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

Qasim Ali 1 · Imran Rashid 1 · Muhammad Zubair Shabbir 2 · Kashif Shahzad 3 · Kamran Ashraf 1 · Neil D. Sargison 4 · Umer Chaudhry 4

Received: 12 June 2018 / Accepted: 13 August 2018
© The Author(s) 2018

**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 individuals of *H. contortus* and 148 of *H. placei* worms 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.
**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 abomasum 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 abomasum. The heads of individual worms were dissected and lysed in single 0.5-μl tube containing 40 μl lysis buffer and protease 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 1 μl 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 complement to 5.8S rDNA coding sequence and biotin labelled reverse primer complement to the 28S rDNA coding sequence. Final PCR conditions were 1× thermapol reaction buffer, 2 mM MgSO4, 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 PyroMark 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 MgSO₄, 100μM 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*ₐ) 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 (*Aₐ*) ranging from 2 to 11 in *H. contortus* and 2 to 10 in *H. placei*. A number of unique alleles (*Aₐ*) was observed in each population. Furthermore, *H. contortus* and *H. placei* showed high levels of mean allele richness (*Aₐ*) ([*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 (*Hₒ*) ranged from 0.125 to 0.937 and expected heterozygosity (*Hₑ*) ranged from 0.125 to 0.892. In *H. placei*, the *Hₒ* ranged from 0.213 to 0.909 and *Hₑ* ranged from 0.118 to 0.947. Overall, there was a high level of mean heterozygosity in both *H. contortus* and *H. placei* ([*Hc*: *Hₒ* 0.636/ *Hₑ* 0.626] (Hp: *Hₒ* 0.629/*Hₑ* 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, *Hₒ* was less than the *Hₑ* and inbreeding coefficient (*Fₛₜ*) values were high (Table 1). The presence of null alleles (*Nₒ*) 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 |   |   |   |   |   |   |   |   |   |   |   |   |
|---|-------------|---|---|---|---|---|---|---|---|---|---|---|---|
|Pop1C (20a) |   |   |   |   |   |   |   |   |   |   |   |   |   |
|N_e | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 2 | 6 |   |   |   |
|H_e | 0.74487 | 0.56795 | 0.64865 | 0.34127 | 1.00000 | 0.70000 | 0.68413 | 0.69701 | 0.34127 | 0.67403 |   |   |   |
|p value | 0.14984 | 0.56519 | 0.46149 | 0.26899 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 | 0.00000 |   |   |   |
|FIS | −0.35472 | −0.24009 | −0.05643 | 0.19048 | −0.07438 | 0.15601 | −0.04762 | 0.15601 | −0.07438 | 0.15601 |   |   |   |
|A_U | 6 (0) | 4 (1) | 4 (0) | 3 (0) | 6 (1) | 3 (0) | 8 (0) | 4.857 C |   |   |   |   |   |
|Pop2C (16a) |   |   |   |   |   |   |   |   |   |   |   |   |   |
|N_e | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 2 | 6 |   |   |   |
|H_e | 0.75806 | 0.27218 | 0.65517 | 0.15942 | 0.81048 | 0.57863 | 0.83065 | 0.58066 |   |   |   |   |   |
|p value | 0.49250 | 1.00000 | 0.66101 | 1.00000 | 0.00277 | 0.00277 | 0.90764 |   |   |   |   |   |   |
|FIS | −0.07438 | −0.15385 | 0.08696 | −0.04762 | 0.15601 | 0.77778 | 0.77778 | 0.77778 |   |   |   |   |   |
|A_U | 5 (0) | 2 (0) | 4 (0) | 2 (0) | 11 (6) | 4 (0) | 8 (0) | 5.143 C |   |   |   |   |   |
|Pop3B (20a) |   |   |   |   |   |   |   |   |   |   |   |   |   |
|N_e | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 2 | 6 |   |   |   |
|H_e | 0.77051 | 0.66154 | 0.63406 | 0.34359 | 0.69487 | 0.71270 | 0.85979 | 0.66815 |   |   |   |   |   |
|p value | 0.18131 | 0.01338 | 0.07325 | 1.00000 | 0.32706 | 0.98828 | 0.33936 |   |   |   |   |   |   |
|FIS | −0.10616 | 0.17228 | 0.05389 | −0.05389 | 0.15601 | 0.00000 | 0.00000 | 0.00000 |   |   |   |   |   |
|A_U | 5 (0) | 3 (0) | 4 (0) | 3 (1) | 6 (2) | 5 (0) | 9 (2) | 5.000 C |   |   |   |   |   |
|Pop9C (8a) |   |   |   |   |   |   |   |   |   |   |   |   |   |
|N_e | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 2 | 6 |   |   |   |
|H_e | 0.81667 | 0.56667 | 0.47253 | 0.12500 | 0.45000 | 0.73626 | 0.83516 | 0.57176 |   |   |   |   |   |
|p value | 0.09344 | 0.08564 | 0.44389 | 0.00000 | 0.39140 | 0.12120 | 0.97897 |   |   |   |   |   |   |
|FIS | 0.40426 | 0.12500 | 0.41463 | 0.00000 | 0.17647 | 0.00000 | 0.00000 | 0.00000 |   |   |   |   |   |
|A_U | 5 (0) | 3 (0) | 3 (0) | 2 (0) | 5 (2) | 4 (0) | 6 (0) | 4.000 C |   |   |   |   |   |
|Pop12B (9a) |   |   |   |   |   |   |   |   |   |   |   |   |   |
|N_e | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 2 | 6 |   |   |   |
|H_e | 0.80220 | 0.63333 | 0.46667 | 0.38462 | 0.69167 | 0.75758 | 0.89167 | 0.66110 |   |   |   |   |   |
|p value | 0.57143 | 0.75000 | 0.42587 | 0.50000 | 0.83333 | 0.75000 | 0.63333 |   |   |   |   |   |   |
|FIS | 0.30435 | −0.20000 | −0.33333 | −0.12500 | 0.29114 | −0.11111 | 0.16832 |   |   |   |   |   |   |
|A_U | 5 (0) | 3 (0) | 3 (0) | 2 (0) | 5 (2) | 4 (0) | 6 (0) | 4.000 C |   |   |   |   |   |

|   | H. placei |   |   |   |   |   |   |   |   |   |   |   |   |
|---|-------------|---|---|---|---|---|---|---|---|---|---|---|---|
|Pop1C (20a) | Hc36 | Hpms43 | Hpms52 | Hpms53 | Hpms102 | Hcms3561 | Hc53265 | All loci |   |   |   |   |   |
|N_e | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 2 | 6 |   |   |   |
|H_e | 0.70000 | 0.86667 | 0.89373 | 0.90000 | 0.85333 | 0.80000 | 0.11765 | 0.66110 |   |   |   |   |   |
|p value | 0.46667 | 0.78161 | 0.87767 | 0.68974 | 0.50000 | 0.50000 | 0.50000 | 0.50000 |   |   |   |   |   |
|FIS | −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 |   |   |   |   |   |

---

Parasitol Res
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<sub>IS</sub> | −0.29545  | −0.05376      | 0.02804       | 0.21552       | −0.27273      | 0.10280        | −0.01200       |         |
| A<sub>U</sub>  | 2 (1)      | 6 (1)         | 10 (1)        | 5 (0)         | 7 (0)         | 4 (0)          | 20 (0)         |         |
| Pop6C (8ª)    |            |               |               |               |               |                |                |         |
| N<sub>e</sub> | 1           | 1             | 0             | 1             | 2             | 0              | 1              |         |
| H<sub>e</sub> | 0.57500     | 0.59477       | 0.89153       | 0.73333       | 0.78879       | 0.70588        | 0.23333        |         |
| p value      | 0.66434    | 0.03911       | 0.01269       | 0.65715       | 0.76947       | 0.22638        | 0.06665        | 0.65895 |
| F<sub>IS</sub> | 0.13846    | 0.45455       | 0.36778       | −0.02439      | 0.05660       | 0.38462        | 1.00000        |         |
| A<sub>U</sub>  | 3 (0)       | 4 (0)         | 10 (0)        | 6 (0)         | 6 (0)         | 5 (0)          | 2 (0)          | 5.143 C |
| Pop7C (20ª)    |            |               |               |               |               |                |                |         |
| N<sub>e</sub> | 0           | 1             | 6             | 4             | 5             | 8              | 0              |         |
| H<sub>e</sub> | 0.52254     | 0.79801       | 0.78307       | 0.69355       | 0.78851       | 0.78261        | 0.45641        | 0.68767 |
| p value      | 0.00533    | 0.67662       | 0.00043       | 0.20411       | 0.01393       | 0.41396        | 1.31847        | 0.66559 |
| F<sub>IS</sub> | 0.1923     | 0.01099       | 0.55326       | 0.19403       | 0.15916       | −0.06796       | −0.32558       |         |
| A<sub>U</sub>  | 2 (0)       | 5 (0)         | 6 (0)         | 6 (0)         | 8 (0)         | 6 (0)          | 3 (0)          | 5.143 C |
| Pop8C (18ª)    |            |               |               |               |               |                |                |         |
| N<sub>e</sub> | 0           | 1             | 7             | 7             | 8             | 5              | 0              |         |
| H<sub>e</sub> | 0.50952     | 0.77718       | 0.83550       | 0.67965       | 0.75789       | 0.79077        | 0.43968        | 0.68432 |
| p value      | 0.59535    | 1.00000       | 0.00043       | 0.11726       | 0.02062       | 0.72083        | 1.00000        | 0.55976 |
| F<sub>IS</sub> | 0.01923    | −0.06161      | 0.89583       | 0.06667       | 0.35252       | −0.17551       | −0.01115       |         |
| A<sub>U</sub>  | 3 (0)       | 6 (1)         | 7 (2)         | 4 (0)         | 6 (0)         | 7 (0)          | 4 (1)          | 5.286 C |
| Pop9C (12ª)    |            |               |               |               |               |                |                |         |
| N<sub>e</sub> | 0           | 1             | 1             | 3             | 1             | 1              | 1              |         |
| H<sub>e</sub> | 0.48913     | 0.71861       | 0.89610       | 0.84314       | 0.83983       | 0.83117        | 0.51948        | 0.73392 |
| p value      | 0.63773    | 1.00000       | 0.00162       | 0.50537       | 0.00032       | 0.01318        | 1.00000        | 0.67388 |
| F<sub>IS</sub> | −0.38583   | −0.14650      | 0.50495       | 0.08197       | 0.47090       | −0.09890       | −0.23894       |         |
| A<sub>U</sub>  | 3 (0)       | 6 (0)         | 10 (1)        | 7 (1)         | 7 (0)         | 7 (0)          | 4 (1)          | 6.286 C |
| Pop10C (19ª)   |            |               |               |               |               |                |                |         |
| N<sub>e</sub> | 0           | 0             | 7             | 3             | 2             | 1              | 6              |         |
| H<sub>e</sub> | 0.56899     | 0.70413       | 0.73188       | 0.67540       | 0.76292       | 0.77619        | 0.45290        | 0.66749 |
| p value      | 0.84211    | 0.94737       | 0.41667       | 0.75000       | 0.58824       | 0.88889        | 0.58333        | 0.71666 |
| F<sub>IS</sub> | −0.02296   | 0.13148       | 0.00433       | 0.68492       | 0.05477       | 0.57634        | 0.63338        |         |
| A<sub>U</sub>  | 3 (0)       | 5 (0)         | 8 (0)         | 7 (0)         | 6 (0)         | 8 (0)          | 3 (0)          | 5.714 C |
| Pop11B (20ª)   |            |               |               |               |               |                |                |         |
| N<sub>e</sub> | 0           | 0             | 7             | 1             | 0             | 10             | 1              |         |
| H<sub>e</sub> | 0.52179     | 0.71282       | 0.83385       | 0.79232       | 0.78462       | 0.71053        | 0.24751        | 0.65763 |
| p value      | 0.70000    | 0.70000       | 0.30769       | 0.73684       | 0.60000       | 0.80000        | 0.26316        | 0.58681 |
| F<sub>IS</sub> | 0.09606    | 0.67413       | 0.00001       | 0.35896       | 0.00120       | 0.94949        | 1.00000        |         |
| A<sub>U</sub>  | 3 (0)       | 6 (0)         | 5 (0)         | 7 (0)         | 9 (0)         | 5 (0)          | 5 (2)          | 5.857 C |
| Pop12B (11ª)   |            |               |               |               |               |                |                |         |
| N<sub>e</sub> | 0           | 0             | 0             | 0             | 1             | 7              | 0              |         |
| H<sub>e</sub> | 0.48485     | 0.70563       | 0.86147       | 0.84948       | 0.83158       | 0.71429        | 0.17749        | 0.66054 |

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

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

To measure the level of genetic differentiation, pairwise FST 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 FST values, ranging from 0.1522 to 0.2364 (Table 2). In *H. placei*, overall FST value also indicated a low level of genetic differentiation ranging 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 1 (continued)

|          | Hc36 | Hpms43 | Hpms52 | Hpms53 | Hpms102 | Hcms3561 | Hc53265 | All loci |
|----------|------|--------|--------|--------|---------|----------|---------|---------|
| *H. contortus* |      |        |        |        |         |          |         |         |
| *H. placei*    |      |        |        |        |         |          |         |         |
| *H. contortus* |      |        |        |        |         |          |         |         |
| *H. placei*    |      |        |        |        |         |          |         |         |

### Table 2

Pairwise FST 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       | −0.0245| −0.0245| −0.0444| −0.0057|       |       |        |        |
| Pop9C       | −0.0284| −0.0284| −0.0449| 0.0212| −0.0249|       |        |        |
| Pop10C      | −0.0081| −0.0090| −0.0486| 0.0108| −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 |       |       |       |       |       |        |        |
| 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. contortus* 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,
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 (TA_C) and F167Y (TA_C) SNPs at the isotype 1 β tubulin locus (Kwa et al. 1994) through either recurrent, or pre-existing mutations; and the single time emergence of the E198A (GA_C) 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 Fst 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 (Gillett 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.

### 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

### Acknowledgments
We are grateful to the Vice Chancellor (Prof. Dr. Talat Naseer Pasha) of the University of Veterinary and Animal Science Lahore.
Pakistan for his great support to collect samples from abattoirs. We would also like to thank Moredun Research Institute Scotland for their kind support to use pyro-sequencer. The article is the sequel of Dr. Umer Chaudhry thesis, which is available in full at the University of Calgary, Canada website.

**Funding information** The study was financially supported by the Higher Education Commission of Pakistan.

**Compliance with ethical standards**

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

**Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

**References**

Ali Q, Rashid I, Ashraf K, N Z, Ashraf S, Chaudhry U (2014) Genetic variation in the rDNA ITS-2 sequence of Haemonchus placei from Cattle Host. J Inf Mol Biol 3:13–18

Archie EA, Ezenwa VO (2011) Population genetic structure and history of a generalist parasite infecting multiple sympatric host species. Int J Parasitol 41:89–98. https://doi.org/10.1016/j.ijpara.2010.07.014

Besier RB, Kahn LP, Sargison ND, Van Wyk JA (2016) The pathophysiology, ecology and epidemiology of Haemonchus contortus infection in small ruminants. Adv Parasitol 93:95–143. https://doi.org/10.1016/bs.apar.2016.02.022

Blouin MS, Yowell CA, Courtney CH, Dame JB (1995) Host movement and the genetic structure of populations of parasitic nematodes. Genetics 141:1007–1014

Brasil BS et al (2012) Genetic diversity patterns of Haemonchus placei and Haemonchus contortus populations isolated from domestic ruminants in Brazil. Int J Parasitol 42:469–479

Chaudhry U, Gilleard J (2015) Molecular Analysis of Benzimidazole Resistance in Haemonchus contortus and Haemonchus placei. PhD Thesis

Chaudhry U, E. M. Redman, Muthusamy Raman, Gilleard JS (2015a) Genetic evidence for the spread of a benzimidazole resistance mutation across southern India from a single origin in the parasitic nematode Haemonchus contortus Int J Parasitol IJPARA-S 15-00127-2

Chaudhry U, Redman E, Abbas M, Muthusamy R, Ashraf K, Gilleard J (2015b) The presence of benzimidazole resistance mutations in Haemonchus placei of US cattle. Int J Parasitol 45:149–159

Chaudhry U, Redman EM, Ashraf K, Shabbir MZ, Rashid MI, Ashraf S, Gilleard JS (2016) Microsatellite marker analysis of Haemonchus contortus populations from Pakistan suggests that frequent benzimidazole drug treatment does not result in a reduction of overall genetic diversity. Parasit Vectors 9:349. https://doi.org/10.1186/s13071-016-1624-0

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

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

Gilleard JS, Beech RN (2007) Population genetics of anthelmintic resistance in parasitic nematodes. Parasitology 134:1133–1147. https://doi.org/10.1017/S0031182007000066

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

Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361–372

Hunt P, Knox M, Le Jambre L, McNally J, Anderson L (2008) Genetic and phenotypic differences between isolates of Haemonchus contortus in Australia. Int J Parasitol 38:885–900

Hussain T, Periasamy K, Nadeem A, Ellahi Babar M, Pichler R, Diallo A (2014) Sympatric species distribution, genetic diversity and population structure of Haemonchus isolates from domestic ruminants in Pakistan. Vet Parasitol 206:188–199. https://doi.org/10.1016/j.vetpar.2014.10.026

Jacquet P, Humbert JF, Comes AM, Cabaret J, Thiam A, Cheikh D (1995) Ecological, morphological and genetic characterization of sympatric Haemonchus spp. parasites of domestic ruminants in Mauritania. Parasitology 110( Pt 4): 483–492

Kaplan RM, Vidyasankar AN (2012) An inconvenient truth: global worming and anthelmintic resistance. Vet Parasitol 186:70–78. https://doi.org/10.1016/j.vetpar.2011.11.048

Kwa MS, Veenstra JG, Roos MH (1994) Benzimidazole resistance in Haemonchus contortus is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol Biochem Parasitol 63:299–303

Luikart G, Cornuet J (1998) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conserv Biol 12:228–237

O’Connor LJ, Walkden-Brown SW, Kahn LP (2006) Ecology of the free-living stages of major trichostrongylid parasites of sheep. Vet Parasitol 142:1–15. https://doi.org/10.1016/j.vetpar.2006.08.035

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539. https://doi.org/10.1093/bioinformatics/bts460

Prichard R (2001) Genetic variability following selection of Haemonchus contortus with anthelmintics. Trends Parasitol 17:445–453

Redman E, Packard E, Grillo V, Smith J, Jackson F, Gilleard JS (2008) Microsatellite analysis reveals marked genetic differentiation between Haemonchus contortus laboratory isolates and provides a rapid system of genetic fingerprinting. Int J Parasitol 38:111–122. https://doi.org/10.1016/j.ijpara.2007.06.008

Redman E et al (2015) The emergence of resistance to the benzimidazole anthelmintics in parasitic nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Negl Trop Dis 9(6): e0003494

Rice W (1989) Analysing tables of statistical tests. Evolution; Int J Organic Evol 43:223–225.

Santos MC, Redman E, Amarante MRV, Gilleard JS, Amarante AFT (2017) A panel of microsatellite markers to discriminate and study interactions between Haemonchus contortus and Haemonchus placei. Vet Parasitol 244:71–75. https://doi.org/10.1016/j.vetpar.2017.07.011

Silvestre A, Sauve C, Cortet J, Cabaret J (2009) Contrasting genetic structures of two parasitic nematodes, determined on the basis of neutral microsatellite markers and selected anthelmintic resistance markers. Mol Ecol 18:771–780. https://doi.org/10.1111/j.1365-294X.2009.04421.x

Trell K, Mattsson JG, Allderborn A, Hoglund J (2003) Pyrosequencing analysis identifies discrete populations of Haemonchus contortus from small ruminants. Int J Parasitol 33:765–771

Yin F et al (2016) Population structure of Haemonchus contortus from seven geographical regions in China, determined on the basis of microsatellite markers. Parasit Vectors 9:586. https://doi.org/10.1186/s13071-016-1864-z