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PROSPECTS OF USING COMPLEX GENOTYPES FOR BETA-CASEIN, PROLACTIN AND LEPTIN GENES IN MARKER-ASSISTED BREEDING IN DAIRY CATTLE

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The analysis of the prospects of using complex genotypes at the loci of beta-casein, prolactin and leptin in marker-assisted breeding programs of Ukrainian Black-and-White and Red-and-White dairy breeds of cattle to create experimental groups of animals producing A2 milk was carried out. Data are given on the peculiarities of the distribution of allele frequencies and genotypes at the loci of beta-casein (c.350C>A, rs43703011), prolactin (c.35333764C>T, rs211032652) and leptin (c.239C>T, rs29004508) in experimental cattle populations. It was established that in both cattle populations, the loci of beta-casein, prolactin and leptin are polymorphic according to experimental mutations. The expediency of using complex genotypes for each of the polymorphic loci for the selection of individuals of both research breeds for the purpose of use in further selection work is substantiated. The number of individuals with the "desired" complex genotypes in each population was determined as $CSN2^{A2A2}PRL^{CC}LEP^{CC}$ *for the Ukrainian Black-and-White dairy breed and* $CSN2^{A2A2}PRL^{TT}LEP^{CC}$ *for the Ukrainian Red-and-White dairy breed. According to the results of the research, it was found out that in the research group of cattle of the Ukrainian Black-and-White dairy breed, the number of individuals with the "desired" complex genotype is 10%, while in the population of the Ukrainian Red-and-White dairy breed it is 17% of the total sample (n =30). It has been proven that the minimum number of individuals required for genetic population studies (n=30, FAO) is insufficient for the effective selection of animals with the desired complex genotypes based on the identified polymorphic loci in both breeds of dairy cattle. Using the obtained data, it is proposed to carry out further marker-assisted selection in order to obtain experimental groups of A2 milk-producing animals based on the selection of individuals with the desired complex genotypes in both experimental cattle breeds using the Gene Pyramiding method. Gene Pyramiding with the markers aims to obtain individuals with the best economic traits according to the optimal breeding scheme, which involves the selection of the desired target alleles and the pyramiding of their most optimal combinations into one complex genotype.*

Key words: polymorphism, population, cattle, allele, genotype, variability.

ПЕРСПЕКТИВИ ВИКОРИСТАННЯ КОМПЛЕКСНИХ ГЕНОТИПІВ ЗА ЛОКУСАМИ БЕТА-КАЗЕЇНУ, ПРОЛАКТИНУ ТА ЛЕПТИНУ В МАРКЕР-АСОЦІЙОВАНІЙ СЕЛЕКЦІЇ У МОЛОЧНОМУ СКОТАРСТВІ

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Проведено аналіз перспективи використання комплексних генотипів за локусами бета-казеїну, пролактину та лептину в програмах маркер-асоційованій селекції української чорно-рябої та червоно-рябої молочних порід корів зі створення експериментальних груп тварин-продуцентів А2 молока. Наведено дані, стосовно особливостей розподілу частот алелей та генотипів за локусами бета-казеїну (c.350C>A, rs43703011), пролактину (c.35333764C>T, rs211032652) та лептину (c.239C>T, rs29004508) у дослідних популяціях корів. Встановлено, що в обох популяціях корів локуси бета-казеїну, пролактину та лептину за дослідними мутаціями є поліморфними. Обґрунтовано доцільність використання комплексних генотипів за кожним з поліморфних локусів для відбору особин обох дослідних порід з метою використання у подальшій селекційній роботі. Встановлено кількість особин з "бажаними" комплексними генотипами в кожній популяції – $CSN2^{A2A2}PRL^{CC}LEP^{CC}$ для української чорно-рябої молочної породи та $CSN2^{A2A2}PRL^{TT}LEP^{CC}$ для української червоно-рябої молочної породи. За результатами досліджень з'ясовано, що у дослідній групі корів української чорно-рябої молочної породи кількість особин з "бажаним" комплексним генотипом становить 10 %, в той час як в популяції української червоно-рябої молочної породи – 17 % від загальної вибірки (n=30). Доведено, що мінімальна кількість особин, необхідна для проведення генетико-популяційних досліджень (n=30, FAO) є недостатньою для ефективного відбору тварин із бажаними комплексними генотипами за виявленими поліморфними локусами в обох породах молочних корів. За використання отриманих даних запропоновано проведення подальшої маркер-асоційованої селекції з метою отримання експериментальних груп тварин-продуцентів А2 молока на основі відбору особин з бажаними комплексними генотипами в обох дослідних породах корів за використання методу Gene Pyramiding. Генний пірамідінг за допомогою маркерів має на меті отримання особин із найкращими економічними ознаками відповідно до оптимальної схеми розведення, яка передбачає вибір бажаних цільових алелів та пірамідування їх найбільш оптимальних комбінацій в один комплексний генотип.

Ключові слова: поліморфізм, популяція, корови, алель, генотип, мінливість.

Despite the clear successes of genomic selection in cattle breeding, the use of specialized types of breeding work based on marker-assisted selection is still relevant worldwide (Gutierrez-Reinoso et al., 2021; Wiggans et al., 2022).

The problems that can be solved due to the use of modern molecular genetic approaches of marker-assisted selection depend on the peculiarities of a specific region/country and, to a large extent, are determined by the specific characteristics of gene pool breeds of local selection (Mrode et al., 2019). In Ukraine, one of the most promising trends in animal husbandry is the production of "organic products", which naturally includes the so-called A2 milk. The uniqueness of this product is due to the genetic characteristics of the producers as different forms of milk, A1 and A2, differ only in the type of beta-casein contained in it (Cieślińska et al., 2019; Sebastiani et al., 2022; Jiménez-Montenegro et al., 2022). At the same time, A1 and A2 are among the most significant, but not the only, allelic forms (Antonopoulos et al., 2021). Alleles A^1 and A^2 differ from each other in the presence of a certain amino acid at position 67 of the beta-casein molecule (Sebastiani et al., 2020). In the case of the A^1 form it is histidine, for A^2 it is proline (Thiruvengadam et al., 2020). Accordingly, different forms of beta-casein are determined by a mutation in the original *CSN2* gene, namely, the presence of adenine in the case of the A^1 allele and cytosine in the case of the A^2 allele (Dai et al., 2016; Kay et al., 2021).

Based on the results of typing, it is possible to select individuals from the population with a specific desired genotype (A^2A^2) for the purpose of obtaining both products (A2 milk) and for use in breeding work (forming populations of A2 milk-producing cattle). From this point of view, Ukrainian breeding cattle populations are quite promising due to the relatively high share of the A^2 allele in the homozygous state (depends on the breed, population, farm, etc.). However, one-time selection of individuals with the A^2A^2 genotype is clearly insufficient for targeted marker-assisted selection. In this case, the genetic environment factor becomes important, which can eliminate all the advantages of obtaining the target product (A2 milk). First of all, this applies to the parameter of the value of the milk yield, which depends on a number of genes. For example, based on the results of molecular genetic examination, it is possible to create experimental populations of cattle of various breeds, which will consist entirely of individuals with the A^2A^2 genotype, which, accordingly, are A2 milk producers. But, despite all the attractiveness of the situation, productive qualities of cattle (milk productivity) can be quite variable and, in general, worse than other groups of animals. Therefore, in order to avoid such a situation, it is necessary to analyze individuals not only for the target gene of beta-casein, but also for a number of loci that belong to the "major" loci, that is, those that are associated with milk yield indicators. In this case, the phenomenon of breed specificity becomes particularly significant as for each individual breed, different genes and allelic variants can contribute to the overall milk productivity. Therefore, determining the possibility of using complex genotypes when creating herds of cattle producing A2 milk is of primary importance for the implementation of marker-assisted breeding programs. From this point of view, prolactin and leptin genes, allelic variants of which are associated with increased indicators of milk productivity of animals, are of considerable interest (along with the beta-casein gene) for Ukrainian Black-and-White and Red-and-White dairy cattle.

The prolactin gene (*PRL*) is one of the most important quantitative trait loci (QTL) in cattle breeding. It encodes protein that belongs to the regulatory hormones of the pituitary gland and takes part in a large number of physiological functions of the body (Dobolyi et al., 2020). It is a key factor in initiating and maintaining the lactation function (Knight, 2001). For the prolactin locus in cattle, a number of promising muta-

tions have been established in various functional elements of the gene (promoter, exon, and others), which, taking into account the physiological value of prolactin as a hormone-regulator of lactation activity, makes it promising for research specifically in dairy cattle breeding (Shah et al., 2021).

The leptin gene (*LEP*) encodes a protein that is directly related to the regulation of fat and energy metabolism. It refers, first of all, to markers of meat productivity of animals (Wang et al., 2020). In addition, allelic variants (for example, A59V mutation) were identified, which are associated with parameters of milk productivity of cattle, as well as with the content of somatic cells in milk (Yazdani et al., 2010; Maletić et al., 2019; Mandefro et al., 2021).

It should be outlined that it is actually impossible to determine all possible variations of complex genotypes based on a large number of loci, due to the unlimited number of variants and the limited number of individuals of this breed in the farm. Therefore, we chose such objects, which have an associative relationship with milk productivity of indicators of Ukrainian Red-and-White and Black-and-White cattle was established by us in previous studies (Kulibaba et al., 2019, 2021).

The purpose of the study. The purpose of the research is to determine the complex genotypes at the loci of beta-casein, prolactin and leptin in the populations of Ukrainian Black-and-White and Red-and-White dairy cattle with further analysis of the possibility of their use in marker-assisted breeding programs to create experimental groups of cattle producing A2 milk .

Research materials and methods. The research was carried out in the laboratory of molecular genetic and physiological and biochemical research in animal husbandry of the Institute of Animal Husbandry of the National Academy of Sciences and in the laboratory of molecular genetic research of the Department of Animal Biology of the National University of Life and Environmental Sciences of Ukraine.

Cattle of the Ukrainian Black-and-White dairy breed (n=30) (SE RH "Hontarivka") and Ukrainian Red-and-White dairy breed (n=30) (SE RH "Hontarivka") were used as the object of research.

Polymorphism of beta-casein, prolactin and leptin loci was determined by mutations of *CSN2* (c.350C>A, rs43703011), *PRL* (c.35333764 C>T, rs211032652) and *LEP* (c.239C>T, rs29004508).

Polymorphism of the beta-casein locus was determined using the allele-specific PCR method according to the algorithm previously presented in the authors' publication (Kulibaba et al., 2023). Polymorphism of the prolactin and leptin loci was determined using the PCR-RFLP method (PCR with restriction analysis).

Appropriate primers were used to amplify experimental genome fragments: for *CSN2* – GCCCAGATGAGAGAAGTGAGG, GATGTTTTGTGGGAGGCTGTTAT and GATGTTTTGTGGGAGGCTGTTAG (Keating et al., 2008); for *PRL* – GTTCTTGCTTTATGTAACACCG and TAGGTCAATCACTCTGAGCA (Kulibaba et al., 2019); for *LEP* – GGGAAGGGCAGAAAGATAG and TGGCAGACTGTTGAGGATC (Haegeman et al., 2000).

In the case of the PCR-RFLP method, appropriate restriction endonucleases were used: RsaI for *PRL* and HphI for *LEP*.

Electrophoretic distribution of amplification/restriction fragments was performed using an agarose gel (1.5%) with ethidium bromide staining. The size of the DNA fragments on the electrophoregram was determined using the GeneRuler 50 bp molecular mass marker (Thermo Fisher Scientific).

Based on the results of genotyping, the main genetic and population indicators were determined: frequencies of alleles and genotypes, the ratio of observed (H_0) and

expected (H_e) heterozygosity values, Wright's fixation index (F_{is}). Research indicators were determined using the GENALEX version 6.5 software package in the Excel 2019 environment (Smouse et al., 2017).

Research results. According to the results of the research, the *CSN2*, *PRL*, and *LEP* loci were found to be polymorphic in each of the certified breeds as individuals with all possible genotypes are present in both populations.

Typing of individuals by experimental loci was carried out using the analysis of the obtained electrophoregrams. Examples of electrophoregrams and features of polymorphism for each of the loci were described in detail by the authors in previous studies (Kulibaba et al., 2019, 2021, 2023)

Beta-casein polymorphism was determined using the allele-specific PCR method. For each allele, there are corresponding amplified fragments, 854 bp in size, on the electrophoregram. The presence of a fragment only for the corresponding allele (A^1 or A^2) indicates the homozygous genotype A^1A^1 or A^2A^2. The presence of both fragments indicates a heterozygous A^1A^2 genotype.

In the case of the prolactin locus, the presence of restriction fragments of size 360 and 56 bp indicates the CC genotype (Rsal-/Rsal-), while the presence of fragments 195, 165, and 56 indicates the TT genotype (Rsal+/Rsal+). The CT heterozygous genotype (Rsal+/Rsal-) is represented by a combination of the above variants – 360, 195, 165 and 56 bp. It should be outlined that each of the PRL alleles additionally contains a monomorphic restriction site for RsaI.

At the leptin locus, the presence of restriction fragments with sizes of 311 and 20 bp indicates the TT genotype (HphI+/HphI+); the presence of one fragment in 331 bp – the CC genotype (HphI-/HphI-). CT heterozygotes (HphI+/HphI-) are presented as fragments 331, 311 and 20 bp.

The frequency values of genotypes and alleles for each of the experimental loci in both breeds of cattle are given in the table.

Table

Values of frequencies of genotypes and alleles at the CSN2, PRL and LEP loci in experimental cattle populations

Locus	Ukrainian Black-and-White dairy breed		Ukrainian Red-and-White dairy breed	
	Genotype frequencies	Allele frequencies	Genotype frequencies	Allele frequencies
CSN2	A^1A^1 – 0.37; A^1A^2 – 0.43; A^2A^2 – 0.20.	A^1 – 0.58; A^2 – 0.42.	A^1A^1 – 0.10; A^1A^2 – 0.57; A^2A^2 – 0.33.	A^1 – 0.38; A^2 – 0.62.
PRL	CC – 0.60; CT – 0.30; TT – 0.10.	C – 0.75; T – 0.25.	CC – 0.46; CT – 0.27; TT – 0.27.	C – 0.60; T – 0.40.
LEP	CC – 0.60; CT – 0.23; TT – 0.17.	C – 0.72; T – 0.28.	CC – 0.47; CT – 0.43; TT – 0.10.	C – 0.68; T – 0.32.

The research results (features of the genetic structure of cattle populations of both breeds) fully correspond to the results obtained earlier (Kulibaba et al., 2019, 2021). No significant changes in genetic and population parameters were found.

Discussion. It should be noted that at the first stage, the goal of our research was to determine the genetic and population parameters in order to analyze the polymorphism of each of the genes in the experimental groups of cattle. That is why, according to FAO recommendations, we used only 30 individuals of each breed for genotyping, as this number is minimal for research. On the basis of the obtained results, an analysis of the possibility of using complex genotypes for the needs of marker-assisted selection was carried out in terms of the perspective of this approach in MAS programs for obtaining herds of cattle producing A2 milk.

According to the results of earlier studies, the formula of promising complex genotypes for the loci of beta-casein, prolactin and leptin for cattle of the Ukrainian Black-and-White dairy breed has the following form $CSN2^{A2A2}PRL^{CC}LEP^{CC}$. In this formula, the $CSN2^{A2A2}$ genotype gives the possibility of obtaining milk that contains only the A2 form of beta-casein (that is, A2 milk directly). In turn, individuals with the PRL^{CC} genotype show higher milk yield values within several lactations compared to individuals with other genotypes (PRL^{CT} and PRL^{TT}). At the same time, the difference in values between individuals with different genotypes can reach 16%. According to the leptin locus, individuals with a genotype homozygous for the C allele are characterized by an increased value of breast milk, up to 19%, which is most denoted during the first lactation.

As we can see, all variants of the loci in the complex genotype formula are in the homozygous state. According to the results of this formula, the animals are milk producers of type A2, while they are characterized by higher milk yield values for different lactations compared to individuals with other genotypes for these loci. The main question, in this context, is whether it is possible to select individuals that meet the formula in the research population of animals? Is the standard sample of 30 heads, which according to FAO recommendations is the minimum for describing genetic-population parameters, sufficient, or should a larger sample be analyzed?

To provide an answer to the above questions, we will carefully consider the results of the analysis of the experimental population of cattle of the Ukrainian Black-and-White dairy breed (n=30), which are listed in the table. As we can see, the limiting factor for selection is the number of individuals with the A^2A^2 genotype at the beta-casein locus (6 heads), provided that a group of A2 milk-producing animals is created. At the same time, the number of individuals with the desired genotype at the prolactin and leptin locus is significantly larger and, in fact, the same (18). Therefore, the minimum value of the number of individuals homozygous for the A^2 allele at the beta-casein locus is a limiting factor for any options for selecting animals from this sample. In addition to everything, the research loci (*CSN2*, *PRL* and *LEP*) are located on different chromosomes (beta-casein gene – on 6; prolactin gene – on 23; leptin gene – on 4, respectively), which makes it impractical to conduct haplotype analysis in contrast to mutations, contained within one locus. This imposes certain restrictions on the possibility of using combinations of genotypes in the form of a single functional unit, which, at the same time, is leveled by the homozygous state of each of the loci in the formula of the desired complex genotypes for the research cattle breeds.

According to the results of typing individuals of the experimental group of animals of the Black-and-White dairy breed with the complex desired genotype $CSN2^{A2A2}PRL^{CC}LEP^{CC}$, only 3 heads were found. Other individuals with the $CSN2^{A2A2}$ homozygous genotype were characterized by the presence of a heterozygous genotype at the prolactin locus and a homozygous/heterozygous TT/CT genotype at the leptin locus ($CSN2^{A2A2}PRL^{CT}LEP^{TT}$ or $CSN2^{A2A2}PRL^{CT}LEP^{CT}$). Thus, under the condition of the established distribution of genotypes by three loci, when analyzing 30 heads of cattle of

the Ukrainian Black-and-White milk breed, the proportion of animals with the desired complex genotype, which can be selected for further breeding work, is only 10%. It should also be noted that it is possible to analyze individuals for further selection for the purpose of creating herds of A2 milk-producing cattle only after bonification (i.e. breeding core), which imposes additional restrictions.

Considering cattle of the Ukrainian Red-and-White dairy breed, the situation is slightly different, which is associated with certain corrections that arise as a result of the peculiarities of the distribution of genotype and allele frequencies by the studied loci (table). It should be noted that in the case of the Ukrainian Red-and-White dairy breed, the formula of the desired complex genotypes has the following form $CSN2^{A2A2}PRL^{TT}LEP^{CC}$. The main difference concerns the TT genotype at the prolactin locus. For the experimental breed of cattle, according to the results of research, the predominance of individuals with the PRL^{TT} genotype compared to PRL^{CT} and PRL^{CC} (which can reach 12%) was established, which led to a change in the formula. In turn, by locus of leptin, as in the case of the Ukrainian Black-and-White dairy breed, CC is the preferred genotype. Individuals with this genotype demonstrate increased values of the standard milk parameter compared to individuals with other genotypes during three lactations (the difference between the indicators of individuals with different genotypes reaches almost 20%). Due to the peculiarities of the distribution of allele frequencies and genotypes, the limiting factor for this breed of cattle is the TT genotype at the prolactin locus. The number of individuals with this genotype is 8 heads. At the same time, the number of individuals with the $CSN2^{A2A2}$ genotype was 10 heads.

According to the results of the analysis, the number of individuals of the Ukrainian Red-and-White dairy breed with the desired complex genotype $CSN2^{A2A2}PRL^{TT}LEP^{CC}$ was 5 heads. Other individuals (with a homozygous genotype for the A^2 allele of the beta-casein locus) were characterized by complex genotypes $CSN2^{A2A2}PRL^{CT}LEP^{CC}$, $CSN2^{A2A2}PRL^{CC}LEP^{CC}$, $CSN2^{A2A2}PRL^{CC}LEP^{CT}$ and $CSN2^{A2A2}PRL^{CT}LEP^{CT}$. Thus, the share of individuals of the Ukrainian Red-and-White dairy breed, which were characterized by the desired complex genotype and are suitable for creating a group of animals producing milk A2, was almost 17% (5 heads).

In a comparative aspect, the selection of individuals with the desired complex genotype by the number of animals is more effective in the population of cattle of the Ukrainian Red-and-White dairy breed (practically twice the number of individuals) in comparison with Black-and-White cattle. However, in any case, for the effective formation of a breeding nucleus, it is necessary to significantly increase the number of individuals for genotyping by experimental loci. The minimum number of individuals (n=30) for genetic population studies is sufficient only to determine the characteristics and ratio of frequencies of alleles and genotypes, but, especially under the condition of increasing the number of research loci, but insufficient for the selection of animals according to complex genotypes.

It should be determined that increasing the number of components of the formula of the desired complex genotype significantly complicates the task, as it leads to an increase in the number of options and, accordingly, to a decrease in the number of individuals with the desired genotype under the condition of a limited sample. We may give an example for a system consisting of two alleles per locus. If individuals are selected based on the results of typing at one locus (A^1 or A^2 allele of beta-casein), there will be 3 different options; for two loci (*CSN2* and *PRL*) – already 9 variants; for three (*CSN2*, *PRL* and *LEP*) – 27 variants of complex genotypes. Accordingly, under the condition of an even distribution of individuals with different complex genotypes in the sample, only 3-4 individuals with the desired complex genotypes can be selected from a population

consisting of 100 individuals. It is quite clear that the peculiarities of the genetic structure of different cattle populations (the ratio of genotype frequencies within groups) lead to certain variations in the number of individuals with complex genotypes, but, in any case, for the selection of individuals, it is necessary to increase the number of analyzed animals.

In addition to all of the above, the need to conduct a primary analysis of the genetic population structure of experimental groups of animals should be noted as an integral component of the general concept of marker-assisted selection, which we have repeatedly emphasized in many publications. Knowledge of the features of the genetic structure (features of the organization of genetic variability in the population) of experimental animal populations makes it possible to predict the prospects of selection at the level of complex genotypes and serves as a foundation for further marker-assisted selection (MAS). This especially applies to the analysis of variations that are not located within the same locus. Taking into account that each of the studied loci (*CSN2*, *PRL*, *LEP*) is in different parts of the animal genome (in different chromosomes), it does not make sense to perform haplotype analysis (as it happens when studying different mutations within the same locus), therefore determination of complex genotypes is, in fact, an alternative option for analysis.

The use of complex genotypes to create herds of A2 milk-producing cattle is also promising for the application of methodological approaches of the Gene Pyramiding system, which, in the context of the conducted research, can become an integral component of the general strategy of MAS – Marker-assisted Gene Pyramiding (Xu et al., 2012). When using such a system, it is necessary to carry out genotyping not only of cattle, but also of breeders according to experimental loci and, in the future, to use only sperm with the specified genotype to obtain offspring of the desired type. Thus, the required number of individuals can be obtained during several generations. It is the combination of different methodological approaches that is the most effective strategy for obtaining high-quality livestock products (A2 milk) against the background of the absence of potential risks of reducing the parameters of total milk productivity due to the influence of "unwanted" alleles of other loci (in the presented work, these are loci of prolactin and leptin).

Conclusions.

1. Based on the results of the research, it was established that the loci of beta-casein (c.350C>A, rs43703011), prolactin (c.35333764 C>T, rs211032652) and leptin (c.239C>T, rs29004508) are polymorphic according to marker mutations in research populations of Ukrainian Black-and-White and Red-and-White dairy cattle.

2. The number of individuals with the "desired" complex genotype $CSN2^{A2A2}PRL^{CC}LEP^{CC}$ in the cattle population of the Ukrainian Black-and-White dairy breed is 10%, while the number of individuals with the "desirable" complex genotype $CSN2^{A2A2}PRL^{TT}LEP^{CC}$ in the cattle population of the Ukrainian Red-and-White dairy breed is 17%.

3. It has been proven that for the effective selection of individuals with the desired complex genotypes based on the identified polymorphic loci in both breeds of dairy cattle, the use of the minimum number of individuals (n=30) is insufficient.

4. For the effective creation of herds of A2 milk-producing cattle, it is recommended to use the proposed complex genotypes for both experimental breeds of cattle using the methodical approaches of the Gene Pyramiding – Marker-assisted Gene Pyramiding system.

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