)	0.312	0.327	0.366	0.039	0.039	0.812	Normal L-shape distribution
Pop5C (*Hp*)	0.642	0.643	0.394	0.468	0.937	0.578	Normal L-shape distribution
Pop6C (*Hp*)	0.597	0.638	0.234	0.375	0.578	0.687	Normal L-shape distribution
Pop7C (*Hp*)	0.016	0.355	0.328	0.007	0.039	0.937	Normal L-shape distribution
Pop8C (*Hp*)	0.141	0.144	0.587	0.039	0.039	0.578	Normal L-shape distribution
Pop9C (*Hp*)	0.401	0.411	0.101	0.054	0.078	0.109	Normal L-shape distribution
Pop10C (*Hp*)	0.383	0.403	0.021	0.578	0.812	0.054	Normal L-shape distribution
Pop11C (*Hp*)	0.376	0.388	0.098	0.375	0.468	0.296	Normal L-shape distribution
Pop12C (*Hp*)	0.386	0.299	0.621	0.369	0.468	1.000	Normal L-shape distribution

**IAM*, infinite allele model, *TPM*, two phase model, *SMM*, stepwise mutation model

and Mauritania. This may be due to different evolutionary rates or population demography (Brasil et al. 2012). The implication of the high level of genetic diversity on the emergence of benzimidazole resistance has been described by number of population genetic studies. The multiple time emergence of the F200Y (T**A**C) and F167Y (T**A**C) SNPs at the isotype 1 ß tubulin locus (Kwa et al. 1994) through either recurrent, or pre-existing mutations; and the single time emergence of the E198A (G**C**A) SNP at the same locus through a single mutation have been reported, associated with high levels of genetic diversity in *H. contortus* populations of small ruminants (Chaudhry et al. 2015a).

In the present study, genetic differentiation occurred in *H. contortus* and *H. placei* from Pakistani buffalo and cattle at low but significant levels. Similar findings have been described in Australia and the UK in *H. contortus* of small ruminants (Hunt et al. 2008; Redman et al. 2015). The consequences of low levels of population genetic structure in *H. contortus* and *H. placei* within the region, at least in part reflects high levels of animal movement (Blouin et al. 1995; Chaudhry et al. 2015a; Chaudhry et al. 2016; Hunt et al. 2008; Redman et al. 2015). Conversely, if the gene flow is high, the anthelmintic resistance mutations potentially spread within regions. Low levels of genetic variation within *H. contortus* and *H. placei* populations also imply that there is no reproductive isolation. Interestingly, the Pop9C population of *H. contortus* from Okara showed a high F_{st} value, which might be related to limited trade, brought about by known lower levels of communication and economic isolation, hence lower levels of animal movement compared to the other regions. Moreover, confounding environmental management, animal breed, host density and climatic conditions may also influence the epidemiological pattern of this population of *H. contortus*.

The free-living stages of parasitic nematodes are influenced by environmental factors, in particular temperature and humidity (O'Connor et al. 2006), which vary depending on geographical location and season, while the parasitic stages are influenced by host responses. The success of parasitic nematodes is due to their adaptation to these factors (Gilleard and Redman 2016). *Haemonchus* spp. are adapted to warm temperatures and plentiful precipitation, while dry, hot and cold weather is usually fatal for the free-living larval stages (Besier et al. 2016). Punjab is a subtropical region of Pakistan, where climatic conditions are warm with moderate precipitation, throughout the year, while winter temperatures are mild (between 10 and 20 °C) providing consistent opportunities for genetic exchange, transmission and dispersal in both *H. contortus* and *H. placei*. Our findings suggest that these conditions may have resulted in little population bottlenecking or genetic drift, potentially even for those parasite populations under drug selection and high gene flow.

Conclusions

This is the first report of the population genetic study of *H. contortus* and *H. placei* from buffalo and cattle hosts using a panel of microsatellite markers. The results of the low level of genetic differentiation among *H. contortus* and *H. placei* populations of Punjab, Pakistan, may be explained by large effective population sizes and low rates of genetic drift. Furthermore, limited animal movement associated with trade may have led to the reduction of gene flow and isolation of *H. contortus* and *H. placei* populations in this region.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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