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Original scientific article

GENOME-WIDE SEARCH POLYMORPHISMS USING ILLUMINA BEADCHIP IN RUSSIAN MEAT MERINO SHEEP FOR FUTURE GENOTYPING BY SEQUENCING

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For the mass use of genotyping by sequencing in sheep of the Russian Meat Merino breed, it is necessary to determine the loci of the genome with a sufficient frequency of occurrence in the population. To identify them, genotyping of Russian Meat Merino sheep was carried out using Ovine Infinium HD BeadChip 600K. As a result of polymorphism evaluation of 606,000 loci, 555 SNPs were selected with a frequency of occurrence of both homozygous variants in the range of 0.2850-0.3149. After excluding substitutions located closer than 1cM, a list of 387 polymorphisms was obtained. The selected substitutions were located on all 26 autosomes. The greatest number of polymorphisms were on the 1, 3, 6, 9, 12 and 22 chromosomes. The least substitutions were found on chromosomes 4, 8, 11 and 19. Only one substitution with the required frequency of occurrence was identified on the X chromosome. The average distance between SNPs was 4,000 to 7,000 kbp. The list of polymorphisms we have chosen can be used to confirm the reliability of the origin in the molecular genetic examination of sheep of the Russian Meat Merino breed.

Keywords: sheep, SNP, genotyping by sequencing, NGS, breeding, Russian Meat merino

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INTRODUCTION

The study of the genome of farm animals is a modern method that increases the efficiency of the breeding process. With the widespread use of marker-associated and genomic selection technologies, serious successes have been achieved in improving the productive qualities of pigs (GAO *et al*., 2021), sheep (XU *et al*., 2021) and cattle (MRODE *et al*., 2019). Currently, the use of data on the structure of the genome in animal husbandry is based on various methods of assessing the structure of DNA and pursues two main goals. This is a study of the genetic structure of the herd to determine the relationship between individuals (AL-ATIYAT, 2015) and the study of molecular markers of economically valuable traits (DE CAMARGO, 2019).

Kinship relationships between animals are most often investigated to confirm the authenticity of the origin in breeding farms. For this purpose, the method of DNA fragment analysis is used with an assessment of the polymorphism of the length of microsatellite loci. This method has a number of advantages, primarily due to the cheapness of the study and the high reliability of the result. However, the scope of microsatellite analysis today ends there. Unfortunately, this method is not applicable for evaluating and predicting productive qualities (AL-ADIYAT, 2015).

Along with microsatellite analysis, the study of single nucleotide polymorphisms (SNP) is used to determine the reliability of the origin (CLARKE *et al*., 2014; HEATON *et al*., 2014). At first, the same devices for fragment analysis were used to identify them, as in the study of microsatellite markers (EDEA *et al*., 2017). They allow to determine up to several dozen SNPs in a single sample in a multiplex format. But if it is necessary to simultaneously identify hundreds and thousands of polymorphisms, the methods of fragment analysis become inapplicable. In such cases, studies studies with DNA SNP chips are used (CIANI *et al*., 2020) and genotyping sequencing technologies using next generation sequencing (NGS) (WILLIS *et al*., 2018).

Due to the fact that SNP markers can have a direct connection with the realization of productive traits by changing the structure of the amino acid chain of the protein, they can be successfully used as molecular markers for predicting productivity (RAHMAN *et al*., 2021). As a result of the conducted studies, a sufficiently large number of single-nucleotide polymorphisms have been identified, which are successfully used for breeding in order to increase productivity in pigs (GAO *et al*., 2021), cattle (GEBREHIWOT *et al*., 2021) and sheep (BRITO *et al*., 2017). Thus, for the genotyping of farm animals, it is necessary to apply a study of a large number of SNPs for each DNA sample. Moreover, the list of these polymorphisms may vary depending on the genotyped breed and the appearance of new SNPs associated with productivity. The use of DNA chips for genotyping in such conditions has a number of limitations associated with the need to customize the chip during production for each specific situation. More promising is the use of next generation targeted sequencing of a new generation, which, moreover, allows you to detect new SNPs, which is impossible for DNA chips. Another important advantage of genotyping by sequencing is the possibility of simultaneous study of hundreds and thousands of SNPs for several hundred animals in just one sequencer run (DE DONATO *et al*., 2013).

Genotyping sequencing is also becoming more widespread due to the appearance of convenient tools for creating panels of the studied loci, such as AgriSeq by Illumina (WILLIS *et al*., 2018). However, in order to develop effective locus panels, it is necessary to perform a preliminary assessment of the genome of a specific animal breed to detect polymorphisms with a

sufficient frequency of occurrence, as well as having a certain relationship with animal productivity indicators. To do this, full-genome sequencing can be performed, but it is more cost-effective to do this using high-density DNA chips. As a result of evaluating hundreds of thousands of polymorphisms, it is possible to select those that meet the established criteria and develop a method for detecting them using genotyping by sequencing (BRAZ *et al.*, 2021).

The main objective of our research was genotyping using DNA chips from Illumina (USA) of one of the promising Russian breeds - the Russian Meat merino to compile a list of SNPs applicable for genotyping by sequencing based on NGS technology.

MATERIALS AND METHODS

Ethics statement

The sample collection and study purpose were approved by the Institutional Animal Care and Use Committee (approval number 2022-0064, 11.02.2022) of the All-Russian Research Institute of Sheep and Goat Breeding, Stavropol, Russian Federation.

Phenotypic data

The object of the study was sheep of the Russian Meat Merino breed at the age of 12 months from breeding farms of the Stavropol Territory (Russian Federation). In total, 50 individuals with the most pronounced breed characteristics were selected for genotyping. They characteristics were included the length, density and tone of the wool, the quality of the grease, the parameters of the exterior, live weight. Wool parameters were evaluated according to the recommendations of HOLMAN and MALAY-ADULT (2012). The exterior was assessed by measurements using a measuring tape and a pelvimeter. Weighing was performed using scales with an accuracy of 10 grams. The live weight was 78.6±0.9 kg, the height at the withers was 77.4±0.8 cm, the length of the wool fibers was 11.1±0.6 cm, the thickness of the wool was 18.6±0.4 μm. All animals were clinically healthy, kept in optimal conditions and received a balanced diet.

Genotyping

Genomic DNA was isolated from whole blood samples taken under aseptic conditions from the jugular vein using the Pure Link Genomic DNA MiniKit (Invitrogen Life Technologies, USA) in accordance with the manufacturer's protocol. Animal genotyping was performed using Ovine Infinium HD BeadChip 600K (Illumina Inc. CA, USA) according to the manufacturer's protocol. Initial processing of the genotyping results was performed using the Genome Studio 2.0 software (Illumina Inc. CA, USA).

Quality control of genotyping

Quality control of genotyping was carried out using PLINK V.1.07 software (PURCELL *et al.*, 2007). The data processing included samples with call rate of detected SNPs more than 0.95. Substitutions with a minor allele frequency (MAF) of less than 0.05 and a missing genotype of more than 0.1 were also excluded. The value $p = 0.0001$ was used as the threshold according to

the Hardy-Weinberg equilibrium criterion. With a positive result, all 50 samples of studied animals underwent genotyping quality control.

Genetic and statistical analysis

The analysis of genotyping results, the study of the frequency of polymorphisms, and the assessment of the distribution of SNPs by genome regions were carried out using Genome Studio 2.0 software (Illumina Inc. CA, USA). Descriptive statistics for distances between SNPs were calculated in Excel for Windows (Microsoft, USA). The Ovis_Aries_3.1 genome assembly was used to map the SNP.

RESULTS

A study of the presence of single-nucleotide polymorphisms in the genome of Russian Meat Merino sheep using Illumina Ovine Infinium HD BeadChip 600K showed that there is a sufficiently large number of substitutions with a frequency of occurrence of both allelic variants in a homozygous form of about 0.3. To develop a genotyping panel by sequencing, we assumed to select up to 400 SNPs with such a frequency of occurrence. In the selection process, polymorphisms with the frequency of occurrence of wild homozygous genotypes in the range of 0.28-0.32 were first selected, among which those with the same frequency of occurrence of mutant genotypes were selected. After that, the total number of polymorphisms corresponding to our criteria was estimated. When choosing a narrow frequency range of 0.2950-0.3049, the number of corresponding SNPs turned out to be too small (Table 1). Increasing the interval to 0.2900-0.3099 also did not allow obtaining a sufficient number of polymorphisms. Further expansion of the frequency range of occurrence showed that the interval 0.2850-0.3149, giving 555 SNP, is most appropriate. An increase in the frequency range of occurrence above the specified limits leads to too many polymorphisms, which significantly exceeds the requirements we have established.

Table 1. Numbers of SNP's in different frequency ranges in Russian Meat Merino rams.

# of selection	WH frequency range (min-max)	WH SNP's (n)	MH frequency range (min-max)	WH+MH SNP's (n)	Heterozygotes frequency (M±m)
1.	0.2950-0.3049	7400	0.2950-0.3049	227	0.396±0.010
2.	0.2900-0.3099	9606	0.2900-0.3099	275	0.395±0.008
3.	0.2850-0.3149	11901	0.2850-0.3149	555	0.389±0.007
4.	0.2800-0.3199	19671	0.2800-0.3199	1795	0.377±0.005
5.	0.2750-0.3249	26872	0.2750-0.3249	2674	0.387±0.004

WH - Wild homozygous genotype; MH - Mutant homozygous genotype

The frequency range we have chosen includes a greater number of polymorphisms in the Russian Meat Merino than should be included in the panel for genotyping by sequencing. But this allows you to choose from them those that will most accurately characterize the genome of the studied animals by removing from the list the nearby and concatenated inherited substitutions. Evaluation of the frequency of occurrence of heterozygous genotypes for the SNPs selected at the first stage of research showed that they are also quite widespread in the population, their average values are in the range of 0.389±0.007. There were no replacements

without physical localization among the ones we selected. On chromosome X, only one polymorphism met our criteria, which we did not exclude from further research, since it is minimal, but still characterizes this chromosome.

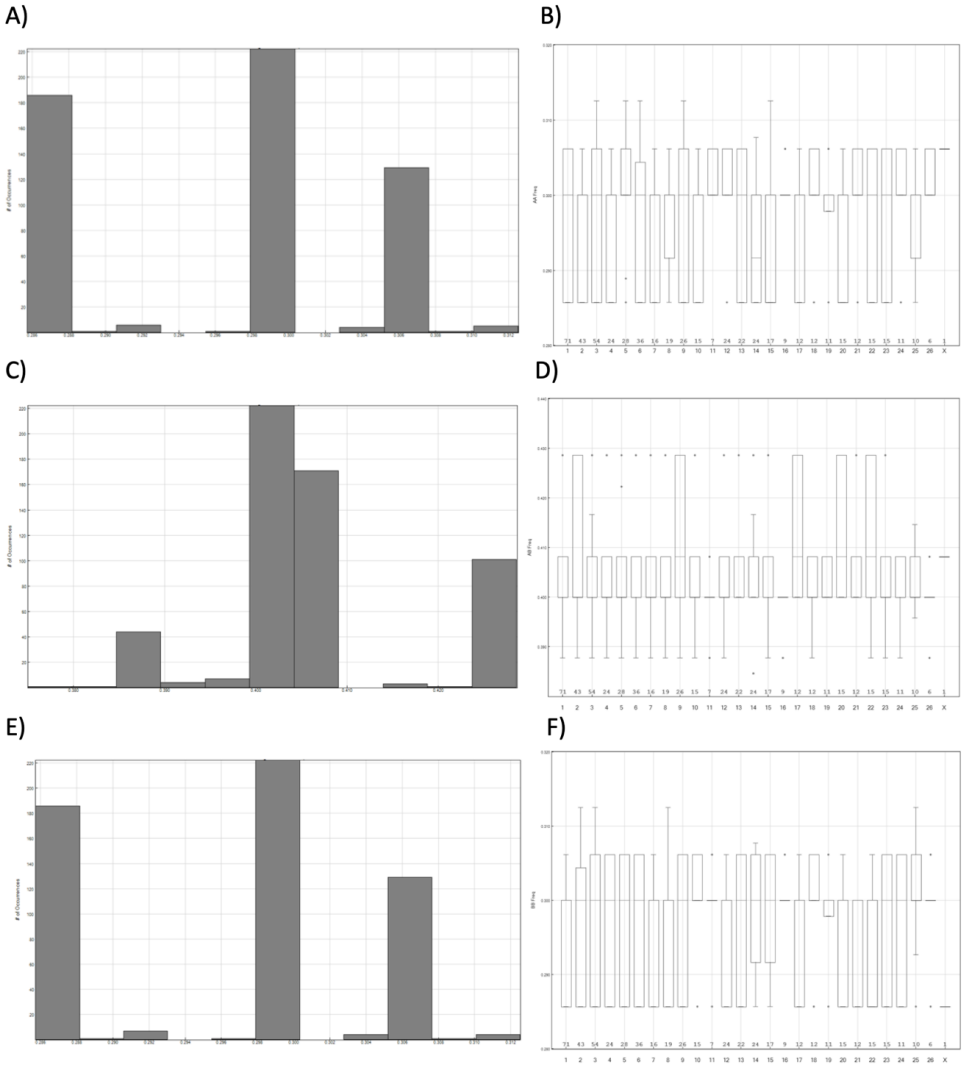

Figure 1. The number of SNPs with different frequency of occurrence and the distribution of the frequency of occurrence of polymorphisms on individual chromosomes in rams of the Russian Meat Merino breed. A, B – wild homozygous genotypes; C, D – heterozygous genotypes; E, F – mutant homozygous genotypes.

The second stage of the research included the study of quantitative parameters of the frequency of occurrence of the polymorphisms we selected in Russian Meat Merino sheep. It showed that for wild homozygous variants of the genotype, the largest number of selected substitutions had a frequency of occurrence in the middle of the range 0.2850-0.3149 (Figure 1, A). Slightly fewer substitutions were at the lower limit, and minima were observed for frequencies 0.294 and 0.302. A comparison of the frequency spread of individual SNPs on chromosomes revealed four chromosomes with a maximum index and 7 chromosomes with a minimum spread (Figure 1, B). For chromosome X, the assessment was not carried out, since it has only one of the substitutions we selected.

Among the heterozygous genotypes of the substitutions we selected, the greatest number had a frequency of occurrence in the central part of the range 0.375-0.430 (Figure 1, C). The lowest number of SNPs was detected in the lower part of the frequency range. About 100 substitutions had a frequency of occurrence near the upper limit of the specified interval. Within individual chromosomes, different types of frequency distribution of heterozygous replacement variants were noted, different from the pattern for wild homozygous genotypes (Figure 1, D). For all chromosomes (excluding X with a single replacement), the average frequency of detection of polymorphisms was about 0.4. For 9 chromosomes, the minimum frequency of occurrence for SNPs located on them were within the same limits, for the remaining chromosomes this indicator was below 0.390. The maximum frequency of occurrence for most chromosomes was at 0.408 and only for 8 chromosomes, this indicator exceeded 0.415, although single substitutions with maximum frequency indicators were found on more than half of the chromosomes.

Mutant homozygous variants of the polymorphisms we found in Russian Meat Merino sheep had a frequency distribution similar to wild homozygous genotypes (Figure 1, E). The maximum number of substitutions (more than 220) occurred with a frequency of about 0.3. Also, a significant number of polymorphisms had a frequency of occurrence at the lower limit of the interval 0.285. Another peak in the frequency distribution was at 0.305. About 130 replacements had this frequency. In the other areas of the interval we selected, the frequency of occurrence was minimal. The distribution of indicators of the frequency of occurrence of SNP by chromosomes showed that on 17 chromosomes out of 26 the average frequency was within 0.285, and on 5 chromosomes there was a minimal spread in the frequency of occurrence.

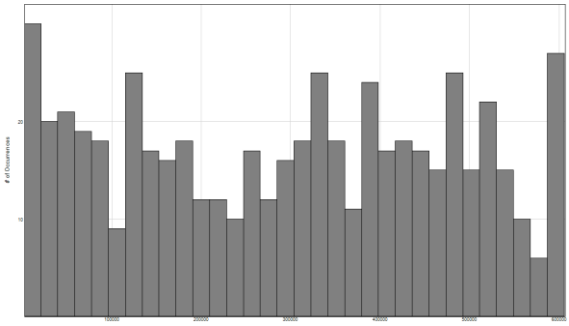

Figure 2. Distribution of SNPs selected for genotyping by genome regions according to the Illumina BeadChip Ovine 600K index from 1 to 606,000 in Russian Meat Merino sheep.

The distributions of the polymorphisms selected by us by genome loci based on conditional SNP indices from 1 to 606,000 according to the nomenclature Illumina Beadchip Ovine 600K were studied (Figure 2). The above indexing has a connection with the distribution of SNP across chromosomes and on its basis, it is possible to judge the number of polymorphisms in the zone of a certain chromosome. The evaluation showed that the largest number of substitutions we selected is in 6 regions of the genome corresponding to chromosomes 1, 3, 6, 9, 12 and 22. The smallest number of polymorphisms was found in the loci located on chromosomes 4, 8, 11 and 19. In general, SNPs proposed for genotyping are available in all regions of the genome, and their number ranges from 6 to 28.

The second stage of the selection of single-nucleotide polymorphisms for use in genotyping by sequencing of Russian Meat Merino sheep included an assessment of the distances between neighboring polymorphisms. Of the two substitutions that are closer than 1000 kbp (one centimorganide), for which the probability of concatenated inheritance is very high, only one was used to compile the final list. Thus, 387 loci were left for the final set of polymorphisms.

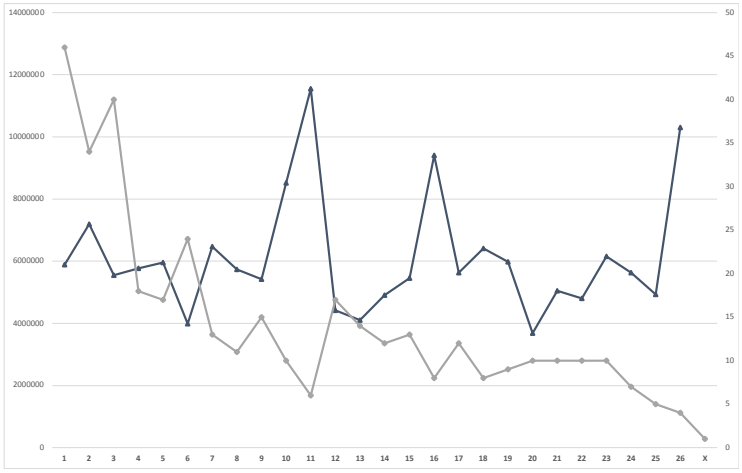

Figure 3. The number and average distance between 387 SNPs selected for genotyping on different chromosomes in Russian Meat Merino sheep. Dark gray, left Y–axis is the average distance between SNPs, bp; Light gray, right Y-axis is the number of SNPs; X–axis is the chromosome.

As a result, the average distance between the genotyped SNPs on the main number of chromosomes turned out to be in the range of 4000 – 7000 kbp. (Figure 3). The largest distances between substitutions are noted on chromosomes 11, 16 and 26. At the same time, the smallest number of polymorphisms was also selected for genotyping on the latter – only 4. There were fewer SNPs only on chromosome X, where only one replacement met the established criteria. The largest number of polymorphisms was selected by us on the first three chromosomes, 46, 34 and 40, respectively.

DISCUSSION

To identify single-nucleotide polymorphisms suitable for genotyping by sequencing in sheep breeds of Russian Meat Merino, we conducted a study of the presence and frequency of occurrence of SNP in more than 600,000 genome loci. The study was carried out on the basis of data from the Illumina Beadchip Ovine 600K consortium chip. Using filters with different frequency intervals of substitutions, a sufficiently large number of polymorphisms were selected, the homozygous variants of which, both in wild and mutant forms, corresponded to the selected intervals. After analyzing the distribution of the SNPs, we found across chromosomes and the genome as a whole, as well as estimating the distance between neighboring polymorphisms, 387 substitutions were selected that met our criteria. Their prevalence in the population of the studied sheep breed allows us to recommend the obtained set of SNPs both for genotyping by sequencing in order to confirm the authenticity of the origin of animals, and for the development of custom SNP chips.

When choosing single-nucleotide polymorphisms for animal genotyping, their set should be suitable for the effective solution of breeding tasks. The first and most demanded task of this type of molecular genetic examination is to determine the authenticity of the origin of individuals of both sexes. Despite the presence of various methods of determination, including immunogenetics and microsatellite analysis, genotyping by SNP allows to confirm the authenticity of the origin with high reliability. According to ISAG recommendations (www.isag.com), the number of polymorphisms to obtain a qualitative result must be at least 100. An increase in the number of substitutions in the set increases the probability of a reliable assessment, but at the same time the price of the studies performed also increases (MCCLURE *et al*., 2018). In the course of our research, we expected to find about 400 substitutions that meet the selected criteria. This would make it possible to both genotypes according to all the proposed polymorphisms, and choose more limited sets from them to maintain a balance between the cost of research and the economic efficiency of breeding work.

When creating a set of polymorphisms sufficient to perform genotyping in accordance with the tasks set to determine the reliability of the origin of animals, we used several criteria. The first of them was the frequency of occurrence of wild and mutant homozygous variants of polymorphisms detected using DNA biochips. This was done by adjusting the data sorting parameters and selecting first wild homozygous variants of polymorphisms, and then mutant homozygotes among them in the Genome Studio 2.0 program. We used several frequency intervals based on the threshold frequency of 0.3 proposed by TORTEREAU *et al*. (2017). However, we could not fully rely on the criterion approach of these authors, since they planned to select almost twice as many substitutions for genotyping as we did. As a result, several intervals of different values were used, using the indicator 0.3 as the center point. Narrow ranges, as the research results have shown, give too few substitutions. Also, wide ranges were excluded, into which a lot of polymorphisms fell. We found the optimal interval to include 555 substitutions. This was less than the set recommended by MCCLURE *et al*. (2018) for cattle genotyping in the amount of 800 polymorphisms, but in our case, we considered it sufficient, since we set the task to select less than 400 substitutions from them. The approach that we have applied, of course, is quite individual and is designed for a specific breed of Russian Meat

Merino. However, the principle of choosing substitutions can also be used to search for SNPs in other sheep breeds.

The set of polymorphisms we obtained for genotyping was evaluated by the frequency distribution of occurrence for different variants of the combination of wild and mutant genotypes. As a result, it was found that the majority of both wild and mutant homozygotes had a frequency of occurrence in the middle and near the lower boundary of the selected intervals. Heterozygotes had peak frequency values on the contrary, in the central part of the interval. The obtained result indicates that further expansion of the interval in the direction of increasing the frequency of occurrence is impractical, since the number of substitutions in this direction of the range is progressively decreasing.

The determination of the number of substitutions with different frequency of occurrence on each of the chromosomes showed that despite the fluctuations in their number, in general, the distribution was relatively uniform. There were no variants of chromosomes on which substitutions occurred only with a low or only with a high frequency relative to the range we selected. Only the X chromosome stands out, on which only one polymorphism was found that meets the established criteria. On the one hand, this indicates a high homogeneity of the genetic composition of the chromosome, and on the other hand, it shows a low informative value of studying polymorphisms on it in relation to identifying the authenticity of the origin of animals. Nevertheless, it was decided to leave this single polymorphism in the list for genotyping, since it at least partially marks this sex chromosome.

The completeness of the genome coverage using the selected SNPs for genotyping was evaluated based on their distribution at individual loci using an individual polymorphism index according to the manifest attached to the Illumina Beadchip Ovine 600K. This index catalogues all 606,000 polymorphisms detected using a chip, conditionally representing the entire DNA chain covered as a single line, not divided into chromosomes. The result showed that the substitutions we selected are present in all loci of the genome. The distribution was not quite uniform, but there were no significant differences in quantity. As shown by the results of HULSEGGE *et al*. (2018), this pattern is quite common when selecting SNPs for genotyping. In their opinion, such fluctuations in the number of polymorphisms in the genome loci do not reduce the reliability of the results of the assessment of the reliability of the origin of animals.

The selection of single-nucleotide polymorphisms, based on the assessment of their frequency of occurrence in the sample of animals, did not take into account the influence of their mutual location. The loci used for genotyping should be located at a sufficient distance from each other to avoid duplication of their contribution to the assessment of the genotype structure. In this case, the probability of their joint inheritance will decrease and the substitutions will not correlate with each other in terms of occurrence in the genome. Therefore, we calculated the distances between neighboring polymorphisms and removed from the initial list those closer to one centimorganide. As a result, the distance between most substitutions ranged from 4000 to 7000 kbp. We consider this sufficient for high-quality genotyping. The final list thus included 387 SNPs, which is one and a half times more than was proposed by TORTEREAU *et al*. (2017).

On some chromosomes (11, 16 and 26), the distances between polymorphisms were significantly larger, which is due to the ratio of the size of these chromosomes and the number of substitutions detected on them. For chromosome X, the distance between SNPs was not

calculated, since there is only one replacement on it. We plan to search for new replacements on the X chromosome in the next generations of Russian Meat Merinos, as the breeding process in the breed continues and new polymorphisms with the required parameters may appear.

In the proposed variant, the list of selected SNPs in the genome of Russian Meat Merino sheep is sufficient to solve the tasks set to confirm the authenticity of the origin of animals, since all substitutions have a sufficient frequency of occurrence, are located at loci throughout the genome and are at a sufficiently large distance from each other.

CONCLUSION

Using the genotyping of sheep of the Russian Meat Merino breed based on the Illumina BeadChip Ovine 600K, loci suitable for genotyping by sequencing animals of this breed were found. Single nucleotide polymorphisms with a high frequency of occurrence in the range of 0.2850-0.3149 homozygotes of both wild and mutant variants were identified. Heterozygous variants of these substitutions occurred with a frequency of 0.389±0.007. The number of polymorphisms corresponding to the selected criteria was 555. Analysis of the location of the detected SNPs in the sheep genome showed their presence along the entire length of the genotyped DNA region, but only one replacement was detected on the X chromosome. After excluding SNPs with a location closer to one centimorganide (approximately 1,000 bp), we recommend 387 single-nucleotide polymorphisms for use in genotyping. Such a set of substitutions will effectively solve the problems of confirming the authenticity of the origin of Russian Meat Merino sheep, accurately identify animals in the process of breeding work, and account for inbreeding in the population. The proposed set of SNPs is recommended both for use in genotyping by sequencing of a new generation, and for customization of SNP biochips.

Data availability

Data are available in supplement file.

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PRETRAGA POLIMORFIZMA ŠIROM GENOMA KORIŠĆENJEM ILUMINA ČIPA U RUSKIM MESNATIM MERINO OVCAMA ZA BUDUĆU GENOTIPIZACIJU SEKVENCIONIRANJEM

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Izvod

Za masovnu upotrebu genotipizacije sekvenciranjem kod ovaca rase Ruski mesni merino potrebno je odrediti lokuse genoma sa dovoljnom učestalošću pojavljivanja u populaciji. Da bi se identifikovali, izvršena je genotipizacija Ruskih mesnih Merino ovaca pomoću Ovine Infinium HD BeadChip 600K. Kao rezultat procene polimorfizma 606.000 lokusa, odabrano je 555 SNP-ova sa učestalošću pojavljivanja obe homozigotne varijante u rasponu od 0,2850-0,3149. Nakon isključivanja supstitucija koje se nalaze bliže od 1cM, dobijena je lista od 387 polimorfizama. Odabrane supstitucije su locirane na svih 26 autozoma. Najveći broj polimorfizama je bio na 1, 3, 6, 9, 12 i 22 hromozomu. Najmanje supstitucija je nađeno na hromozomima 4, 8, 11 i 19. Na X hromozomu je identifikovana samo jedna supstitucija sa potrebnom učestalošću pojavljivanja. Prosečna udaljenost između SNP-ova bila je 4.000 do 7.000 kbp. Spisak polimorfizama koji smo odabrali može poslužiti za potvrdu pouzdanosti porekla u molekularno-genetskom ispitivanju ovaca rase Ruski mesni Merino.

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