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Whole Genome Resequencing of Jordanian Awassi Rams (*Ovis aries*) Using Hiseq Sequencing Technology: The First Step Towards Sheep Genomic Selection

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ABSTRACT. We report the whole ram genome of Jordanian Awassi (*Ovis aries*), screened using the power of Illumina HiSeq sequencing technology, providing insights into the ram's genomic structure. Generated data will help in the assessment of naturally occurring genetic variation and population structure of this sheep breed using different structure variation markers. Also, it will help in the established marker-trait associations that can be used in marker-assisted breeding for qualitative and quantitative productive traits. A total of 23,812,247 single

nucleotide polymorphism (SNPs) were identified, of which 177,117 (0.74%) were in the coding regions, as well as 3.77 million insertions/deletions and 3357 frame-shifting mutations in the coding region. The re-sequencing revealed 38,900 structure variation types distributed along genome, including 45 insertions and 16,643 deletions types. Also, there were 13,689 copy number variations, of which 3743 were up-regulated, and 9946 were down-regulated. These results will help in describing SNPs and the distribution of structural variations types used in genetic mapping and breeding programs of sheep breeds.

Key words: *Ovis aries;* Sheep; SNPs; Insertions / deletions; Copy number of variations

INTRODUCTION

Awassi sheep (*Ovis aries*) is the dominant fat tail sheep breed in the Mediterranean countries, including Jordan. It has many unique characteristics, including acceptable performance under harsh conditions and its preferred meat quality by Mediterranean consumers (Tabbaa et al., 2001). Also, it has various contributions to the vast majority of the livelihood of the Bedouins, making it a superior sheep breed compared to imported sheep. Awassi sheep in Jordan are well characterized for their carpet wool, body weight, and morphological characteristics at different ages (Tabbaa, 1998 and 2003). Semen of Awassi rams and reproductive characteristics of Awassi ewes have been characterized (Tabbaa et al., 2006 and 2008).

Over the last three decades, reports on releasing and developing sheep genome have appeared. Crawford et al. (1995) showed a detailed genetic linkage map of sheep covering 2070 cM of the sheep genome. Two years later, Barendse et al. (1997) reported a new genetic linkage sheep map with 446 highly informative markers. In 2001, a sheep genic linkage map was generated with more than 1000 loci (Maddox et al., 2001). In the year 2009, the sheep genome announced and identified expressed genes associated with the biology of the rumen and lipid metabolism (Jiang et al., 2014). In the same year, a linkage map for males from a single-family of Awassi-Merino backcross progeny reported (Raadsma et al., 2009). Studying the genome of the male sheep (Ram) is a characteristic of interest to sheep breeders and geneticists.

Evidence from Mediterranean archaeozoological sites suggested that sheep were first domesticated in the nearby Arabian Peninsula in the Fertile Crescent region in Southwest Asia 11,000 years ago (Zeder, 2008) and spread out from these domestication centers into the world during the subsequent few thousand years (Chessa et al., 2009). Humans reared sheep for s meat before wool and milk were used (Chessa et al., 2009). Following their domestication and selection, they have been adapted to a diverse range of environments. The implementing of quantitative genetics and the use of artificial insemination to prioritize superior rams has resulted in a vast increase of genetic gain in sheep breeding (Kijas et al., 2012)

Various types of molecular genetic evidence show that Jordan Awassi has common haplotype groups (B and A) and group C with Saudi, Kuwaiti, Egypt, and Yemen sheep considering mitochondrial DNA (mtDNA) and microsatellite-based evidence (Al-Atiyat et al., 2018; Jawasreh et al., 2018). The National Agricultural Research Center in Jordan set up to do whole genome sequencing for 100 Jordanian native species, including plants, animals, and insects. The main aims were to facilitate access to and use data incorporated in the international domain (NCBI) and to find genetic variants in the populations studied. The project started sequencing the native honeybee; *Apis mellifera syriaca* (Wallberg et al., 2014; Haddad et al.,

2016), the Oriental Hornet, *Vespa orientalis* (Haddad et al., 2017), and in 2019 three genomes were completed, which were the Awassi ram, Awassi ewe, and ancient Jordanian olive.

Jordan Awassi revealed a high level of genetic variability based on molecular markers (Al-Atiyat et al., 2012). Jawasreh et al. (2011) reported for the first time, the new Sagri separate Awassi strain reported to be of distinct characteristics found in Jordan Valley and started to distribute in Jordanian governorates. In Jordan, studies on Awassi sheep involved genetic diversity and genetic structure and its relatedness with other sheep breeds (Al-Atiyat et al., 2014; Jawasreh et al., 2018), genetic parameters of some essential genes and their association with some economic traits (Jawasreh et al., 2007, 2010, 2012, 2017, 2018 and 2019).

National sheep breeding strategy in Jordan is based on ram selection, including an advanced genotyping test for economic traits and identified day blindness mutation found in imported improved Awassi (Jawasreh et al., 2007, 2010, 2012 and 2017). Genetic mutation of reported Microtia (earless) controlled by GATA-6, was also investigated using genome-wide association (GWAS) (Jawasreh et al., 2016). Advanced genome selection techniques will be required to speed-up Awassi sheep improvement, putative selection, understanding disease inheritance, and get insights into the genetic control of milk, meat, and wool production. Overall, increasing our knowledge of Awassi sheep evolution and breeding was the principal motivation for the current resequencing of Jordanian Awassi (*Ovis aries*) Ram whole genome.

MATERIAL AND METHODS

Animal Samples

Animal care and use approved all experimental protocols involving animals with the ethical approval of relevant national legislation (Approval Code No. (G/6) 2006): Instructions and conditions for the acquisition of test animals and testing them).

Whole-blood samples (10 mL) collected from 13 rams from three main Awassi sheep producing areas in Jordan kept in the freezer till DNA extraction. Genomic DNA extracted using the DNA purification kit (Promega, Wisconsin, USA) following instructions of the manufacturer. Genomic DNA was fragmented, and after electrophoresis, DNA fragments of desired length was gel purified. Adapter ligation and DNA cluster preparation were performed and subjected to the Ilumina Hiseq 2500 sequencing system (Figure 1).

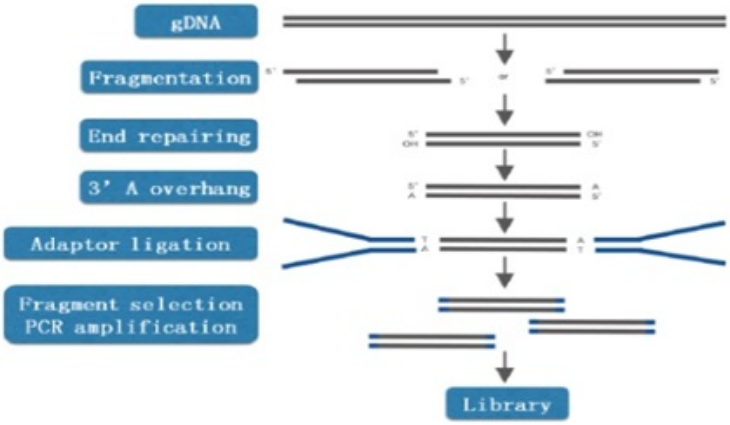

Figure 1. The detailed workflow of the "Quantification Pipelines" module in Panel used in the whole genome resequencing of Jordanian Awassi sheep.

The pipeline of Bioinformatics Analysis

The sequencing data generated from the Illumina pipeline started with the removing of the adapter sequence, and the low-quality reads discarded to produce clean data. The next step was to use Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/) (Li and Durbin, 2009) for aligning reads to the reference sequence. BAM format files used to store the alignment information. After fixing mate-pair information, adding read group information, and marking duplicate reads caused by polymerase chain reaction, variant calling, and detecting of Single Nucleotide Polymorphisms (SNPs) and small Insertion/Deletions (InDels) by GATK software (https://www.broadinstitute.org/gatk/) (McKenna et al., 2010). BreakDancer software (http://breakdancer.sourceforge.net/) (Chen et al., 2009) used for identification of Structure Variants (SVs) and Copy Number Variants (CNVs). At each stage of the analysis, quality control applied for the alignment and the called variant (Figure 2).

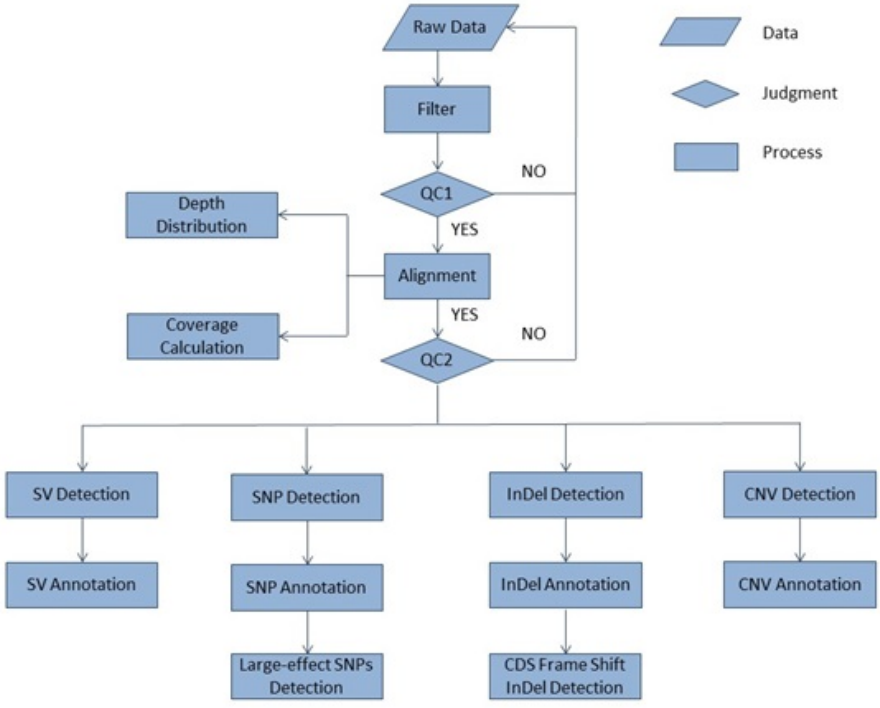

Figure 2. The pipeline of Standard Bioinformatics Analysis. The pipeline based on the family of SOAP software.

Filter Reads

Raw reads contain adapters, unknown or low-quality bases discarded. The parameters of SOAP nuke (unreleased) software for this project is: "-n 0.05 -l 20 -q 0.2 -G -Q 2". The filter steps included removing reads more than half of the bases' qualities are less than five and get clean reads.

Alignments

Sequencing reads aligned using BWA software (http://biobwa.sourceforge.net/bwa.shtml) (Li and Durbin, 2009) to the reference genome. The short sequence aligns against long the reference providing the output of the sequence in SAM (Sequence Alignment/Map) format. Picard-tools (v1.118) were used to sort the SAM files by coordinate and converted them to BAM files. Genome Analysis Toolkit (GATK) ignored duplicate reads in later analyses. Picard tools software (v1.118) was also used to mark these duplicates.

SNP and Short InDel Detection

Single Nucleotide Polymorphisms (SNPs) and short InDels in our pipeline detect using GATK. After the identifying of SNPs or/and InDels, we use the programs developed by BGI to do annotation and classification of SNPs in gene regions. It provides statistics to present the distributing

Structure variation (SV) Detection

The read-pair method used to detect the structural variation. We use BreakDancer/CREST to detect SVs. A detailed description of the method used by BreakDancer found at http://breakdancer.sourceforge.net (Chen et al., 2009). Using reference genome our reads disturbed, which could be because of existing of deletions or insertions between the two mapped reads. The result is a list of structure variations detected at the whole genome level.

Copy Number Variation Detection

According to the method of CNVs detection (Zheng et al., 2011), based on the result of SOAP alignment, the depth of each base calculated and standardized by the mean depth of its chromosome to calculate the Copy Number Variation. Based on this theory, CNVs detected according to standard criteria, where, based on the base depth of each fragment, P-value ≤ 0.35 and mean depth < 0.5 or > 2.0 for probability to be CNV.

The genome raw data sequences of Jordanian Awassi sheep have been deposited in the NCBI under SRA (sequence reads archives) under the following accession numbers: PRJNA574879. SRA records will be accessible with the following link after the release date: https://www.ncbi.nlm.nih.gov/sra/PRJNA574879.

RESULTS

Sequencing Assessment

For ram samples, libraries with 64 paired-end sequencings with an insert size around 300 bp constructed. The resequencing yielded 100.09 G as clean databases in total. To ensure quality, the raw data changed by deleting the adapter pollution in reads and then the reads, which 1,929,009 contain over 50% low-quality bases (quality value ≤ 12)

removed. The summaries of data and quality control shown in Table (1) and Figures 3 and 4. The Q20 base rate of each lane is above 98.73%, so the data quality is very high.

Table 1. Summary of the sequencing results data clean reads in the Jordanian Awassi ram Sheep.

Sequence	Value
GC rate	43.35%
Q20 rate	98.73%
Q30 rate	93.95%
Reads	1000.95 Mb
Bases	100.09 Gb
Clean Data/Raw	94.63 %

Mb and Gb; mega and giga base pairs.

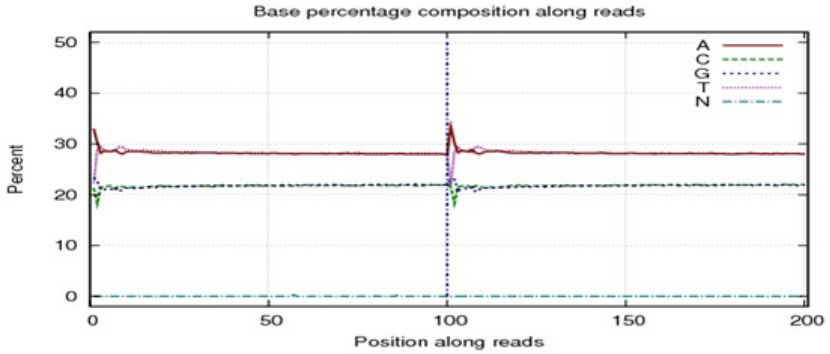

Figure 3. Distribution of base composition on clean reads, X-Axis positions, along with reads. Y-Axis means the base content rate. A curve overlapped with the T curve while the G curve overlapped with the C curve except for the first several bp positions (For Illumina sequencing platform, the random hexamer-primer used to synthesize the cDNA could cause PCR bias. So there are significant fluctuations in the first several bp positions along with reads, which is a typical situation). If an abnormal condition happens during sequencing, it may show an unbalanced composition

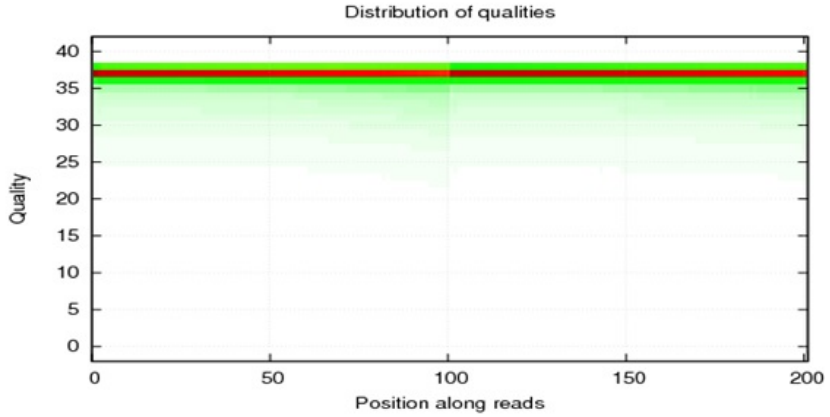

Figure 4. Distribution of base quality on clean reads, X-axis positions along with reads. Y-axis is quality value. E8, 181,200ach dot in the image represents the quality value of the corresponding position along with reads. If the percentage of the bases with low quality (< 20) is very high, then the sequencing quality of this lane is inadequate.

The reference genome, Sheep. Fa (Jiang et al., 2014) has taken for this project. The genome size is 2,584,832,510 bp and the effective size is 2,560,768,911 bp. Burrows-Wheeler Aligner (BWA) software used for sequence reads alignment to the reference genome. The mapping rate and the final effective mapping depth in different accessions was 99.28%, and 36.32 (Figure 5). Figures 6 and 7 show the distribution of sequencing depth and its cumulative depth besides the insert size distribution based on the result of the alignment.

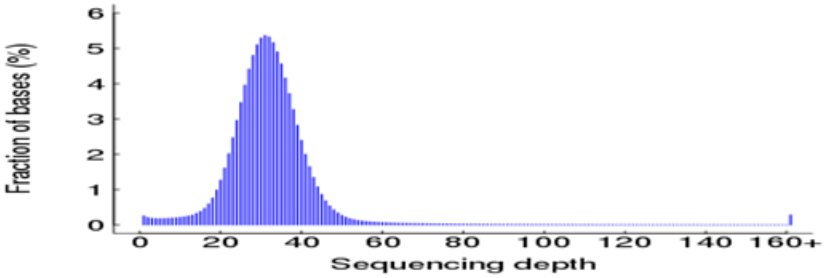

Figure 5. Sequencing depth histogram. The X-Axis is the sequencing depth, and Y-axis means the rate of the sequencing depth.

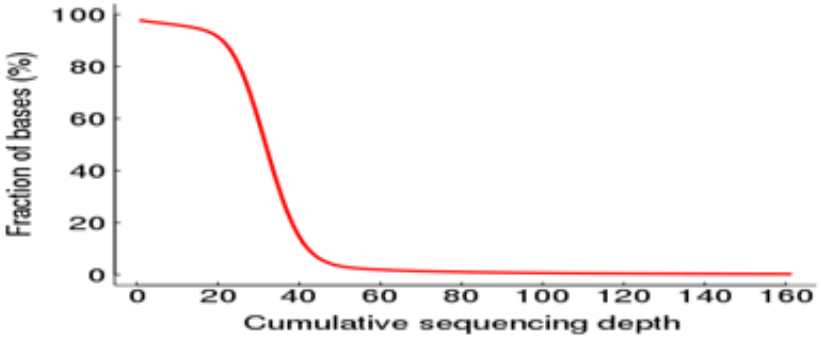

Figure 6. Distribution of cumulative sequencing depth. The X-Axis is the cumulative sequencing depth, and Y-axis means the rate of the cumulative sequencing depth.

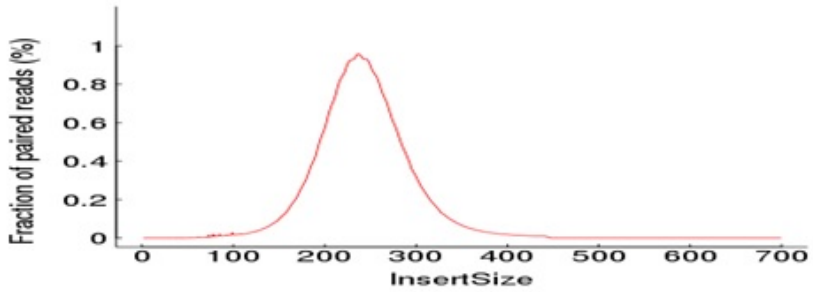

Figure 7. Insert size Distribution of Paired Reads. The X-axis represents the insert size of paired reads, and Y-axis shows the fraction of paired reads with an insert size.

SNP detection and annotation

SNPs are small differences but with great impact on the variation of genomes and the biological traits. Based on the consensus sequence (all SNP we got), the polymorphic loci between the identified genotype and the reference can filter, and a high fidelity SNP data set generated (Table 2). 23,812,247 SNPs among this sheep genome, of which 177,117 (0.74%) SNPs in the coding regions identified. The SNP results listed as CDS, exon, and genes. The distribution of SNP in the reference genome presented in Figure 8.

Table 2. Summary of genome-wide SNPs and the annotation distribution in the Jordanian Awassi ram Sheep.

SNP distribution	Value
SNP	20,039,103
Homozygous	2,403,946
Heterozygous	17,635,157
Synonymous CDS	161,420
Nonsynonymous CDS	107,116
Exons	842,118
genes	8,181,200
mRNA	23,235,273

CDS: Coding sequence.

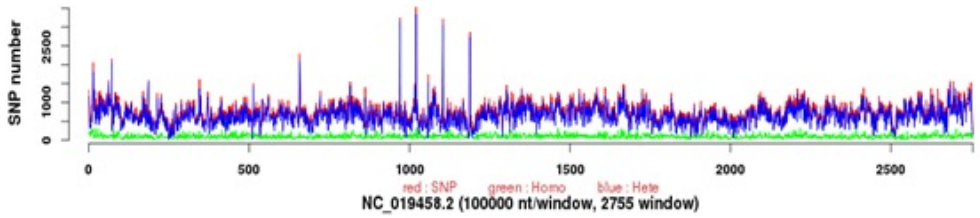

Figure 8. The distribution of SNPs in genome NC_019458.2. The X-axis represents the window numbers, and SNP numbers of the Y-axis mean the number of SNPs in each window.

In this project, 89,956 synonymous and 76,120 non-synonymous SNPs were annotated in this sheep genome. We went further to analyze the distribution of so-called large-effect SNPs, which are predicted to have a potentially disabling effect on gene function. The analysis showed that 1,035 SNPs were expected to induce premature stop codons, a 1,213 to disrupt splicing donor or acceptor sites, a 2,448 to alter initiation methionine residues, and 366 SNPs to remove the annotated stop codons, resulting in longer open reading frames.

Short InDel detection and annotation

The Genome Analysis Toolkit (GATK) was used to detect InDels, and the InDels result outputted in 'vcf' format. 3,769,565 InDels generated and distributed as 1,840,556 insertions, and 1,929,009 were deletions (Table 3 and Figure 9). After InDels identified, we annotated the result of InDel (Table 4). InDel annotation can reveal more details about InDels in some specific DNA regions, in the coding region. The effects of frame-shifting (e.g., 1-, 2-, 4-, 5-, 7-, 8-, 10-bp.) short InDels more significantly explain the different trait than

those of non-frame-shift (e.g., 3-, 6-, 9-bp.) ones, so we further calculated the frame-shift InDel based on the InDel annotation result (Table 3).

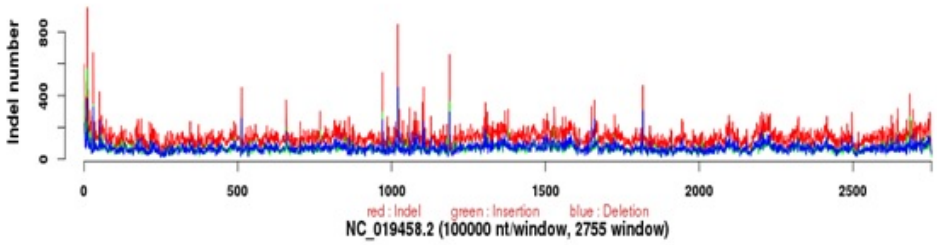

Figure 9. The distribution of InDel in genome NC_019458.2. 'InDel number' means the number of InDels in each window.

Table 3. Insertion and Deletion, annotation , distribution and frame shift mutation in CDS in the genome of Jordanian Awassi ram Sheep.

Insertion and Deletion	Value
InDel	3,769,565
Insertion	1,840,556
Deletion	1,929,009
CDS	4,087
C- gene segment	3,182
V- gene segment	17,961
cDNA match	187,524
Exon	523,983
Gene	11,654
mRNA	483,879
Frame-shift Mutation CDS	3,357
3X-shift Mutation CDS	705
3X-shift Mutation CDS Phase 0	234
3X-shift Mutation CDS PhaseNo0	471

*CDS: Coding Sequence

Structure copy number variation detection and annotation

Structure variation is an important difference among individuals of the same species. The exist of structural variation between the individual sequences and the reference; the alignment might not match. Such unusual paired-end alignment can thus use for detecting structure variation. The structural variation detected includes deletion, insertion, duplication, inversion, and transposition. 42.8% were deletion type of SV. The SV results presented in Table 4 and Figure 10.

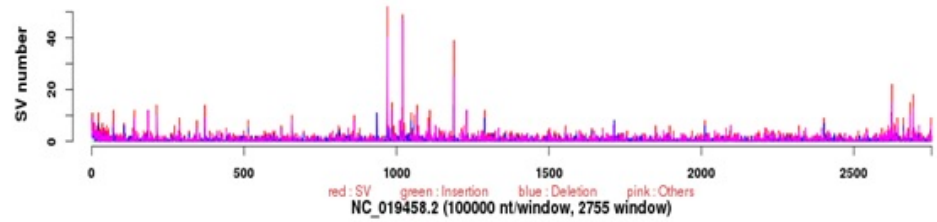

Figure 10. The distribution of structure variants (SV) in genome NC_019458.2. X-axis Y-axis: 'SV number' means the number of SVs in each window.

Table 4. Structure variation annotation , distribution in the genome of Jordanian Awassi Sheep rams.

Structure variation	Value
SV	38,900
Insertion	45
Deletion	16,643
Others	22,212
CDS	3,455,409
C- gene segment	41
V- gene segment	480
cDNA match	223,503
Exon	3,925,821
Gene	186,625
mRNA	34,524

*CDS: Coding Sequence

Copy Number Variation (CNV) is an important form of structural variation among individuals of the same species. The regions of the genome that have deleted or duplicated on some chromosomes between the sequencing individual and the reference correspond to CNVs. The CNV results listed in Table 5 and Figure 11 that is, over 72% of the CNV were down-regulated.

Table 5. Copy number variation (CNV) distribution and annotation distribution in the genome of Jordanian Awassi Sheep rams.

Copy number variation	Value
CNV	13,689
Up-regulation	3,743
Down-regulation	9,946
CDS	3,862
C gene segment	1
V gene segment	48
cDNA match	979
exon	5,070
gene	4,970
mRNA	11,987

*CDS: Coding Sequence

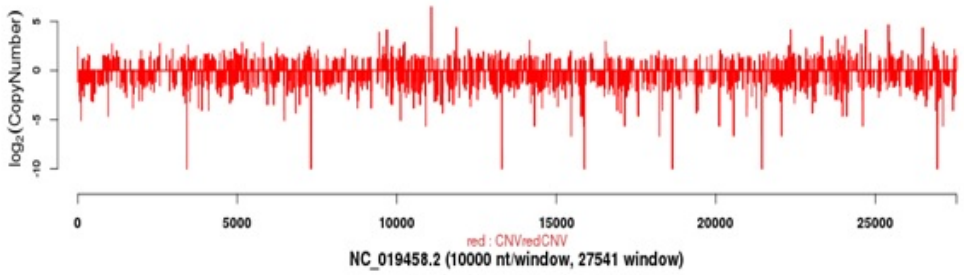

Figure 11. The distribution of copy number variations (CNVs) in genome NC_019458.2. 'Log 2 (Copy Number)' means a base-2 logarithmic copy number variation in each window.

DISCUSSION

In this project, we reportthe first Jordanian Awassi sheep whole-genome resequencing after the first simple sequencing (0.64-kb sequences) reported by (Al-Atiyat

and Aljumaah, 2014; Jawasreh et al., 2019a and 2019b). Copy Number Variation (CNV) is an important form of structural variation among individuals of the same species. SNPs have a great impact on the variation of genomes and the biological traits, so it is helpful to look into the SNP annotations and paid particular attention to those in genic regions. Non-synonymous SNPs believed to contribute to phenotypic variation among individuals or within populations (Jawasreh et al., 2019). They are also useful candidate mutations that may explain the different phenotypes among different animals.

Several SNPs that in different ovine genomic regions investigated and their associations with some economic Awassi sheep traits also tested. Jawasreh et al. (Jawasreh et al., 2017 and 2019) targeted a region between exons 61 bp 1C, 88 bp 1D, and 473 bp of the Awassi calpastatin gene and detected two non-synonymous variants that associated with growth performance and meat characteristics. Through simple sequencing procedure, Jawasreh et al. (2019) observed non-synonymous SNPs in the Awassi Beta-lactoglobulin, Prolactin, and Kappa Casein genes, the single effect and the interaction between the studied gene's SNPs genotypes analyzed and found to be of significant effect on Awassi milk production and composition. A non-synonymous mutation found in the Prolactin gene that associated with the birth weights of Awassi sheep explored (Jawasreh and Ismail, 2019a) and recommended using in selection to avoid dystocia. In the Awassi Calpastatin gene, individuals heterozygous in SNP were healthier than homozygous individuals (Jawasreh and Ismail, 2019b). It also associated the prolactin gene variants with prolificacy of Awassi sheep; BB genotype had 0.16 and 0.11 more lambs at birth than AA and AB, (Jawasreh et al., 2014). A SNP at base pair 34, 647, 499 on OAR23 detected through Genome-wide to show for Microtia in Awassi sheep (Jawasreh et al., 2016). Kijas et al. (2012) developed and used a set of 49,034 SNPs from a diverse collection of domestication and selection of sheep breeds in genotyping. The genome scan revealed 31 regions containing genes for coat pigmentation, skeletal morphology, body size, growth, and reproduction. They provide a high-density map of genetic variability viewed as an in-depth of the genetic history for sheep breed. Using Illumina Ovine 50K SNP BeadChip assay, Ahbara et al. (2019), identified putative candidate genes related to skeletal structure and morphology, fat deposition, and adaptation to environmental selection pressures in Ethiopian indigenous sheep. They suggested that Ethiopian fat-tail sheep could be a valuable animal genetic resource for understanding the genetic control of skeletal growth, fat metabolism, and associated physiological processes. Furthormore, to explore the genetic diversity and genetic structure of Ethiopian fat-tailed and Awassi sheep breeds. Using high density (50K) SNPs assay, Getachew et al. (2020), showed that the intense selection pressure on the Awassi sheep, resulted in a significant differentiation from local Awassi, and less within-population variation and high level of runs of homozygosity based genomic inbreeding. They suggested using runs of homozygosity as a tool to control the level of inbreeding, which can help to widen the genetic base of the Ethiopian Awassi crossbreeding program.

The insertion or deletion of a particular region in the Ovine genome may cause overexpression or effect on some economic traits. Lassoued et al. (2017) reported a mutation in BMP15 gene called FecXBar in Barbarian sheep that associated with a single nucleotide substitution, deletion, and insertion this gene cDNA leading to a frameshift at protein position 101. This mutation affected the prolificacy of barbarian sheep and suggested using in selection for improving the prolificacy of this sheep breed. A nonmutant FecB mutation found in Awassi while they screened GDF9 gene in Awassi sheep and was

polymorphic within the Awassi sheep breed without affecting Awassi ewes prolificacy (Jawasreh et al., 2014). In cattle, sheep, and pig, structural variation reported to linked to copy number variations (CNVs) for productive traits and diseases. SVs artificially selected in livestock such as a duplication of the agouti signaling protein gene that causes white coat color in sheep (Bickhart and Liu 2014).

In mammalian genomes, CNVs play a vital role in identifying their impact on natural and disease phenotypes. By using computational and experimental methods, it can catalog CNVs for critical functional characteristics with those in other mammalian genomes such as humans' genome (Guryev et al., 2008). Identifying genes based on the SNP variation requires additional validation for population structure. The potential of combining SNP variation and transcriptome data to identify putative genes that play a role in the improving of Awassi production or in the control of diseases that may affect sheep improvement.

CONCLUSIONS

The first whole Genome Resequencing of Jordanian Awassi Rams sheds light on various genetics and genomic studies. The results of this study focus in-depth on genetic variation, population structure, the distribution of (SNPs), the detected SNPs, and genetic variations in the whole genome could be used in marker assisted selection programs. Further investigations to determine the phylogeny, evolution, and gene pathways will be essential to expand our understanding of the evolutionary biology of economic traits such as milk, meat, wool production, and disease tolerance in Awassi sheep breeding and genetic improvement based on genomic selection.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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