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EXPLORING THE STRUCTURE OF HAPLOTYPE BLOCKS, RUNS OF HOMOZYGOSITY AND EFFECTIVE POPULATION SIZE IN KHUZESTANI RIVER BUFFALO

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ABSTRACT

Buffalo is considered as one of the most important species of livestock in many developing countries for milk and meat production. Knowledge about the characterization of haplotype block structure and about the genetic diversity of a population are fundamental factors for the success of genome-wide association and genomic selection studies. Parameters such as effective population size (N_e), Heterozygosity, runs of homozygosity (ROH) and inbreeding based on ROH (F_{ROH}) can give new insight about the level of genetic diversity for the population under selection. The main objective of this study was to investigate the haplotypic structure and genetic diversity in Iranian river buffalo (n = 123) using the Axiom Buffalo 90 K Genotyping Array. Analysis revealed 1726 haplo-blocks spanning 8.2 % of the genome and containing 12.4 % of all Single-nucleotide polymorphism (SNPs). The contemporary (5 generations ago) effective population size was approximately 240 animals. Totally, 992 ROH were identified, most of which were short (59 %) and had a length less than 10 Mb. Average observed heterozygosity and ROH-based inbreeding were 0.387 and 0.045, respectively. Our results will provide practical information to assist the genomic selection (GS) and genome-wide association study (GWAS) in buffaloes. Furthermore, the results of N_e, heterozygosity and ROH analyses displayed new knowledge about the level of genetic diversity in the Khuzestani river buffalo population.

Key words: haplotype block; runs of homozygosity; effective population size; inbreeding; buffalo

INTRODUCTION

Buffalo (*Bubalus bubalis*) is considered as an indigenous animal in the southwest and north of Iran for more than 3000 years, especially for milk and meat production (Safari *et al*., 2018). The distribution of this ecotype is more common in Khuzestan, Lorestan province and in southern parts of Iraq. The Khuzestani ecotype has the highest milk and meat production among the buffaloes of the country (Shokrollahi and Hasanpour, 2014; Safari *et al.*, 2019). The desirable adaptability of this ecotype to some conditions such as poor quality of food sources, high temperature and humidity variations, irregular rainfall and the prevalence of various diseases, has made it a source of milk and meat production in difficult conditions, thereby playing an important role in the supply of protein products (Warriach *et al.*, 2015).

Although studies on the haplotype block structure are not as extensive as linkage disequilibrium (LD) studies, the detection of the haplotype block structure can transform the information of several SNPs into haplotype block information, which allows

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a set of SNPs to be selected as a coherent way (Johnson *et al.*, 2001). Haplotype blocks are regarded as the part of genomes, in which the LD level is high, and the haplotype diversity and recombination rate are low (Khatkar *et al.*, 2007).

Among different factors, N_e is one of the most important parameters in population genetics, which determines the genetic diversity, inbreeding and genetic drift in the population (Frankham, 2005). The N_e not only provides information on the evolutionary history of species and breeds but also develops the understanding and modelling of the genetic architecture underlying complex traits (Tenesa *et al.*, 2007). In cases where N_e is small, the genetic diversity will be limited within the population, which influences genetic gain in breeding programs. Prior to any action in relation to the conservation of the genetic resources of a livestock population, required information should be obtained from the N_e and its genetic diversity (Zhao *et al.*, 2014).

Moreover, genetic diversity can be estimated with the help of other methods using marker data, including observed and expected heterozygosity, runs of homozygosity (ROH) and Wright's F statistic (F_{ST}). The contiguous lengths of homozygous genotypes in an animal are the runs of homozygosity (ROH), which occur because of the parents transmitting identical haplotypes to their offspring. Loss of genetic diversity due to population bottleneck or founder effect as well as recent inbreeding in a population result in longer ROH. The time of inbreeding can be estimated using the frequency and extent of ROH. In addition, it is possible to improve the mating systems and to minimize inbreeding through detecting the ROH. Therefore, the objectives of this study were to assess the characterization of haplotype block structure and the within-population diversity in Khuzestani river buffalo, using the Axiom Buffalo 90K Genotyping Array, the only commercial SNP genotyping array that can be applied for obtaining the genome-wide SNP data in buffalo.

MATERIAL AND METHODS

Samples, genotyping and quality control

The present study was conducted using 123 animals collected from different cities of Khuzestan province, Iran. Hair samples with hair roots were obtained from parts of the animal body with less contamination. After DNA extraction, the samples were transferred to the Genomics Laboratory of Parco Tecnologico Padano in Italy for subsequent genotyping. In the next step, the genotyping was performed using the Axiom Buffalo 90 K Genotyping Array in Affymetrix, according to their standard protocol. Initially, the SNP chip contained 89980 markers. Primary qualifications of raw genotypes were determined by Affypipe (Nicolazzi *et al.*, 2014), and all polymorphic and monomorphic high-resolution genotypes including 62495 SNP were initially retained (Ghoreishifar *et al.*, 2018). Finally, additional quality control was done using the PLINK v1.7 (Purcell *et al.* 2007) software. The animals were eliminated with more than 5 % missing genotype. Then, minor allele frequency (MAF) and call rate (CR) factors were calculated for each SNP. The SNPs that totally had MAF and CR less than 1 % and 95 %, respectively, were excluded and then significantly deviated from Hardy-Weinberg equilibrium (HWE) (P-value < 0.000001) were filtered out. In the current study, sex chromosomes and loci without an assigned position in the Cattle Genome Assembly UMD 3.1 (Zimin *et al.*, 2009) were discarded. Although chromosome-level assembly of the water buffalo genome (UOA_WB_1) has been published recently (Low *et al.*, 2019), we used the UMD3.1 (Zimin *et al.*, 2009) assembly in our study because it is more reliable and has better gene annotation information (Ghoreishifar *et. al.*, 2020).

Haplotype blocks

The haplotype phase was reconstructed with the BEAGLE Version 3.3.1 software (Browning and Browning, 2011) for each autosome with the default parameters and 10 iterations. Based on the method proposed by Gabriel *et al.* (2002), we detected haplotype blocks in Khuzestani river buffalo using Haploview (Barrett *et al.*, 2004).

Effective population size

The historical and current effective population size of Khuzestani river buffalo was estimated using the SNeP software v1.1 (Barbato *et al.*, 2015) using the following formula suggested by Corbin *et al.* (2012):

$$Ne_{(t)} = \left[\frac{1}{4f(c_t)}\right]\left[\frac{1}{E\left(r^2_{adj}|c_t\right)} - \alpha\right]$$

Where, $Ne_{(t)}$ is the effective population size t generations ago, c_t is the recombination rate for a specific physical distance between SNPs in t generations age, and t = 1/(2c); and α is a constant in the equation to correct for the occurrence of mutations (if required). However, instead of assuming 1 cM = 1 Mb, a recombination rate modifier in the following formula was used to calculate c (Sved, 1971):

$$c = d\frac{1-(d/2)}{(1-d)^2}$$

where d is the linkage at distance c, which could be estimated using r^2 adjusted for sample size (adjustment required if the sample size is small). In this study, the default parameters of "1" and "no correction" were used for α and sample size adjustment, respectively.

Runs of homozygosity

Runs of homozygosity were obtained using PLINK v1.7 (Purcell *et al.*, 2007) with the following criteria: (i) an ROH should have at least 1 SNP per 1 Mb; (ii) the minimum length that form a ROH was ≥ 1 Mb; (iii) a sliding window under examination are permitted to contain at most 1 heterozygous SNP, and (iv) examining window size set to 20 SNPs. We determined the distribution of ROH across the following five length (Mb) categories: < 5 Mb, 5-10 Mb, 10-20 Mb, 20-30 Mb, 30-40 Mb, and > 40 Mb. The equation introduced by McQuillan *et al.* (2008) was applied to calculate the inbreeding coefficients (F_{ROH}) as follows: $F_{ROH} = L_{ROH} / L_{auto}$

$$F_{ROH_i} = \frac{L_{ROH}}{L_{auto}}$$

where, L_{ROH}: total length of all ROHs in the individual genome, L_{auto}: total length of SNP-covered autosome. PLINK v1.7 (Purcell *et al.*, 2007) software was used to compute the genetic diversity index [observed heterozygosity (Ho)] and the inbreeding coefficient based on ROH (F_{ROH}).

RESULTS

Data qualification

After initial filtration by Affypipe software (Nicolazzi *et al.*, 2014), 117 animals passed the quality control process based on DQC and call rate. After final quality control, two animals were excluded due to the animal call rate < 95 %; and eventually, 115 animals were included for subsequent analyses followed by exclusion of 336 SNPs due to Hardy-Weinberg disequilibrium and 2019 SNPs for having MAF less than 5 %.

Eventually, 60140 SNPs on autosomal chromosomes, covering 2500.57 Mb of the bovine genome assembly, were retained for further analysis.

Table 1 shows the statistical analysis of existing SNPs. The autosomes differed in size, with BTA1 being the longest chromosome (158.1 Mb) and BTA25 the shortest (42.7). The average distance between the adjacent SNPs was 41.5 Kb and the average total MAF was 0.309. The MAF has been introduced as one of the most influential factors in estimating r^2, so that especially higher MAF in shorter marker distances (less than 10 Kb) leads to an increase in the average values of r^2 (Zhu *et al.* 2013).

Haplotype block structure

For all autosomes, we characterized the haplotype block structure, including SNPs located at a maximum distance of 500 kb. The summary of size, number and SNPs involved in haploblocks, block length and percentage of blocks per chromosome are provided in Table 2. The total number of blocks identified in the present study was 1726, consisted of 7613 SNPs, and covered 209.09 Mb (8.2 % of the total genome) on 29 autosomes (Table 2). BTA1 showed the highest number of SNPs (539) and haplotype blocks (130), while BTA26 presented the smallest number of SNPs (101) and haplotype blocks (26).

Figure 1 clarifies the distribution of the block size frequency for all autosomes. Blocks between 75 and 150 kb carried most weight and blocks with more than 600 kb happened at a low frequency.

Effective population size

The N_e estimated against the previous generations is shown in Figure 2. The contemporary (5 generations ago) effective population size of the Khuzestani river buffalo is approximately 240 animals. Figure 2 also displays that the reduction in N_e was stronger during the past 40 generations than before.

Table 1. Genome-wide summary of marker, heterozygosity and minor allele frequency (MAF) for 29 autosomes in Khuzestani buffalo population

Chr	Number of SNP	BTA length (Mb)	MAF	Observed Heterozygosity	Distance between adjacent markers (Kb)		
					Average	SD	Median
1	3837	158.13	0.310	0.393	41.2	24.5	32.2
2	3307	136.49	0.301	0.379	41.3	27.0	32.0
3	2906	121.20	0.307	0.378	41.7	29.8	31.6
4	2887	120.40	0.315	0.390	41.7	25.4	32.8
5	2868	120.53	0.307	0.387	42.0	31.7	31.6
6	2816	119.39	0.313	0.390	42.4	32.7	32.3
7	2678	112.40	0.308	0.388	42.0	37.8	31.3
8	2678	113.14	0.306	0.381	42.3	30.8	32.2
9	2497	105.48	0.313	0.388	42.3	26.2	32.7
10	2526	103.86	0.310	0.387	41.1	42.4	31.5
11	2580	107.13	0.307	0.390	41.5	24.8	32.7
12	2036	91.01	0.306	0.391	44.7	61.8	32.5
13	2072	83.69	0.308	0.386	40.4	28.7	31.4
14	2096	83.34	0.314	0.386	39.8	24.0	31.1
15	1985	84.90	0.314	0.394	42.8	39.0	32.1
16	1917	81.19	0.311	0.396	42.4	37.0	31.6
17	1796	74.98	0.313	0.387	41.8	29.9	31.9
18	1576	65.60	0.304	0.376	41.7	35.3	31.7
19	1550	63.50	0.314	0.396	41.0	27.0	30.9
20	1701	71.78	0.308	0.384	42.2	25.3	33.0
21	1639	71.04	0.308	0.385	43.4	41.2	32.1
22	1538	61.24	0.303	0.386	39.8	24.4	32.1
23	1245	52.03	0.309	0.388	41.8	34.0	31.2
24	1581	61.99	0.314	0.384	39.2	21.6	31.4
25	1122	42.71	0.295	0.372	38.1	23.7	30.4
26	1277	51.23	0.311	0.384	40.2	22.6	32.0
27	1113	45.33	0.304	0.381	40.8	36.0	30.6
28	1135	46.04	0.316	0.392	40.6	33.4	31.2
29	1181	50.84	0.302	0.387	43.1	40.6	32.4
Total	60140	2500.57	0.309	0.386	41.5	24.5	32.2

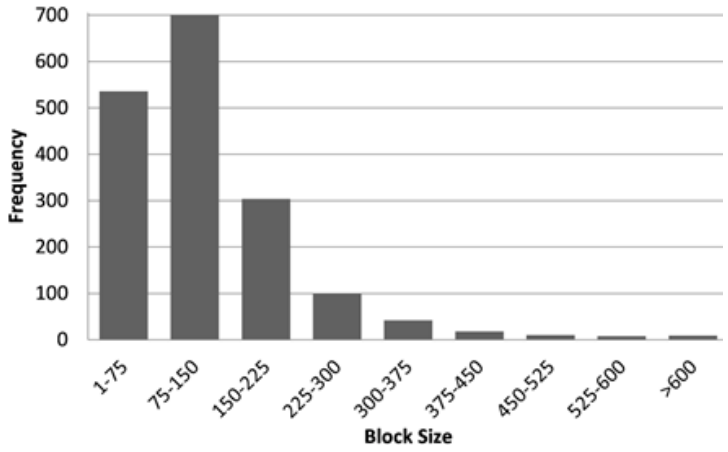


Figure 1. Size distribution of predicted haplotype blocks across all autosomes

Table 2. Haplotype block summary per autosome

BTA	Number of block	Block length (kb)	Percentage BTA in block	MAX_BlockSize (Kb)	Numer of SNP in Block	Percentage SNP in block
1	130	13644	8.6	488	539	14.0
2	117	14412	10.6	1120	479	14.5
3	87	9488	7.8	380	383	13.2
4	97	11149	9.3	383	418	14.5
5	88	12293	10.2	565	409	14.3
6	83	10459	8.8	563	375	13.3
7	71	8552	7.6	873	320	11.9
8	82	11867	10.5	673	400	14.9
9	61	7321	6.9	311	271	10.9
10	72	7595	7.3	265	301	11.9
11	82	9422	8.8	417	349	13.5
12	57	7035	7.7	496	304	14.9
13	49	5264	6.3	358	207	10.0
14	53	5401	6.5	606	212	10.1
15	45	5312	6.3	461	184	9.3
16	53	6739	8.3	568	228	11.9
17	50	5923	7.9	512	230	12.8
18	44	4382	6.7	300	166	10.5
19	38	8277	13.1	2908	234	15.1
20	37	5387	7.5	595	171	10.1
21	51	6368	9.0	498	232	14.2
22	50	6298	10.3	760	228	14.8
23	39	6807	13.0	1169	199	16.0
24	47	4357	7.0	324	183	11.6
25	30	2739	6.4	309	121	10.8
26	26	2503	4.9	262	101	7.9
27	26	2179	4.8	240	103	9.3
28	26	3320	7.2	383	116	10.2
29	35	4604	9.1	710	150	12.7

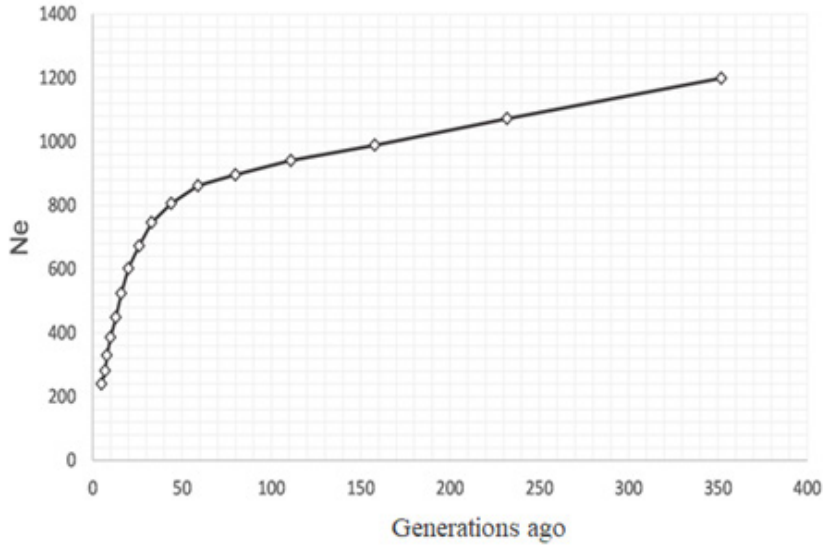


Figure 2. Estimates of effective population size (N_e) over time up to 5 generations based on linkage disequilibrium calculations from 29 autosomes

Runs of homozygosity (ROH) and ROH based inbreeding (F_{ROH})

Figure 3 shows the number of ROHs presented on each chromosome and the percentage of autosome chromosomes covered by ROH. The highest coverage of ROH was observed in BTA25 (31.77 %) and the lowest in BTA1 (7.97 %). Totally, 992 ROH were identified, with the highest in BTA2 (n = 63) and the lowest in BTA29 (n = 14). In this study, the frequency of ROH varied between different distances. Most ROHs were shorter in the range of 0-10 (Supplementary Figure S1). Although the average inbreeding (F_{ROH}) was 0.045, the inbreeding varied from 0.001 to 0.32 among animals (Supplementary Figure S2). In addition, as shown in Table 1, the observed heterozygosity (Ho) in autosomes was in the range of 0.372 to 0.396 and the mean Ho was obtained to be 0.387.

DISCUSSION

There was a significant difference in the extent of haplotype blocks among different autosomes. The average block coverage of the genome in the Khuzestani river buffalo (8.2 %) was higher than in several studies on the cattle. For example, Qanbari

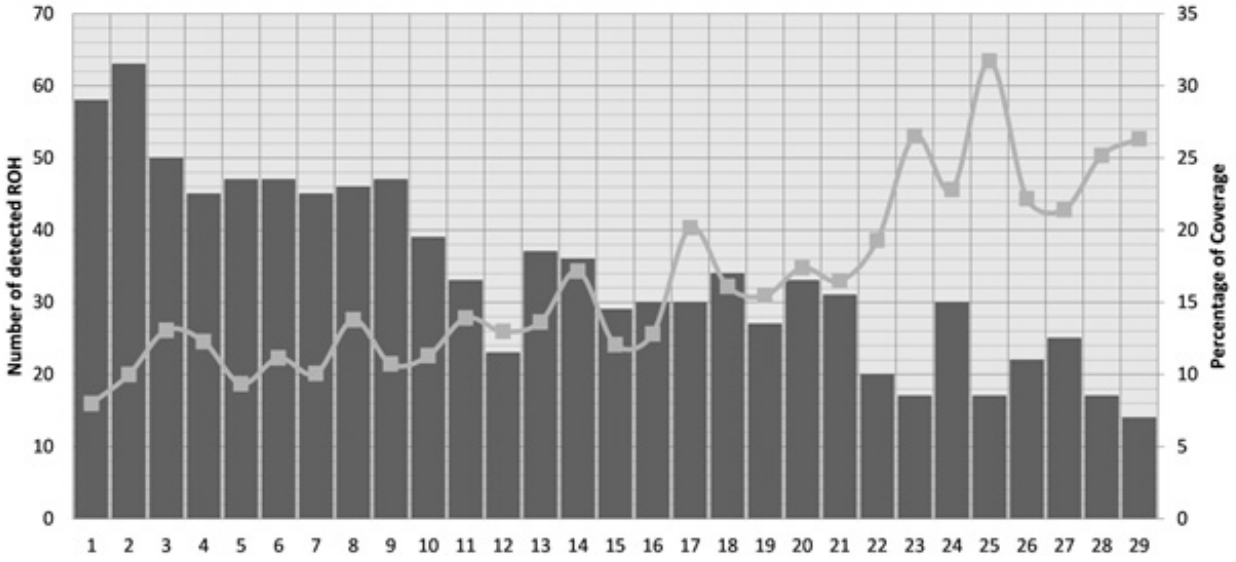
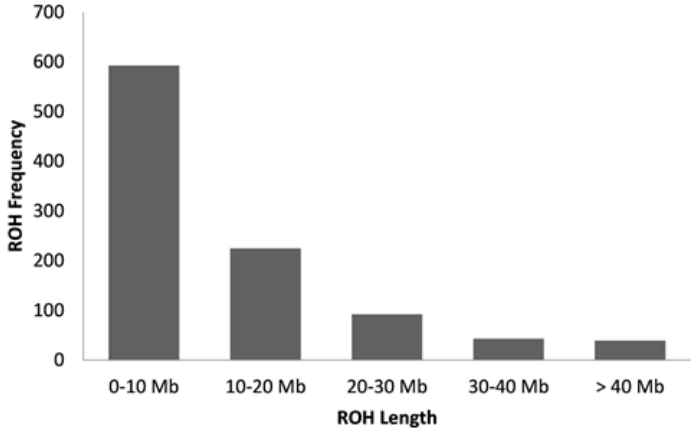
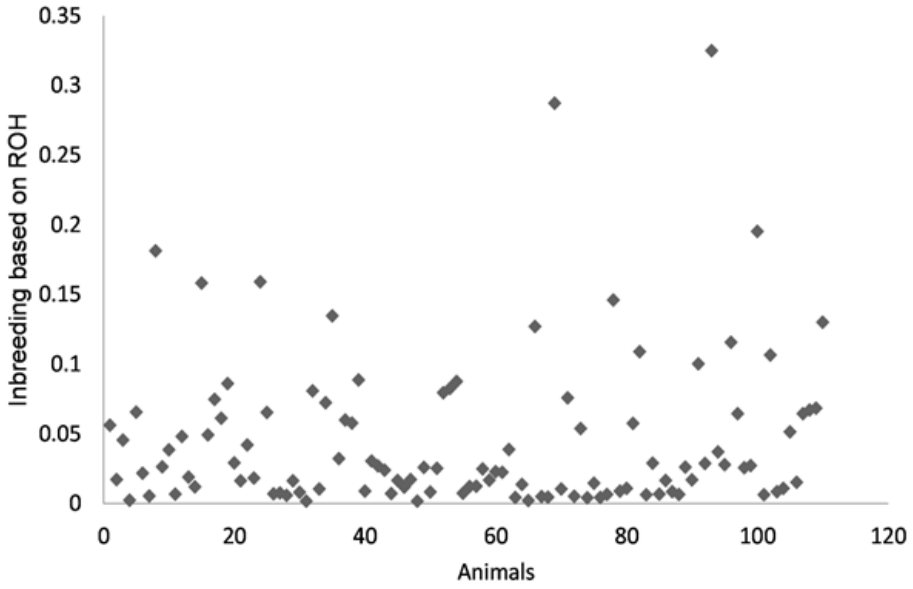


Figure 3. Number of detected ROH and percent of coverage per autosome. The bars display the total number of ROH per autosome and the line display the average percentage of ROH for each autosome



Supplementary Figure S1. Frequency distribution of the number of ROH categorized into six groups according to their length (Mb)



Supplementary Figure S2. Distribution of inbreeding coefficients based on ROH (F_{ROH}) for each animal.

et al. (2010) showed that 4.7 % of the genome was covered by haplotype blocks in the German Holstein cattle breed; Salem *et al.* (2018) reported that this rate was 6.2 % in the Portuguese Holstein cattle breed. The total number of blocks identified in this study (n = 1726) was somewhat higher than in the Portuguese Holstein (n = 969) (Salem *et al.*, 2018), and German Holstein (n = 712) (Qanbari *et al.*, 2010). According to previous findings, there are multiple factors affecting the features of the haplotype blocks, including breed, marker types, marker density, chromosome region and the method of haplotype block definition. The extent of haplotype block structures in Buffalo breed is very rare in order to allow a whole comparison with the results of the current study. Our results, however, were closely similar to those obtained by Fallahi *et al.*, (2019) by studying on Azerbaijani river buffalo with the same technology of the Axiom Buffalo 90K Genotyping Array. For instance, the total number blocks detected across the genome reported to be 1693 in the study of Fallahi *et al.* (2019), which was very close to the present study (1726).

Since the haplotypes, in comparison with individual SNPs, have a stronger LD with Quantitative trait loc (QTL), the use of haplotype blocks in the genomic and GWAS studies has always been recognized as valuable methods (Sun *et al.* 2016). The GWAS and GS studies can be successfully designed and interpreted in livestock population by understanding the structure of haplotypes. The GWAS studies showed that the accuracy of detecting candidate genes could be improved by the haplotype association test rather than the single SNPs analysis model. According to a study by Hess *et al.* (2017), the prediction accuracy in the GS could be increased using fitting covariates for haplotype alleles compared to the SNPs, which may be attributed to enhanced genetic gain by changing the ranking of selection candidates.

The N_e is one of the most important parameter in the management of conservation of genetic resources, the success of breeding programs and the improvements in the design of artificial selection. Frankham *et al.* (2014) suggested that the N_e should be at least 100 in the short term (five generation ago) for retention of genetic variation and prevent inbreeding depression in different populations. Moreover, the Ne should be more than 1000 in order to maintain initial evolutionary potential in perpetuity. As shown in Figure 2, the N_e decreases from 1704 in 884 generation ago gradually, and this slope begins to increase from

40 generation ago to get 240 for 5 generation ago. Most studies on N_e estimates in livestock have focused on cattle, especially dairy cattle. Qanbari *et al.* (2010) reported that the Ne was close to 103 in the German Holstein breed in four generations ago. Biegelmeyer *et al.* (2016) showed that the N_e was 153 and 220 respectively in the Herford and Braford breeds. Zhu *et al.* (2013) showed that the N_e was 73 in the Chinese Simmental breed. In order to estimate the N_e in indigenous cattle breeds of Iran, Karimi *et al.* (2016) reported this value varied from 13 (Sarabi) to 107 (Mazandarani).

Deng *et al.* (2019) reported the decreased pattern of N_e from 1,000 to 100 generations ago across the purebred and crossbred buffalo population, showing the effect of historical process of domestication, breed formation and artificial selection. On the contrary, Fallahi *et al.* (2019) showed N_e of 422 in current population of Azerbaijani river buffalo, indicating enough diversity in the population. Santana *et al.* (2011) studied N_e on dairy buffalo in Brazil (Murrah buffalo) using pedigree data, and reported a very low amount for N_e (n = 40). Despite this low amount in the N_e in the Murrah buffalo, Malhado *et al.* (2013) reported that the N_e is 10 in the other buffalo breeds in Brazil (Jafarabadi buffalo). It seems that particular mating system should strictly be considered to prevent highly inbreeding in these populations.

The difference in N_e reflects the events occurred in the population throughout history. Therefore, the N_e describes as a part of the evolutionary history of the population and provides valuable information for conservation, especially in the indigenous breeds. Although the N_e in this study (n = 240) is higher than the appropriate threshold proposed by Frankham *et al.* (2014) (n = 100), given the fact that breeding buffalo has been more considered in the country due to the high yield of milk and meat production in recent years, the more severe decline has been observed in the N_e in recent years. For this reason, it is necessary to consider various solutions such as designing appropriate mating systems and creating gene pools.

Although a new insight has been displayed for understanding the genetic diversity of water buffalo through the results of ROH analyses, it was nearly impossible to compare our results with other studies because there is no study concerning buffalo breed and other studies have different sampling strategies and methods in different breeds.

In this study, 59 % of the observed ROH had a length less than 10 Mb, and 41 % of ROHs had a length over 10 Mb. Hence, it seems that both ancestral and recent inbreeding occurred in the Khuzestani buffalo population over the years. As mentioned before, despite obtaining the average inbreeding of 0.045 for this population, this value was different among animals in the population, so that some animals showed higher inbreeding than the average. It seems that these values should be considered in the design of mating system and selection. Ghoreishifar *et. al*. (2019) recently reported relatively low average inbreeding (0.023) in Zandi sheep. Mastrangelo *et al.* (2018) examined the average FROH in Italian sheep breeds. They showed that the Valle del Belice breed had the highest inbreeding (0.099) and the Comisana breed had the lowest inbreeding (0.016). Various studies on the cattle breeds show that selection intensity for bull, artificial insemination and embryo transfer in some breeds have been widely used in recent years, influencing N_e, genetic diversity and homozygosity levels. Shokrollahi *et al.* (2009) using microsatellites, reported that the expected heterozygosity in the population of Iranian buffalo varied from 0.69 (Guilani) to 0.78 (Khuzestani). Colli *et al.* (2018) recently implemented one of the most comprehensive studies on genomic diversity in buffalo by integrating the buffalo populations around the world, including the Khuzestani river buffalo. The average observed heterozygosity in their study in different buffalo populations was varied from 0.302 (swamp buffalo) to 0.481 (Aza-Kheli). High heterozygosity (0.386) and low-to-medium inbreeding (0.045) in the Khuzestani river buffalo may be due to the absence of intense artificial selection, suitable effective population size and type of commuting small-holing breeding system in the indigenous buffalo populations in Iran. However, because of low sample size, the estimated parameters should be interpreted with caution.

CONCLUSION

This study has reported the characterization of haplotype blocks and the level of genetic diversity

for Khuzestani river buffalo. Our results can give extra precious information in order to help performing the GS and GWAS in buffaloes perfectly. Furthermore, the results of distribution of ROH revealed that ancient and recent inbreeding have had an influence on the genome of the Khuzestani buffalo population. Although the average of inbreeding was low-to-moderate, some animals showed much higher inbreeding rather than the average and, therefore, this information should be considered in the design of mating system and selection.

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