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Microsatellite analysis reveals extensive gene flow, and lack of population structure in the farm populations of *Haemonchus contortus* in northern China

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ABSTRACT

The parasitic nematode *Haemonchus contortus* is economically an important parasite of small ruminants across the globe. China is the world's largest producer, consumer, and importer of mutton. With ubiquitous distribution across the country *H. contortus* is one of the potential candidates to cause huge economic losses to small ruminant farming industry in China. We herein investigated genetic diversity and population structure of six farm populations of *H. contortus* in northern China, and also compared them to *H. contortus* isolates from UK and Australia. We first prepared individual DNA samples from 240 adult worms, and generated genotyping data using eight microsatellite markers. Obtained data was then subjected to allelic frequency and population genetic analyses. The overall allelic richness (mean/locus/pop = 7.375 ± 0.844–10.125 ± 1.109), and expected heterozygosity (mean/locus/pop = 0.646 ± 0.040–0.735 ± 0.025) indicated high degree of population genetic variation across the Chinese isolates. Low level of genetic differentiation (Fst = 0.010–0.066) was observed across all the populations. AMOVA results showed high level of variation (93%) within the populations. PCA analysis revealed mixed clustering of all the populations with no visible geographical sub-structuring. Finally the population admixture analysis resulted in extensive admixing of genotypes across all the populations. With these findings we conclude that there is no obvious population genetic structure with extensive gene flow across all the farm populations of *H. contortus* in northern China.

1. Introduction

The nematode parasite *Haemonchus contortus* is economically an important parasitic species that infects the small ruminants worldwide. This parasite feeds on blood in the abomasum of goats, sheep, and cattle [1]. The *H. contortus* infection leads to a pathological condition called haemonchosis, where infected animals show a series of symptoms, including anemia, diarrhea, weight loss, or even death in case of severe infection. Damages due to haemonchosis can lead to economic losses in billions of dollars to the breeding industry of small ruminants [2,3].

According to the FAO (2017) statistics for live animals (http://www.fao.org/faostat/en/#data), China is the largest producer of sheep (161.3 millions), and goats (139.9 millions) in the world. In the same year about 260,000 tons of sheep meat were also imported by China to fulfil the country's need. Beside the traditional herd raising, there are thousands of sheep farms across the country [4]. In 2018 the sheep and cattle industry contributed about 100 billion dollars to revenue in China. With these statistics the small ruminants industry worth in hundreds of billions of dollars. With the globe's largest human population the China is not only the world's largest mutton producer but also consumer and importer. The prevalence of *H. contortus* has also been reported in China with a ubiquitous distribution across the country [5–8]. *H. contortus* is considered a potential candidate to cause huge economic losses in China [9].

Genetic structure of a parasite in a country is determined by the partition of genetic diversity within and across the various populations of the parasite. For the assessment of genetic diversity, a variety of molecular markers is available. Microsatellites are highly polymorphic markers which are commonly used in population genetic studies [10]. Microsatellite loci in *H. contortus* have been identified and analysed in

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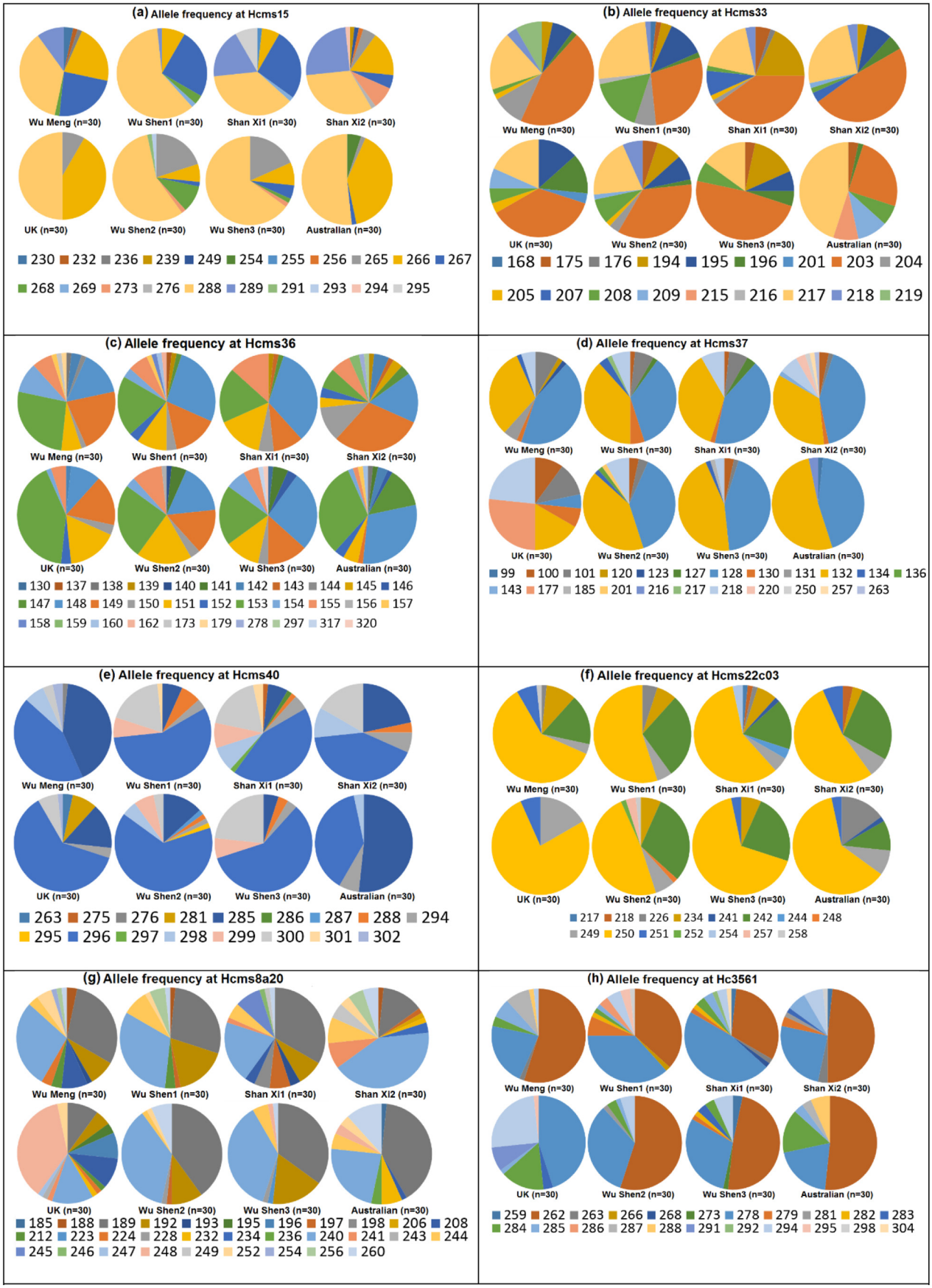

Fig. 1. Graphical representation of gene pool analysis of eight microsatellite loci in eight populations of *Haemonchus contortus* during the present study. Eeach pai chart represents the distribution of allele frequency for a single locus in a single population. Locus name, population name, sample size, and length of alleles are shown for every individual locus analysis.

various studies across the globe [11–18].

The north of China, especially the Inner Mongolia is the largest region for sheep, wool, and meat production [19,20], and it adds 1.6 trillion RMB to country's economy. Given its economic importance, understanding the *H. contortus* genetic diversity is essential for developing the control strategies against haemonchosis, and to control the emergence of drug resistance. Lack of population genetic structure reflects a high degree of gene flow among the populations, and offers a great opportunity to rare local drug resistant alleles to spread across other populations. Various population genetic studies on *H. contortus* have been carried out in various parts of the world [11,21–25]. A few studies on the field isolates of *H. contortus* have also been conducted in China [16,26,27]. Here, we continue to provide further insights in understanding the genetic diversity and population structure of *H. contortus* in China. We investigated six farm populations of *H. contortus* from northern China, using microsatellite markers. We also included two foreign populations of *H. contortus* (one from Australia, and one from UK) in the study, to compare them with Chinese farm isolates of *H. contortus*.

2. Materials and methods

2.1. Worm collection, identification, and DNA extraction

A total of 240 adult worms from eight different populations (for each population, n = 30) of *H. contortus* were analysed in this study. Of these, six populations were from two provinces (Inner Mongolia and Shanxi) in northern China (Fig. S1), one was originated from UK (Moredun Institute), and one was originated from Australia (Haecon-5 strain from the Gasser Laboratory, University of Melbourne). Worms of six Chinese populations were collected from abomasa of sheep, at different farms (Wu Meng, Wu Shen 1-3, and Shan Xi 1 & 2) in northern China. After obtaining from UK (Moredun Institute), and Australia, worms from both of these foreign populations of *H. contortus* were maintained in labs in China by serial passage in helminth free sheep or goats for few years. Total genomic DNA was extracted from individual worms using Qiagen DNeasy Blood & Tissue Kit, Germany (cat no. 69506), according to the kit's protocol. Concentration of DNA was analysed using a nanodrop-2000c spectrophotometer (Thermo Scientific, USA). Identification of *H. contortus* at molecular level was done by sequencing the ITS-2 rDNA as described elsewhere [28]. Screening of SNPs at three conserved sites (24, 205, and 219) established the species of *H. contortus* (Supplementary file S1).

2.2. Microsatellite genotyping

A total of eight microsatellite markers: Hcms15, Hcms33, Hcms36, Hcms37 [17]; Hcms40, Hcms22co3, Hcms8a20 [12]; and Hc3561 [15], were selected. The PCR was carried out in a total reaction volume of 25 µl containing 12.5 µl of 2× Taq PCR Master Mix (TaKaRa, Japan), 50–100 ng of DNA template, 10 pmol of each primer, and 9.5 µl of dd-water. Thermocycling conditions included an initial denaturation at 95 °C for 3 min, followed by 35 cycles of: 94 °C for 30 s, 54 °C/51 °C for 30 s, and 72 °C for 60 s. This was followed by a final extension step for 10 min at 72 °C. All forward primers of each primer pair, were 5′ end labelled with the fluorescent dye FAM. The amplicons were run (capillary electrophoresis) with GeneScan 500 LIZ internal size standard on ABI Prism 3730XL genetic analyser. The software GeneMarker V2.2.0 (SoftGenetics LLC) was used to analyse the individual chromatograms in order to assign the exact allele fragment lengths to the amplicons.

2.3. Data analysis

Implemented in GenAlEx 6.51b2 [29,30] the various analyses: allelic frequencies and distributional patterns, observed and expected heterozygosities (H_e and H_o), estimates of F_{IS} and F_{ST}, and deviations from Hardy–Weinberg equilibrium (HWE), were performed. Analysis of Molecular Variance (AMOVA) was carried out in Arlequin ver 3.5.2.2 [31]. PCA was conducted using GenAlEx 6.51b2. The Bayesian-based analysis software package “Structure ver 2.3.4” [32] was used to analyse the genetic structure of populations. Analysis was conducted for each K (6–8) with 10,000 burn-in and half million Markov Chain Monte-Carlo (MCMC). Results of the structure analysis were visualised and extracted using online web tool “POPHELPER Structure Web App v1.0.10” available for us at (http://www.pophelper.com/).

3. Results

3.1. Allele frequency analysis

A total of eight microsatellite loci (Hcms15, 33, 36, 37, 40, 22co3, 8a20, and HC3561) were amplified to investigate their allelic patterns across the eight *H. contortus* populations. Adult worms (n = 240) were genotyped at all of these loci. High degree of polymorphism was observed at all the loci, with number of alleles ranging from 15 to 32 per locus across all the populations (Fig. 1). The most polymorphic loci were Hcms36 and Hcms8a20. Hcms22co3 was the least polymorphic locus. The population ShanXi2 possessed maximum number of alleles (mean = 10.125 ± 1.109) across all the loci (Table 1). Minimum numbers of alleles (mean across all loci = 7.00 ± 1.210) was observed in Australian population. Maximum numbers of private alleles were observed in ShanXi2 followed by ShanXi1 and WuMeng populations, while minimum numbers of private alleles were recorded in WuShen3 and Australian populations (Fig. 2).

3.2. Genetic diversity and Hardy-Weinberg equilibrium

Genetic diversity was assessed using allele frequencies and heterozygosity analyses (Table 1). A considerable variation was observed in the allelic patterns across all the microsatellite loci. Minimum numbers of alleles (3) were observed at two loci (Hcms15 & 22co3) in UK population. Maximum numbers of alleles (15) were recorded at locus Hcms36 in WuShen1, and ShanXi2 populations. Average numbers of alleles (and expected heterozygosity, H_e) across all the loci were observed as: WuMeng, 9.250 ± 0.921 (0.713 ± 0.031); WuShen1, 9.250 ± 1.146 (0.710 ± 0.035); ShanXi1, 9.875 ± 0.766 (0.733 ± 0.024); ShanXi2, 10.125 ± 1.109 (0.735 ± 0.025); UK, 7.250 ± 1.236 (0.676 ± 0.054); WuShen2, 8.500 ± 0.535 (0.678 ± 0.036); WuShen3, 7.375 ± 0.844 (0.646 ± 0.040); and Australian, 7.00 ± 1.210 (0.649 ± 0.033). Locus wise maximum value (0.851) of the H_e was observed in ShanXi2 at locus Hcms36, which was followed by WuShen1 (H_e = 0.847) at the same locus, while the minimum value of the H_e (0.380) was observed in UK population at locus Hcms22co3. Values of Shannon's information index (I) supported the distribution of allelic patterns and the H_e across all the populations (Fig. 2). Maximum pairwise Nei genetic distance (0.373) was recorded for UK vs ShanXi2, while minimum (0.037) for WuShen2 vs WuShen3. For each of the all loci, there was a significant departure (α threshold after Bonferroni correction = 0.006) from HWE in 1–6 populations with heterozygosity excess or deficiency.

3.3. Genetic differentiation, population structure and clustering

In an attempt to explore the population dynamics, we subjected our data to various population genetic analyses. To ascertain the extent of gene flow, we first calculated the pairwise Fst values across all the populations (Table 2). Low genetic differentiation (Fst = 0.010–0.066) was observed across all the populations. Low Fst values indicate high gene flow among the populations. To further explore the extent of genetic sub-structuring between the populations we conducted PCA (Fig. 3a). In PCA plot the two axes accounted for 23.75% of the

Table 1
Genetic diversity index of eight *Haemonchus contortus* populations, using a panel of eight microsatellite markers, during present study. Deviations from Hardy-Weinberg equilibrium (significant deviations are indicated by asterisk; α threshold after Bonferroni correction = 0.006) in eight *H. contortus* populations, are presented. Minus sign (−) indicates heterozygote deficiency, and a plus sign (+) indicates heterozygote excess.

Population		Hcms 15	Hcms 33	Hcms 36	Hcms 37	Hcms 40	Hcms 22co3	Hcms 8a20	Hc 3561	Mean across all loci ± SD
Wu Meng (n = 30)	A	8	10	13	9	6	7	13	8	9.250 ± 0.921
	H_e	0.752	0.739	0.838	0.699	0.632	0.596	0.808	0.637	0.713 ± 0.031
	H_o	0.967	0.633	0.867	0.933	0.667	0.433	0.833	0.867	0.775 ± 0.064
	P value	+*					-*			
	F_{is}	−0.285	0.144	−0.034	−0.335	−0.055	0.273	−0.032	−0.360	−0.086 ± 0.081
Wu Shen 1 (n = 30)	A	6	11	15	9	7	5	10	11	9.250 ± 1.146
	H_e	0.569	0.809	0.847	0.717	0.631	0.608	0.780	0.721	0.710 ± 0.035
	H_o	0.767	0.967	0.767	0.900	0.367	0.433	0.833	0.633	0.708 ± 0.076
	P value		+*	-*	+*	-*	-*	+*		
	F_{is}	−0.348	−0.195	0.094	−0.255	0.419	0.287	−0.068	0.121	0.007 ± 0.095
Shan Xi 1 (n = 30)	A	7	10	9	7	11	10	13	12	9.875 ± 0.766
	H_e	0.749	0.761	0.802	0.679	0.756	0.622	0.822	0.677	0.733 ± 0.024
	H_o	0.633	0.733	0.667	0.933	0.400	0.433	0.867	0.633	0.663 ± 0.066
	P value			-*		-*	-*			
	F_{is}	0.154	0.036	0.168	−0.375	0.471	0.303	−0.054	0.064	0.096 ± 0.089
Shan Xi 2 (n = 30)	A	11	9	15	11	6	6	13	10	10.125 ± 1.109
	H_e	0.796	0.691	0.851	0.696	0.736	0.633	0.784	0.692	0.735 ± 0.025
	H_o	0.733	0.900	0.633	0.967	0.500	0.467	0.600	0.900	0.713 ± 0.068
	P value		+*	-*	+*	-*	-*	-*		
	F_{is}	0.079	−0.303	0.255	−0.390	0.321	0.263	0.235	−0.301	0.020 ± 0.106
Wu Shen 2 (n = 30)	A	8	11	10	8	9	8	8	6	8.500 ± 0.535
	H_e	0.623	0.808	0.838	0.669	0.551	0.666	0.690	0.582	0.678 ± 0.036
	H_o	0.767	1.000	0.933	1.000	0.333	0.667	0.967	0.900	0.821 ± 0.081
	P value	+*	+*		+*	-*		+*		
	F_{is}	−0.230	−0.238	−0.113	−0.494	0.395	−0.002	−0.401	−0.546	−0.204 ± 0.108
Wu Shen 3 (n = 30)	A	6	7	12	7	6	4	8	9	7.375 ± 0.844
	H_e	0.534	0.709	0.845	0.607	0.596	0.496	0.712	0.668	0.646 ± 0.040
	H_o	0.700	0.767	0.800	1.000	0.267	0.400	1.000	0.900	0.729 ± 0.095
	P value				+*	-*				
	F_{is}	−0.311	−0.082	0.053	−0.648	0.553	0.193	−0.405	−0.348	−0.124 ± 0.136
UK (n = 30)	A	3	8	9	7	7	3	14	7	7.250 ± 1.236
	H_e	0.569	0.787	0.756	0.816	0.583	0.380	0.814	0.704	0.676 ± 0.054
	H_o	1.000	0.900	0.767	1.000	0.433	0.000	0.867	0.833	0.725 ± 0.121
	P value	+*	+*		+*	-*	-*		+*	
	F_{is}	−0.756	−0.143	−0.015	−0.225	0.257	1.000	−0.064	−0.184	−0.016 ± 0.177
Australian (n = 30)	A	5	7	14	4	4	6	10	6	7.00 ± 1.210
	H_e	0.570	0.712	0.785	0.544	0.581	0.579	0.761	0.664	0.649 ± 0.033
	H_o	0.900	1.000	0.900	0.967	0.800	0.533	0.933	0.833	0.858 ± 0.052
	P value	+*		+*	+*			+*		
	F_{is}	−0.579	−0.404	−0.146	−0.777	−0.378	0.079	−0.226	−0.255	−0.336 ± 0.093

Abbreviations: A, number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; F_{is}, inbreeding coefficient. Note: The actual p-values for all the dataset are shown in Supplementary Fig. S4.

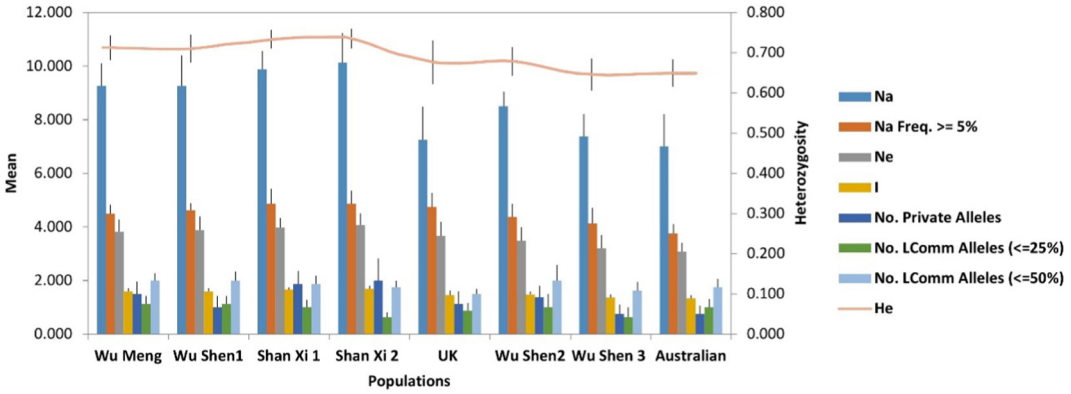

Fig. 2. Allelic patterns across populations. *Na*, numbers of different alleles; *Na (Freq ≥5%)*, numbers of different alleles with a frequency of ≥5%; *Ne*, numbers of effective alleles; *I*, Shannon's Information Index; *No. Private Alleles*, numbers of alleles unique to a single population; *No. LComm Alleles (≤25%)*, numbers of locally common alleles (Freq. ≥ 5%) found in 25% or fewer populations; *No. LComm Alleles (≤50%)*, numbers of locally common alleles (Freq. ≥ 5%) found in 50% or fewer populations; *H_e*, expected heterozygosity (scale for H_e is shown on the right side).

Table 2
Pairwise F_{ST} values among all the populations of *Haemonchus contortus* in the present study.

Population	WM	WS1	SX1	SX2	UK	WS2	WS3	AS
WM	0.000							
WS1	0.022	0.000						
SX1	0.021	0.013	0.000					
SX2	0.015	0.022	0.020	0.000				
UK	0.057	0.053	0.054	0.061	0.000			
WS2	0.022	0.013	0.022	0.023	0.060	0.000		
WS3	0.026	0.012	0.019	0.023	0.060	0.010	0.000	
AS	0.024	0.038	0.041	0.037	0.066	0.036	0.041	0.000

Population codes: WM, Wu Meng; WS1, Wu Shen 1; SX1, Shan Xi 1; SX2, Shan Xi 2; UK, United Kingdom; WS2, Wu Shen 2; WS3, Wu Shen 3; AS, Australian. Note: Fst values were significant ($p < .05$) for all the comparisons except two (WS3 vs WS2, and WS3 vs WS1) where p value was > 0.05.

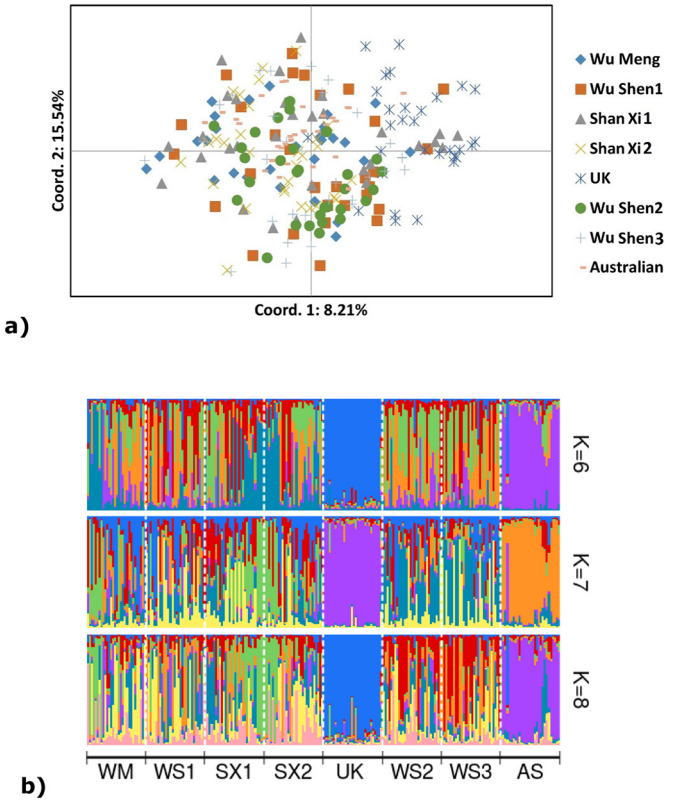

Fig. 3. Population structure analysis of eight different populations of *Haemonchus contortus* in present study. a) Principal coordinate analysis (PCoA). b) Genetic admixture analysis by STRUCTURE software. Population codes: WM, Wu Meng; WS1, Wu Shen 1; SX1, Shan Xi 1; SX2, Shan Xi 2; UK, United Kingdom; WS2, Wu Shen 2; WS3, Wu Shen 3; AS, Australian.

variation. All populations formed an overlapping cluster with no marked geographical subdivision. The UK samples were somewhat distinct, but not fully. All the populations were overlapping in the cluster, and there were many shared genotypes among the various isolates. To analyse the genetic admixture across all the populations we performed Bayesian based admixture analysis using STRUCTURE software (Fig. 3b). We explored admixture for different values of K between six and eight. Extensive admixing across the Chinese populations indicated the presence of shared genotypes between the various isolates. Results of admixture analysis further supported the extensive gene flow among the populations. To further refine the population structure analysis we performed the AMOVA (Table 3). The results showed that 93% of total variation was laying within populations, 2% was among populations, and 5% was among regions. Overall, these findings suggest extensive gene flow and very low populations sub-structuring of *H. contortus* farm populations in northern China. Similar findings have been observed before in the field populations of *H. contortus* from China [16].

4. Discussion

In this study we analysed the genetic diversity and explored the population structure of *H. contortus* farm isolates in northern China using the microsatellite markers. The overall allelic richness and expected heterozygosity indicate high degree of within population polymorphism across all the isolates. This is in agreement with previous studies [16,26]. Genetic diversity in parasites helps them to avoid eradication by the host immune system [33]. *H. contortus* has shown extremely high levels of within population genetic diversity across the globe, which is associated to its large effective population size, and provide it a high adaptive capacity [21,34,35]. Significant deviation from HWE at some loci in some populations was observed, which could be a result of null allele, or it could be caused by temporal fluctuation in allele frequencies. We frequently encountered with homozygotes at some loci in our data set, which might indicate the presence of null alleles. The presence of null alleles has also been reported in previous studies in *H. contortus* [11,12] and other trichostrongylid nematodes [36–38].

We observed low level of genetic differentiation across all the Chinese populations of *H. contortus* as well as in their global comparison with those of UK and Australian isolates. Low level of population differentiation indicate high gene flow across the populations. Low genetic differentiation has also been observed previously in the field isolates of *H. contortus* from China [16,26]. In a global comparison the Chinese isolates of *H. contortus* have shown low genetic differentiation against *H. contortus* populations from Pakistan, and Malaysia [39]. Lack of genetic differentiation was subsequently supported by population structure analyses. Results of AMOVA showed that majority of the variations (93%) was laying within the populations. This indicates high gene flow and no population structure. PCA plot revealed mixed clustering of all the populations with no visible geographical sub-structuring. Extensive admixing was observed in population admixture analysis, which indicates many shared genotypes across all the populations. Overall these results suggest no obvious population genetic structure, and extensive gene flow across all the farm isolates of *H. contortus* in northern China. These findings are in agreement with those for field isolates of *H. contortus* in China [16,26].

Population genetic structure describes the patterns of genetic diversity within and among the populations. Understanding these patterns of genetic variation across the parasite populations is essential to get insights into the parasite's genetic responses to selective pressures

Table 3
Analysis of molecular variance (AMOVA) in eight populations of *Haemonchus contortus* using eight microsatellite markers during present study.

Source of variation	d.f	Sum of squares	Variance	% of total	F-statistic (p-value)
Among regions	2	46.897	0.173	5%	F_{CT} = 0.057 (< 0.05)
Among populations	5	32.789	0.065	2%	F_{SC} = 0.023 (< 0.05)
Within populations	240	719.000	2.996	93%	F_{ST} = 0.078 (< 0.05)

[40]. The question, whether there are non-overlapping populations of a parasite across the country, has practical implications for parasite control. For *H. contortus*, a substantial global, and a low but discernible regional (within countries) population structure has been reported [21,34]. However, we didn't observe it in case for China, where extensive admixing among the Chinese isolates of *H. contortus* was found. We also found the admixing of Chinese populations of *H. contortus* with that of foreign populations of *H. contortus* analysed here. The previous studies also reported similar patterns for Chinese isolates of *H. contortus* [16,26,39]. The high gene flow provides the great opportunity to even rare variants associated with some important trait e.g. drug resistance [14] to spread widely across the populations under the influence of selection. Thus, restricted anthropogenic movements of animals with effective trade barriers, backed by agricultural policies can cut down the gene flow across the *H. contortus* populations in northern China.

5. Conclusion

In summary, the results of this study based on analyses of eight microsatellite loci, show that there is an extensive gene flow among the various farm populations of *H. contortus* analysed herein from China. There is no population sub-structuring, the geographically distinct populations are not genetically distinct, and there are many shared genotypes among the various isolates.

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Declaration of Competing Interest

None.

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