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Research Article

Genomic Diversity and Autozygosity-Based Signatures of Selection in Kangal Akkaraman Sheep via Genotyping-by-Sequencing

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Abstract

In this study, genome-wide variability and selection signatures in Kangal Akkaraman sheep were assessed by 238.103 bi-allelic single nucleotide polymorphisms (SNPs) recovered from genotyping-by-sequencing (GBS) libraries processed in Illumina HiSeq X Ten instrument. Summary statistics of genetic diversity such as minor allele frequency (MAF), observed (H_O) and expected (H_E) heterozygosity were estimated at 0.32, 0.29, and 0.30, respectively. A declining trend in effective population size was observed through generations in which the current population was estimated at 978 individuals 150 generations ago. 608 of 647 runs of homozygosity (ROH) islands were classified into ≤2 Mb. Strong selection signals were identified in thirteen genomic intervals overlapped with 17 protein-coding genes. The sheep quantitative trait locus (QTL) database confirmed that these genomic regions were associated with economically important traits such as milk content *(KCNH5, KCNH7, LRP1B, SNAPC1,* and *SYT16)* and fleece yield *(CCDC85A*, *EFEMP1*, and *PPP4R3B),* parasite resistance *(MMS22L* and *KLHL32),* fat deposition in the tail *(JAZF1, TAXIBP1, EVX1,* and *HOXA13),* and water-holding capacity *(KLHL1* and *DACH1).* This study implies that the Kangal Akkaraman sheep will play a vital role in developing some genotypes tolerant to environmental challenges, parasite infections, fat deposition, and water-holding capacity in the future. Still, the other native sheep should be screened to identify genomic regions under selection practices using high-density genetic data obtained from next-generation sequencing (NGS) platforms.

Keywords: Genetic variation, Genotyping-by-sequencing, Kangal Akkaraman, Molecular genotyping, Selection signals, Selective breeding

Introduction

Mainly distributed in Central Anatolia and nearby places, Akkaraman is the most raised breed with an approximately 40-45% proportion among the native Turkish small ruminant population [1]. Both systematic and non-systematic selection applications have been done in the Akkaraman breed by breeders in which Kangal Akkaraman has been derived from systematic selection management [2]. Kangal Akkaraman breed has been developed to increase body weight phenotype [3], whilst it is raised for meat and milk production in Sivas and nearby provinces [4]. There are ongoing debates about whether Akkaraman-derived sheep are distinct enough from their ancestral population in terms of morphology and genetic structure to categorize them as "variety" or "breed". For example, a documentary published by the General Directorate of Agricultural Research and Politics (GDARP) has categorized Kangal Akkaraman as a distinct breed [4], whereas several studies have stated that this population remains a variety of Akkaraman [2,3]. It seems that further studies using both detailed phenotypic records and whole genome-based phylogenetic analyses are required to enlighten this obscurity.

Variations in the genome are the main reason causing differences among individuals in terms of morphological, psychological, behavioral, and adaptive traits. These variations have been utilized by human beings for diverse purposes throughout history. As highlighted by the Food and Agriculture Organization of the United Nations (FAO) [5], thanks to the manipulation of genetic variations for environmental requirements and economic interests, approximately 8.774 breeds belonging to 38 major animal species have been developed since domestication. Variations in a population including all breeds and varieties are called genetic diversity. Farmers exert great efforts to keep genetic diversity at an optimal level to maintain

their production for current and future demands. In fact, genetic variations in related genes not only improve survival traits in livestock species but also play a pivotal role in selection studies to enhance economically important traits such as meat and milk. On the contrary, intensive selection practices conducted for a specific purpose across several generations may lead to pressure over the genome. This kind of selection is of potential to significantly decrease genetic variations, known as selection signatures, not only in the corresponding genes but also in nearby neutral genomic regions [6].

Periodically monitoring genetic diversity is a beneficial way to shape conservation studies as well as selection practices against environmental factors negatively affecting sustainable animal production in the future. Depending on the type of genetic data, genetic diversity could be estimated via numerous statistics such as the number of alleles, number of effective alleles, nucleotide diversity, minor allele frequency (MAF), observed heterozygosity *(H_O),* expected heterozygosity *(H_E),* and inbreeding level [7]. On the other hand, selection signatures studies are promising to obtain a deeper knowledge of the past breeding history of farm animals and detect regions subjected to recent and long-term selection pressure. Several alternative approaches are available to scan selection signatures at the genome-wide level within and between populations in which runs of homozygosity (ROH) is one of the popular approaches. ROH is defined as the DNA segments holding consecutive homozygous genotypes in an individual due to parents transmitting identical haplotypes to their offspring [8]. Longer ROH segments indicate recent inbreeding, whereas shorter consecutive homozygous segments are considered a sign of long-term selection pressure [9]. However, both analyses (genetic variability and selection signatures) require high-density genomic data to effectively carry out selection and conservation studies.

Fortunately, thanks to rapid advances in molecular genetics, single nucleotide polymorphism (SNP) arrays and next-generation sequencing (NGS) platforms have been developed to recover genetic data across the whole genome. SNP arrays are commonly used for genotyping farm animals due to their simplicity, whereas they possess some disadvantages. Indeed, as highlighted by Bilginer et al.[7], SNP arrays have been developed based on some reference breeds and numerous variations related to environmental adaptation in local breeds could be neglected. Unlike, NGS platforms may overcome this bias since genetic data are recovered randomly across the whole genome. Of these platforms, genotyping-by-sequencing (GBS), one of the reduced representations of genomic libraries, relies on a single restriction enzyme following the barcode ligation and pooling processes [10]. Short reads including barcode information could be processed via different sequencing platforms with a single-end or paired-end mode. Restriction enzyme-based techniques including GBS not only allow for facilitating the complexity of the whole genome but also create an opportunity for higher sample multiplexing which significantly decreases sequencing costs [11].

Although NGS platforms are becoming cost-efficient and more applicable, there is a lack of studies in the literature to focus on screening genetic diversity and selection signatures in Kangal Akkaraman sheep. In this context, this study aims to reveal genetic diversity, ROH characterization, and selection signatures in Kangal sheep via high-density SNP data obtained from GBS library preparation combined with the Illumina HiSeq X Ten sequencing platform.

Material and Methods

Ethical Statement

This study was approved by the Akdeniz University Animal Experiments Local Ethics Committee (Approval no: 1691/2024.04.004).

Sampling and DNA Extraction

Twenty-five animals (5 male and 20 female) belonging to the Kangal Akkaraman breed were sampled from four different herds reared in Sivas province. Oral interview with breeders was utilized to select unrelated animals. Blood samples taken from the jugular vein were subjected to the GeneJET Genomic DNA Purification Kit (Thermo K0721) following the manufacturer's recommendations in order to isolate DNA. DNA quality and quantity were checked by NanoDrop 2000 (Thermo Scientific) and Qubit 4™ (ThermoFischer Scientific), respectively, in which DNA quality ranged from 1.79 to 1.83 at 260/280 OD, while DNA concentrations varied between 34.7 ng/µL and 38.2 ng/µL across the samples. Isolated DNA was optimized for all samples at 30 ng/µL before genomic library preparation was performed.

GBS Library Preparation and Illumina Sequencing

GBS libraries were prepared by using the Eco*RI* (New England Biolabs) restriction enzyme and 25 universal indexed adapters recommended by the Illumina. Briefly, DNA was digested overnight with restriction enzyme at 37°C. Adapters were ligated to DNA fragments using T4 DNA ligase (Invitrogen) at 22°C for an hour and cleaned with AmPureXP (Beckman Coulter) beads. DNA libraries were enriched with the Polymerase Chain Reaction (PCR) technique and pooled libraries were sequenced via single-end mode in the Illumina HiSeq X Ten platform (1x150 base pair).

Variant Calling and Filtering

The Stacks 2 program [12] was employed to assign short reads to individuals according to their barcode information. Assigned reads were processed by the fastp software [13] with default parameters for quality trimming and adapter removal. Burrows-Wheeler Aligner [14] was run with default settings to align clean reads to the reference genome of *Ovis aries* (ARS-UI_Ramb_v3.0). BCFtools pipeline [15] was chosen to call the variants in which only bi-allelic SNPs passing the criteria of read depth ($20 \leq D \leq 500$) and base quality score ($Q \geq 20$) were kept. On the contrary, all the InDels and SNPs not located on autosomal chromosomes were excluded from the data set. The remaining SNPs were processed via PLINK 1.9 software [16] in order to recover SNPs with high genotyping rates (--geno 0.1) and MAF values (--maf 0.05). In the last step, animals with a low genotyping rate (--mind 0.1) were excluded to obtain the final data set.

Statistical Analysis

Genetic diversity parameters such as MAF, H_O, and H_E, in the Kangal Akkaraman breed were calculated in PLINK 1.9 software [16]. The historical effective population size was estimated via SNeP v.1.1 tools with default parameters described by Barbato et al.[17]. The results of effective population till 150 generations ago were visualized by the *plot* function implemented in the R environment [18]. The detectRUNS package [19] implemented in the R environment [16] was run with a consecutive runs approach to analyze genomic inbreeding value derived from ROH (F_{ROH}), ROH characterization, and selection signatures. ROH islands were defined according to the following criteria: i) the minimum number of consecutive SNPs was optimized at 15, ii) the minimum length of a ROH was set to 1 Mb, iii) the maximum gap between consecutive homozygous SNPs was 1 Mb, iv) the maximum two SNPs with missing genotypes and up to one heterozygous were allowed in a ROH. Based on their physical length, each ROH island was categorized into 0 to <2 Mb, 2 to <4 Mb, 4 to <8 Mb, 8 to <16 Mb, and ≥16 Mb clusters. The number of ROH island per each aforementioned ROH length class and chromosomes were calculated. SNPs passing ROH characterization were visualized for all autosomes in the Manhattan plot command of the "*qqman*" package [20] implemented in the R environment [18]. 0.1% of SNPs based on empirical distribution were considered to be under selection pressure Genomic windows of 200 kb (100 kb upstream and 100 kb downstream of the significant SNPs) were screened to detect overlapping protein-coding gene segments. The genes overlapping these segments were confirmed via the *Genome data viewer* module of the *National Institutes of Health (NCBI)* platform [21] by choosing the options of ARS-UI_Ramb_v3.0 assembly. To validate the effects of genes under selection pressure on phenotypic traits, the sheep QTL database *(SheepQTLdb)* [22] was checked.

Results

In this study, nearly 185 million cleaned reads ranging from 5.7 to 12 million per individual were recovered by the GBS technique. A large part of clean reads (94%) were mapped to the reference genome successfully. A total of 1.337.343 SNPs and 86.656 InDels were obtained in the variant calling process, whereas only 238.103 bi-allelic SNPs and 22 individuals passed the filtering criteria. Genotyping rate was 100% indicating that 22 animals possessed alleles regarding 238.103 SNPs without missing genotypes.

Genome-wide analysis revealed that observed heterozygosity (0.29) was slightly lower than expected heterozygosity (0.30) in Kangal Akkaraman sheep. MAF value was estimated at 0.32, while the ROH-based inbreeding coefficient (F_{HOM}) was close to zero (0.01).

The historical changes in population size were assessed via linkage disequilibrium (LD) in which a declining trend was detected across generations. For example, the current 22 individuals turned out to be descended from 100 individuals 13 generations ago *(Fig. 1)*. Moreover, 54 generations ago, the ancestral population was estimated at 378 individuals, whereas the current population was represented by 978 animals 150 years ago *(Fig. 1)*.

Via consecutive runs algorithm, a total of 647 ROH islands were detected. According to their physical length, 608 and 39 were clustered into 0-2 and 2-4 Mb classes, respectively. No ROH islands were detected to be higher than 4 Mb in the Kangal Akkaraman breed. At the chromosome level, the lowest (2) and highest (97) numbers of ROH islands were detected in autosome 19 and 12, respectively. Strong selection signals were detected at thirteen different genomic intervals distributed to ten chromosomes *(Fig. 2)*.

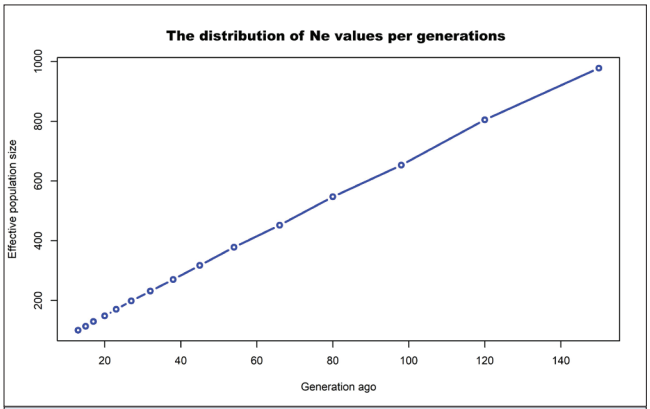

Fig 1. The estimated effective population size in the Kangal Akkaraman sheep for the past 150 generations

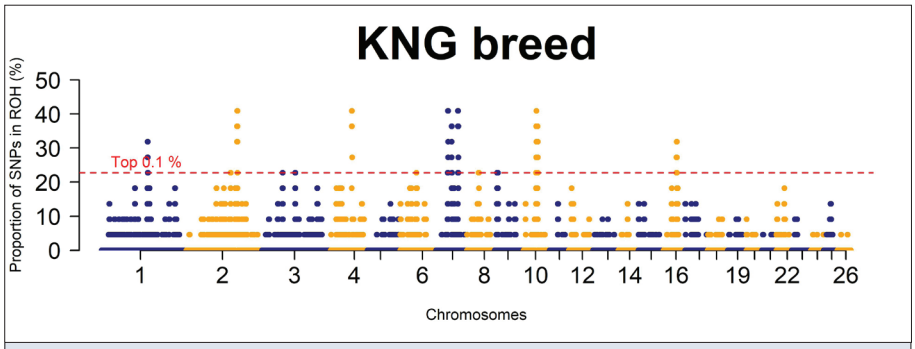

Fig 2. Manhattan plot of the distribution of ROH segments across the autosomal chromosome in Kangal Akkaraman sheep (red line indicates the threshold of top 0.1% SNPs in each breed)

Table 1. *ROH-based genes under selection pressure in Kangal Akkaraman sheep and their effects on phenotype*

Chr	SP	EP	NS	Corresponding Gene(s)	QTL-Related SNPs	Effect(s) on Phenotype
1	151258693	152646935	18	-	-	-
2	146570469	146940231	16	*KCNH7*	2	Milk content
	167861018	169409445	25	*LRP1B*	6	Milk content, fat density, and total protein level in blood
3	67843043	68475055	35	*CCDC85A*, *EFEMP1*, and *PPP4R3B*	5	Fleece yield and bone density
	109115550	110132697	37	-	-	-
4	69828415	71081065	28	*JAZF1*, *TAX1BP1*, *EVX1*, and *HOXA13*	6	Tail fat deposition
6	53545871	53772381	15	-	-	-
7	38305455	39658987	25	*MDGA2*	-	-
	71989011	73094768	23	*SNAPC1*, *SYT16*, and *KCNH5*	6	Milk content, bone density, and body circumference
8	38930308	39921843	15	*MMS22L* and *KLHL32*	2	Fecal egg count
9	10156994	11032974	34	-	-	-
10	41451793	47763646	63	*KLHL1* and *DACH1*	3	Water holding capacity
16	43064491	44451481	29	-	-	-
Total	-	-	363	17	30	-

Chr: *Chromosome,* ***SP:*** *Start Position,* ***EP:*** *End Position,* ***NS;*** *Number of detected SNPs*

Based on the empirical distribution of the proportion of SNPs in ROH islands, a total of 363 SNPs passed the top 0.1% criterion *(Table 1)*. The lowest number of SNPs (15) under selection pressure was observed in chromosomes 6 and 8, while 63 SNPs were identified in chromosome 10. Although strong selection signals were detected in chromosomes 1, 6, and 9, no protein-coding genes were identified in the related genomic intervals *(Table 1)*. On the other hand, a total of seventeen protein-coding genes *(CCDC85A, DACH1, EFEMP1, EVX1, HOXA13, JAZF1, KCNH5, KCNH7, KLHL1, KLHL32, LRP1B, MDGA2, MMS22L, PPP4R3B, SNAPC1, SYT16,* and *TAX1BP1)* were present in the remaining genomic intervals *(Table 1)*. It has been confirmed via the sheep QTL database that a total of 30 QTL-associated SNPs overlapped with genomic regions under selection pressure which turned out to be associated with several phenotypes such as survival traits (water holding capacity and fat deposition in tail), resistance to parasites, morphology (bone density and body circumference), and economically important traits (milk content and fleece yield).

Discussion

The genetic diversity parameters in native Turkish sheep breeds have been mainly monitored via microsatellite markers [2,3], while two studies utilizing the bi-allelic SNP

data have been recently published to assess genome-wide genetic variability in several Anatolian sheep populations [23,24]. An Illumina OvineSNP50 array-based genotyping revealed that heterozygosity ranged from 0.34 to 0.35 with negative inbreeding values among three native sheep populations known as Sakız, Karakaş, and Norduz [23]. Another recent study conducted by Karsli [24] confirmed that the average MAF value was 0.31, whereas heterozygosity ranged from 0.29 to 0.31 in four Anatolian sheep populations (Akkaraman, Güney Karaman, Karakaş, and Morkaraman) which were genotyped via ddRADseq libraries sequenced with Illumina HiSeq X Ten instrument. Besides, negative inbreeding coefficient values were reported for all sheep populations [24]. The current study showed consistent results in terms of inbreeding value (0.01) with previous studies. It is not surprising to detect a low inbreeding in this study because unrelated animals were chosen based on oral interviews with farmers. A lower heterozygosity (0.29) was detected in the Kangal Akkaraman breed compared to findings reported by Bayraktar [23], while similar values of MAF and heterozygosity were observed with findings declared by Karsli [24]. This finding could be attributed to differences between genotyping tools in which SNP arrays scan the previously known variations while the methods of the reduced representation of the whole genome such as GBS and ddRADseq randomly detect genetic variations which could be less polymorphic across the studied populations. As highlighted by Bilginer et al.[7], who comprehensively reviewed several molecular genotyping methods for revealing genetic diversity, NGS platforms such as ddRADseq and GBS are advantageous over microsatellites and array technologies due to covering a larger part of the genome and allowing for variations specific to local populations. Indeed, Bayraktar [23] estimated genomic diversity via 46.314 SNPs in Sakız, Karakaş, and Norduz, while the current study (238.103 SNPs) and Karsli [24] (296.097 SNPs) benefit from higher-resolution genetic data to calculate genome-wide genetic variability in Anatolian sheep.

It was concluded from LD-based analysis that the effective population size of the Kangal Akkaraman breed has decreased from one generation to another. The current 22 individuals were validated to be represented by approximately 1000 animals 150 generations ago. Unfortunately, the historical effective population size of native Turkish sheep breeds has not been calculated via genetic data till now. However, several studies mentioned that the effective population size of native Anatolian sheep breeds has decreased due to uncontrolled breeding systems [2,25], while some breeds such as Güney Karaman and Çine Çaparı have been reported to be on the brink of extinction [26,27].

Compared to genetic diversity studies, revealing genomic regions under selection pressure is a new field of study in Türkiye. Indeed, Demir et al.[6] have recently identified several genes related to visual modality *(LGSN),* olfaction *(MOXD2, OR4C1F,* and *OR4C1E),* and immune response *(TRBV3-1* and *CLDN10)* were under selection pressure in six native Turkish cattle breeds which were genotyped with 211.119 SNPs recovered from ddRADseq technique. On the contrary, NGS-based studies aiming to assess selection signals in numerous livestock species such as sheep, goats, chickens, and geese reared in Türkiye are required to obtain deeper knowledge about their past breeding practices. Therefore, the current study is of significant potential to enlighten genomic regions under selection practices. Indeed, strong selection signals were detected in seventeen protein-coding genes in the Kangal Akkaraman breed which were further confirmed to possess a total of 30 QTL-associated SNPs. A survey of the sheep QTL database validated that some of these genes *(KCNH5, KCNH7, LRP1B, SNAPC1,* and *SYT16)* were associated with economically important traits such as milk content and fleece yield. On the other hand, it is known that native Turkish sheep breeds including Kangal Akkaraman are well-adapted to environmental challenges [28]. Indeed, this study revealed that several genes under selection pressure cover some fixed SNPs related to parasite resistance *(MMS22L* and *KLHL32),* fat deposition in the tail *(JAZF1, TAXIBP1, EVX1,* and *HOXA13),* and water-holding capacity *(KLHL1* and *DACH1).* Moreover, a large part of ROH islands (93.97%) were shorter than 4 Mb indicating that the corresponding genes have been subjected to selection practices for the long term which allowed animals to develop adaptation against environmental challenges. Of these environmental challenges, parasite infections negatively affect health, welfare, and productivity in susceptible animals [29], whereas tolerant animals are of the ability to maintain their production level regarding economically important yields. Fat deposition and water storage capacity play a vital role in surviving in animals reared in grassland. Indeed, as highlighted by Xu et al.[30], fat deposition is an indispensable element for animals thereby harsh environmental stressors such as drought seasons, extreme cold winters, and food shortages could be tolerated by conserving a valuable energy reserve. Due to ongoing global warming, on the other hand, water scarcity will be one of the most threatening environmental challenges in arid and semi-arid regions by causing negative effects on health and reproduction in sheep [31].

In conclusion, the Kangal Akkaraman breed was screened at a genome-wide level to investigate genomic diversity, effective population size, and selection signatures. It was observed that the Kangal Akkaraman breed conserves

sufficient genetic variability across the genome, while effective population size is declining through generations. The authorities should take solid action to prevent this trend in the future. Moreover, the Kangal Akkaraman breed should be subjected to comprehensive conservation programs due to their adaptability to harsh environmental conditions. Indeed, several studies have mentioned that native Turkish sheep populations are well-adapted to their environment. This study conducted at the genome-wide level confirms that several genes associated with environmental adaptation such as parasite resistance, fat deposition, and water storage capacity have become fixed in the Kangal Akkaraman breed. Since the negative impacts of global warming will become more threatening in the future, fat deposition and water storage capacity will be an indispensable part of selection studies. In this context, the Kangal Akkaraman breed will play a vital role in developing selection strategies against environmental challenges. It is noteworthy that the other native Turkish sheep breeds may hold some advantageous genotypes related to survival traits. Therefore, it is recommended that further studies should focus on unraveling fixed genomic regions in other native Anatolian sheep via high-density SNP data obtained from NGS platforms.

Declarations

Availability of Data and Materials: Genomic data used in this study are available from the corresponding author (E. Demir) upon a scientific request.

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