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Analysis of genotyping features of bovine cattle individuals at the *CSN2* locus using ACRS-PCR methods

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Abstract. In the context of solving the problem of obtaining high quality dairy products from livestock, the issue of determining the type of beta-casein (A1 and A2) in the protein fraction of milk becomes essential. Purpose – to analyse the use of ACRS-PCR methods for differentiation of A^1 and A^2 alleles of bovine beta-casein locus. Genotyping features were analysed using the artificially created restriction site polymerase chain reaction method utilising TaqI and DdeI restriction endonucleases. The electrophoretic distribution of DNA fragments in agarose gels of various concentrations was used to analyse restriction patterns. Based on the results of bioinformatic analysis of the nucleotide reference sequences of the experimental fragment of the beta-casein gene, it was found that the primer system for the ACRS-PCR DdeI method is characterised by higher parameters of flanking efficiency of the target DNA site compared to the ACRS-PCR TaqI system due

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to significantly greater effectiveness of hybridisation of oligonucleotides on the target DNA. Based on the results of laboratory tests of both methods, it is proposed to use an additional procedure for analysing the fluorescence intensity of individual elements of restriction patterns, which allows reducing the number of false genotyping that occurs in both cases (based on the results of using both methods) due to the appearance of non-specific amplification/restriction fragments within the size of target restrictions. The application of the ACRS-PCR DdeI method provides more differentiated patterns of the corresponding genotypes in agarose gel compared to the ACRS-PCR TaqI method, but leads to higher material costs for conducting research. These disadvantages of using primer systems for ACRS-PCR of the beta-casein locus determine the relevance of developing alternative methods for typing A^1 and A^2 alleles which include allele-specific PCR. The use of results is promising for solving the problems of genotyping cattle individuals of different breeds by A^1 and A^2 alleles of the beta-casein locus

Keywords: polymorphism; restriction; electrophoresis; allele; amplification

Introduction

High quality of dairy products is the ultimate goal of practical work in cattle breeding. As noted by S.A. Ibrahim *et al*. (2021), one of the main factors that negatively affect the consumption of milk and dairy products is lactose intolerance syndrome, which is believed to be caused primarily by genetic factors and is associated with insufficient lactase activity. However, as the results of numerous studies have shown, according to the review of S. Pal *et al*. (2015), the negative effects of milk consumption are associated not only with lactose, but also with individual protein fractions.

According to M. Macedo *et al*. (2020), the total protein fraction of milk consists of 80% casein fraction, with the largest being the beta-casein fraction, which reaches 40% of the total amount. As indicated by Bisutti *et al*. (2022) and De Vitte *et al*. (2022), in the context of the negative effects of milk consumption, the focus is on beta-casein, some variants of which are associated with the manifestation of various diseases. According to the study by S. Kay *et al*. (2021), a special substance, 7 beta-casomorphine, which is very closely related to beta-casein, is involved in the development of pathological effects from the use of dairy products.

A. Summer *et al*. (2020) report that casomorphin is a short peptide consisting of 7 amino acid residues. The functions of this peptide include the ability to model the effect of a number of different hormones of the gastrointestinal tract and central nervous system. It has an opioid effect that is directly related to the manifestation of allergic reactions. The situation is complicated by the fact that casomorphine is able to pass through the blood-brain barrier, which is of greatest importance for children.

According to C. Sebastiani *et al*. (2020), beta-casein molecule can exist as several variants that differ in certain amino acid residues in the molecule – A^1 and A^2 forms of beta-casein. The differences between the two types of molecules are in one amino acid (only for A^1 and A^2 forms). As indicated in the paper by Thiruvengadam *et al*. (2021) in A^2 this is proline at position 67, and in A^1 – histidine. The presence of histidine at position 67 determines the entire further fate of the molecule. When A^1 beta-casein enters the gastrointestinal tract, cleaving occurs at the

position containing histidine, which leads to the formation of beta-casomorphins. In turn, this is not happening in the case of type A^2 beta-casein. Thus, the consumption of milk containing beta-casein type A^1 leads to the formation of beta-casomorphins, which causes various pathological effects, the most prominent manifestation of which is digestive system disorders.

Understanding the nature of a point mutation in the beta-casein gene that determines the existence of allelic variants A^1 and A^2, which enabled the creation of effective tools for its detection. In the last few years, a number of different molecular genetic research methods are used to differentiate A^1 and A^2 alleles. As stated by C. Sebastiani *et al*. (2022), M. Sodhi *et al*. (2021) and Ivankovic *et al*. (2021), despite the attractiveness of modern sequencing methods, which are also used for typing cattle individuals at the beta-casein locus, the most common are methodological approaches for using certain restriction endonucleases. However, despite the widespread use of methodological approaches based on the use of restriction endonucleases, the analysis of technical features of typing cattle individuals was rather neglected (in a comparative aspect).

Thus, the purpose of this study is to analyse the features of genotyping of cattle individuals at the *CSN2* locus (alleles A^1 and A^2) using ACRS-PCR methods.

Literature Review

According to Yamada *et al*. (2021), methods for detecting allelic variants of the beta-casein gene were developed, which use not just analyse a product – milk, but directly analyse the producer – in this case, a cow. One of the main obstacles to the effective genotyping by an experimental mutation using one of the most common genotyping methods, PCR-RFLP, or restriction analysis, is the fact that the SNP (point mutation) that determines the presence of variants A^1 and A^2, is not located at the restriction site for any endonuclease. The lack of variations in the restriction site makes it impossible to use PCR-RFLP, which necessitates the development of alternative methods. As noted by Smiltina *et al*. (2018) and Antonopoulos *et al*. (2021), one of the most common alternative methods for detecting various allelic variants *CSN2* gene is ACRS-PCR.

According to the study by Pauciullo *et al*. (2021), ACRS-PCR (artificially created restriction site polymerase chain reaction) is based on the use of restriction endonuclease, but, unlike classical PCR-RFLP, the site for the enzyme is present only in the amplicon, and not in the original fragment of the gene. Dąbrowski *et al*. (2019), report that the creation of an artificial restriction site in an amplicon occurs through the use of a special primer in a standard pair, which at the 3' end contains a "false" nucleotide (usually at position 2 of the 3' end), which, in the case of one of the alternative alleles, forms a restriction site during amplification. It is the presence of a mismatch nucleotide in combination with the copied sequence of one of the alleles that leads to the formation of a restriction site in the amplicon. After amplification, a standard restriction analysis is performed, followed by electrophoretic separation of fragments in the gel. The method allows genotyping of cattle individuals at the beta-casein locus with high success.

One of the first modifications of this method was proposed by S. Lien *et al.* in 1992 based on the use of TaqI as a restriction endonuclease. In the following years, a number of modifications appeared, one of the most common is the method developed by McLachlan (2006), based on the use of DdeI restriction endonuclease. The variability of the effectiveness of the above methods underlies the need to search for

new and optimise existing methods for typing beta-casein alleles, which is particularly important in the context of genotyping cattle individuals to solve commercial issues.

Materials and Methods

The research was conducted in the laboratory of molecular genetic research of the Department of animal biology of the National University of Life and Environmental Sciences of Ukraine.

To investigate the effectiveness of genotyping of cattle individuals by A^1 and A^2 alleles, DNA samples from cows of the Ukrainian Black-and-White dairy breed were used as a model object of the beta-casein locus. Hair follicles were used as a source of biological material. DNA isolation was performed using a commercial set of reagents "DNA-sorb-B" ("AmpliSens").

Genotyping of experimental cattle breeds was performed using ACRS-PCR methods (2 methods – using TaqI and DdeI restriction endonucleases), the ACRS-PCR method allows creating an artificial restriction site in the amplicon, which, in turn, allows using appropriate restriction endonucleases for typing individuals. The authors used ARRIVE instructions as a checklist during experiments. Certain primer systems were used to amplify a fragment of the seventh exon of the beta-casein gene. The nucleotide structure and names of primers are shown in Table 1.

Table 1. *Method, labelling, and nucleotide structure of the primers used*

Method	Primer	Source	Nucleotide sequence
ACRS-PCR (DdeI)	DdeI F	(McLachlan, 2006)	ccttctttccaggatgaactccagg
	DdeI R		gagtaagaggagggatgttttgtgggaggctct
ACRS-PCR (TaqI)	TaqI F	(Lien *et al.*, 1992)	cctgcagaattctagtctatcccttccctgggcccatcg
	TaqI R		gagtcgactgcagattttcaacatcagtgagagtcaggctctg

The following amplification programmes were used to amplify the experimental fragment of the beta-casein gene: 1 cycle – denaturation at 94°C, 5 min.; 35 cycles – denaturation at 94°C, 30 s, annealing (60°C for ACRS-PCR TaqI and 56°C for ACRS-PCR DdeI) 30 s, elongation – 72°C, 30 s.

Polymerase chain reaction (PCR) was performed using a commercial DreamTaq PCR Master Mix reagent kit (ThermoScientific). The volume of the final reaction mixture was 10 µL, the primer concentration was 0.2 µM.

Size of restriction fragments in the case of ACRS-PCR DdeI (restriction site C↓TNAG) is 121 bp for A^1 allele; 86 and 35 bp for the A^2 allele.

Size of restriction fragments in the case of ACRS-PCR TaqI (restriction site T↓CGA) is 251 bp for A^2 allele; 213 and 38 bp for the A^1 allele.

For the purpose of electrophoretic separation of amplification/restriction products, a 3% agarose gel for the ACRS-PCR DdeI method and a 1.5% gel for the ACRS-PCR TaqI method were used. Restriction products were separated in agarose gels at a voltage of 150 V for 40-60 minutes.

Visualisation of DNA fragments in the gel was performed using ethidium bromide in the ultraviolet spectrum (312 nm). The molecular weight marker GeneRuler 50 bp (Thermo Scientific) was used to determine the size of the amplification/restriction fragments.

The online Nucleotide Blast toolkit was used to analyse nucleotide sequences. To determine the amplification efficiency when using bioinformatic analysis, the X14711.1 bovine beta-casein gene nucleotide sequence was used as a reference.

Results and Discussion

A comparative analysis of the effectiveness of ACRS-PCR methods was carried out, considering the bioinformatic analysis of nucleotide sequences in the GenBank database.

Based on the results of bioinformatic analysis, it was found that the ACRS-PCR DdeI primer system (primer sequences are given above) is more preferable in terms of hybridisation efficiency (total number of matches according to Watson-Crick nucleotide interaction) with the reference sequence compared to the ACRS-PCR TaqI system. The analysed fragment of the beta-casein gene is shown in Figure 1.

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7981 attataactg gattatggac tcaaagattt gtttccttc tttccaggat gaactccagg
8041 ataaaatcca ccccttgcc cagacacagt ctctagtcta tcccttccct ggacccatcc
8101 ataacagcct cccacaaaac atccctcctc ttactcaaac ccctgtggtg gtgccgcctt
8161 tccttcagcc tgaagtaatg ggagtctcca aagtgaagga ggctatggct cctaagcaca
8221 aagaaatgcc cttccctaaa tatccagttg agccctttac tgaaagccag agcctgactc
8281 tcactgatgt tgaaaatctg caccttcctc tgcctctgct ccagtcttgg atgcaccagc
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Figure 1. Fragment of the beta-casein gene that is flanked by the use of appropriate primer systems
Note: (Bovine beta-casein gene. GenBank: X14711.1 as amended). Hybridisation sites for ACRS-PCR (DdeI) primers are marked with rectangles, and for ACRS-PCR (TaqI) – in grey.
A target mutation C>A is indicated in bold (position 8101 "a")

The efficiency of forward and reverse primer hybridisation (ACRS-PCR DdeI) was 100%, excluding the nucleotide mismatch (position 8105 C > G, Figure 1). In turn, for the ACRS-PCR TaqI method, the hybridisation efficiency of forward and reverse primers was 55 and 82%, respectively. In each case, the presence of a kind of "tail" of nucleotides was observed (positions 8162-8171 and 8303-8308, Figure 1), which can lead both to a decrease in PCR efficiency and to the formation of non-specific amplification products due to suboptimal temperature conditions for annealing primers on the DNA matrix. The established features are also reflected in the electrophoregrams.

Figure 2 shows an electrophoregram of restriction products of the seventh exon of the beta-casein gene using TaqI. Individuals with all possible genotypes were found in the experimental group: A^1A^1, A^1A^2, and A^2A^2.

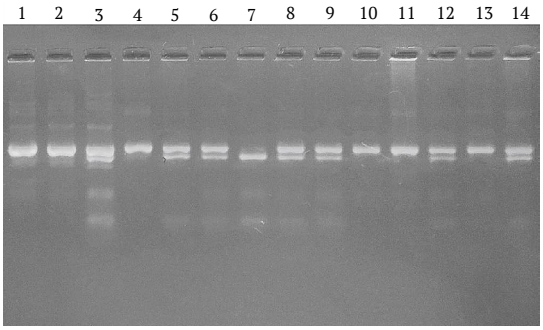

Figure 2. Results of typing of cattle individuals using the ACRS-PCR (TaqI) method
Note: 1 – amplicon; 2, 4, 10, 11, 13 – A^2A^2; 3, 5, 6, 8, 9, 12, 14 – A^1A^2; 7 – A^1A^1

Genotype A^1A^1 is represented on the electrophoregram by fragments 213 and 38 bp; A^1A^2 – 251, 213, and 38 bp; A^2A^2 – 251 bp. The presence of non-specific amplified DNA fragments on the electrophoregram is considered undesirable phenomena for several reasons. First, non-specific amplified fragments can potentially contain a restriction site for the endonuclease used, which will significantly complicate both the effectiveness of the reaction and the interpretation of the resulting patterns. Second, the size of non-specific fragments may coincide with the restriction patterns for the target object. In the case of using the ACRS-PCR TaqI method, this pattern is observed (Figure 2, samples 1-4). Optimisation of the PCR protocol (first of all, the temperature regime of primer annealing) allows reducing the formation of non-specific fragments to a minimum. However, in the case of the pronounced disadvantages of the primer system mentioned above, the formation of non-specific products still occurs. This leads to the need to analyse the intensity of the fluorescence of fragments in a comparative aspect with the target restrictions. Target restriction fragment (allele A^2, size 251 bp) has a much higher luminous intensity in the ultraviolet spectrum (ethidium bromide is used as an intercalating dye) due to the larger amount of DNA. Based on the analysis of the fluorescence intensity of fragments, it is possible to distinguish between "true" and "false" bands on the electrophoregram. However, the situation significantly worsens as a result of a general decrease in the amplification efficiency. In this case, it is very difficult to distinguish between target and non-specific products, which further leads to difficulties in genotyping individuals.

In some samples, it is possible to observe the formation of non-specific fragments that have a size within the target restrictions, which can lead to erroneous typing of individuals as heterozygous. In this case, it is necessary to analyse the fluorescence intensity of various fragments of the pattern. The presence of significant shortcomings in the above-mentioned method (the impossibility of unambiguous and error-free identification of various genotypes in the absence of additional procedures) leads to the need to develop new methods for typing by alleles of the beta-casein locus. Figure 3 shows a photo of the electrophoregram of the results of typing bovine individuals using the ACRS-PCR DdeI method. The electrophoregram shows individuals with all possible genotype variants: A^1A^1, A^1A^2, and A^2A^2. Genotype A^1A^1 shown on the electrophoregram as a single fragment, measuring 121 bp; A^1A^2 – 121, 86, and 35 bp; A^2A^2 – 86 and 35 bp.

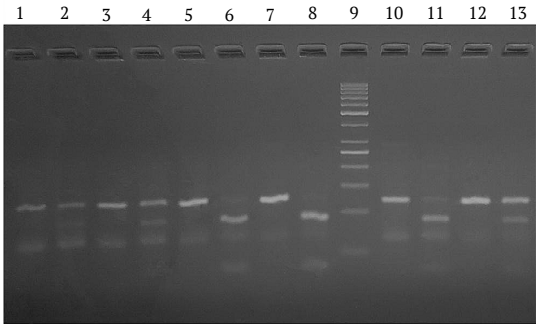

Figure 3. Results of typing of cattle individuals using the ACRS-PCR (DdeI) method
Note: 1 – amplicon; 3, 5, 7, 10, 12 – A^1A^1; 2, 4, 13 – A^1A^2; 6, 8, 11 – A^2A^2; 9 – molecular weight marker GeneRuler 50 bp

The ACRS-PCR method, using DdeI restriction endonuclease, has proven itself quite well as a routine tool for typing cattle individuals at the beta-casein locus, but despite all the advantages, it also has a number of disadvantages, some of which lead to errors in genotyping. The main disadvantage of this method is the need to use DdeI restriction endonuclease, which leads to an increase in time and material costs. In a fairly large number of cases, the presence of non-specific fragments on the electrophoregram was noted, which, given the rather small size of the original amplicon (121 bp), significantly hinders the identification of the A^2 allele and the heterozygous genotype A^1A^2. This occurs based on the results of the presence of a non-specific fragment in the identification zone of the A^2 allele in the restriction pattern. The presence of a non-specific fragment that is sufficiently close to the restriction (86 bp) may be a factor in misinterpreting this genotype as heterozygous, which may lead to a decrease in the number of identified homozygous for the A^2 allele, individuals in the population under study. An additional factor that significantly complicates the identification of alleles is the need to analyse the fluorescence intensity (staining) of restriction fragments. In the case of the absence of inhibitors in the PCR mixture, the intensity of staining of the fragment of the A^2 allele must be smaller than that of a fragment of the A^1 allele, due to a smaller amount of DNA (amplicon of the A^2 allele contains a restriction site for DdeI). Ignoring these facts on the part of researchers can lead to confusion in the analysis of genotypes in the case of insufficient restriction endonuclease activity.

As follows from the results of research and analysis of these electrophoregrams, the detection method based on the use of DdeI restriction endonuclease is more convenient and effective for the needs of cattle typing compared to the TaqI method. The results of experimental studies fully confirm the conclusions that were made during the bioinformatic analysis (given above). The greater efficiency of primer hybridisation on the target DNA sequence, which was demonstrated by analysing the correspondence of the nucleotide structure of the study objects (Watson-Crick interactions between the forward and reverse primer sequences and the target DNA), resulted in greater PCR efficiency. First of all, by increasing the specificity of the reaction, and secondly, by increasing the efficiency of amplification. The increase in the specificity of the reaction is directly caused by the structural features of the oligonucleotides used, which, in the case of ACRS-PCR DdeI, hybridise with the experimental fragment as efficiently as possible. In turn, unambiguous positioning of the primer on the total genomic DNA (flanking the experimental fragment of the genome) leads to a high efficiency of the amplification itself.

Despite the above advantages, the ACRS-PCR method for using DdeI restriction endonuclease has certain disadvantages. In in some cases, the formation of non-specific reaction products is observed. At the same time, the presence of non-specific DNA fragments is recorded in the zone that overlaps with the existing restriction fragments, which can potentially affect the effectiveness of typing (to increase the number of heterozygous individuals in the experimental group of animals). The use of DdeI restriction endonuclease leads to a significant increase in the cost of conducting research, which, along with the standard disadvantages of the ACRS-PCR method that include the need for additional stages of analysis (requires significant time and reagents), determines the relevance of developing alternative methods for typing A^1 and A^2 alleles, the use of which would not have the above-mentioned disadvantages,

which was proposed by a number of researchers in the form of allele-specific PCR techniques.

The allele-specific PCR (AS-PCR) method is devoid of additional costs associated with restriction analysis, although it requires setting up 2 amplification reactions (for each of the alleles). In addition, as practice shows, the AS-PCR method also has disadvantages caused by the hybridisation features of primers that differ by 1 nucleotide at the 3' end, which can also affect the accuracy of genotyping. If PCR protocols are optimised, this method shows fairly accurate results. However, despite the existing advantages, the allele-specific PCR method requires compliance with specific requirements for the accuracy of maintaining temperature conditions with an amplifier (thermal cycler). Any variability or technical impossibility of using certain temperatures during different PCR cycles leads to non-specific amplification of alleles, which can lead to errors in genotyping (first of all, to an increase in the total number of heterozygous individuals in the sample). Therefore, in almost every case, for genotyping different targets, it is necessary to carefully adapt the amplification protocols of experimental fragments, provided that an additional method of individual typing is used. In the vast majority of studies using the allele-specific PCR method, the authors actually ignore the need to optimise protocols and use the amplification programmes available in other literature sources without any modifications (Rahimi *et al.*, 2015).

In turn, the use of a bioinformatic approach, which is based on the analysis of the GenBank nucleotide sequence database, allows not only identifying specific primer hybridisation sites on the target DNA and the size of the flanked fragment, but also establishing potential non-specific hybridisation sites, which can significantly help in optimising PCR protocols. In both cases (ACRS-PCR TaqI and DdeI), the results of the analysis of the reference nucleotide sequence (X14711.1) established a high specificity of the selected primer systems. According to the results of the analysis, only two potential (additional to the target) variants were identified for the ACRS-PCR TaqI primer system, while they were completely absent for ACRS-PCR DdeI. The potential possibility of amplification of non-specific fragments during PCR along with the target fragment of the genome led to the appearance of additional DNA bands (false bands) on the electrophoregram.

In addition to the general information "contamination" of the image, the presence of non-specific fragments significantly complicates the process of restriction of amplicons, since additional amplified sections of DNA can potentially contain a restriction site for the restriction used, which leads not only to a large number of different fragments, but also to a significant decrease in the effectiveness of the restriction reaction. All of the above negatively affects the quality of typing, so, if possible, any potential non-specific hybridisation of primers in other parts of the genome should be avoided (this is especially true in the case of non-specific annealing in different parts of the genome that coincide with the target for the animal species). In view of the above, it becomes clear that PCR protocols need to be optimised to prevent nonspecific amplification. The best approach is to use the maximum possible (highest possible) annealing temperature, as well as (which is less effective) variations in the duration and number of amplification cycles. The results of the bioinformatic analysis are confirmed by the results of electrophoretic separation of restriction fragments, which is shown in Figures 2 and 3 – in the case of the ACRS-PCR TaqI method, additional fragments are available on the electrophoregram, while when using the ACRS-PCR DdeI method, there are significantly less

non-specific amplification/restriction products. Thus, the use of bioinformatic analysis of amplification efficiency is fully confirmed by direct laboratory studies on typing cattle individuals at the experimental locus.

Given the above, it would be advisable to use AS-PCR as the main (simpler and less expensive) method for conducting routine studies of beta-casein locus polymorphism. In turn, to resolve controversial issues related to the accuracy of genotyping, it is necessary to use an alternative method based on ACRS-PCR, in particular, for the use of DdeI restriction endonuclease.

Additional aspects of the problem of animal typing by A^1 and A^2 alleles of the beta-casein gene refer to confusion regarding the use of certain terminology. The methods mentioned in this paper relate specifically to ACRS-PCR, which is caused by the fact that there is no restriction site for any endonuclease in the target fragment (which contains a polymorphic nucleotide). Accordingly, the use of the ACRS-PCR method (for both DdeI and TaqI) leads to the formation of an artificial restriction site in amplicon (amplified fragment), and not in the experimental fragment of the gene (genome). Therefore, the use of the PCR-RFLP designation, in this case, is completely incorrect, which, unfortunately, is not considered by some researchers (Pandey *et al*., 2020; Vougiouklaki *et al*., 2020; Mokhnachova, 2021).

Conclusions

The effectiveness of ACRS-PCR methods (for the use of DdeI and TaqI restriction endonucleases) for typing cattle individuals by the beta-casein locus (typing alleles A^1 and A^2) was analysed. According to the results of bioinformatic analysis of nucleotide reference sequences (X14711.1) of the experimental fragment of the beta-casein gene, it was found that the primer system for the ACRS-PCR DdeI method is characterised by higher parameters of flanking efficiency of the target DNA section compared to the ACRS-PCR TaqI system, due to significantly greater effectiveness of hybridisation of oligonucleotides on the target DNA and almost the complete absence of non-specific amplification products. The results of the theoretical analysis are fully confirmed by laboratory studies on animal typing at the beta-casein locus by two methods. Based on the results of laboratory tests of both methods, it is proposed to use an additional procedure for analysing the fluorescence intensity of individual elements of restriction patterns, which allows reducing the number of false genotyping that occurs in both cases (based on the results of using both methods) due to the formation of non-specific amplification/restriction fragments within the size of target restrictions. Considering these shortcomings of primer systems (ACRS-PCR) for typing cattle individuals at the beta-casein locus, it is advisable to consider alternative methods of genotyping, in particular, the allele-specific PCR method, which, despite certain features of its application, is quite promising for conducting routine large-scale studies in cattle populations of different breeds. In addition to all of the above, ACRS-PCR methods based on the use of DdeI and TaqI restriction endonucleases can be very useful for resolving controversial issues as a reference verification method for typing in order to avoid errors in genotyping cattle individuals by the beta-casein locus. The use of ACRS-PCR methods for typing cattle individuals by allelic variants of the beta-casein locus, both as the main method of typing and for solving technical problems in PCR formulation, allows not only conducting genetic and population studies of different cattle breeds of cows, but also serves as a reliable tool for further implementation of methodological approaches to marker-associated selection (MAS) in dairy

cattle breeding. The use of the proposed methodological approaches allows effectively and accurately differentiating different genotypes (A^1A^1, A^1A^2, and A^2A^2) by the beta-casein locus, conducting individual typing of cattle individuals, and selecting animals with the desired genotype for use in further breeding to create herds of cows producing A2 Milk. Other promising areas include the development of alternative methods for typing cattle individuals by allelic variants A^1 and A^2 of beta-casein locus using ACRS-PCR as a reference method.

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None.

Conflict of Interest

None.

References

[1] Antonopoulos, D., Vougiouklaki, D., Laliotis, G.P., Tsironi, T., Valasi, I., Chatzilazarou, A., Halvatsiotis, P., & Houhoula, D. (2021). Identification of Polymorphisms of the CSN2 Gene Encoding β-Casein in Greek Local Breeds of Cattle. *Veterinary Science,* 8, article number 257. doi: 10.3390/vetsci8110257.

[2] Bisutti, V., Pegolo S., Giannuzzi, D., Mota, F.M., Vanzin, A., Toscano, A., Trevisi, E., Marsan, P.A., Brasca, M., & Cecchinato, A. (2022). The β-casein (CSN2) A2 allelic variant alters milk protein profile and slightly worsens coagulation properties in Holstein cows. *Journal of Dairy Science,* 105, 3794-3809. doi: 10.3168/jds.2021-21537.

[3] Dąbrowski, A., Ułaszewski, S., & Niedźwieck, K. (2019). Rapid and easy detection of the five most common founder mutations in BRCA1 and BRCA2 genes in the Polish population using CAPS and ACRS-PCR methods. *Acta Biochimica Polonica*, 66(1), 33-37. doi: 10.18388/abp.2018_2654.

[4] De Vitte, K., Kerziene, S., Klementavičiūtė, J., De Vitte, M., Mišeikienė, R., Kudlinskienė, I., Čepaitė, J., Dilbiene, V., & Stankevičius, R. (2022). Relationship of β-casein genotypes (A^1A^1, A^1A^2 and A^2A^2) to the physicochemical composition and sensory characteristics of cows' milk. *Journal of Applied Animal Research*, 50(1), 161-166. doi: 10.1080/09712119.2022.2046005.

[5] Ibrahim, S.A., Gyawali, R., Awaisheh, S.S., Ayivi, R.D., Silva, R.C., Subedi, K., Aljaloud, S.O., Siddiqui, S.A., & Krastanov, A. (2021). Fermented foods and probiotics: An approach to lactose intolerance. *Journal of Dairy Research*, 88(3), 357-365. doi: 10.1017/S0022029921000625.

[6] Ivankovic, A., Pecina, M., Ramljak, J., & Pasic, V. (2021) Genetic polymorphism and effect on milk production of CSN2 gene in conventional and local cattle breeds in Croatia. *Mljekarstvo*, 71(1), 3-12. doi: 10.15567/mljekarstvo.2021.0101.

[7] Kohut, M., Petryshyn, M., Sedilo, G., & Fedak, N. (2022). Exterior of cows of the Ukrainian black-spotted dairy breed, obtained under various selection options. *Scientific Horizons,* 25(9), 19-29. doi: 10.48077/scihor.25(9).2022.19-29.

[8] Lien, S., Alestrom, P., Klungland, H., & Rogne, S. (1992). Detection of multiple β-casein (CASB) alleles by amplification created restriction sites (ACRS). *Animal Genetics*, 23, 333-338. doi: 10.1111/j.1365-2052.1992.tb00155.x.

[9] Macedo, M., Pegolo, S., Bisutti, V., Bittante, G., & Cecchinato, A. (2020). Genomic Analysis of Milk Protein Fractions in Brown Swiss Cattle. *Animals*, 10(2), article number 336. doi: 10.3390/ani100203.

[10] McLachlan, CN. (2006). *Breeding and milking cows for milk free of β-casein A1*, United States Patent No. 7094949.

[11] Mokhnachova, N.B. (2021). Genotyping of "Ukrainian" water buffaloes according β-CN (A2-milk), CSN3 and βLG genes. *Proceedings of the National Academy of Sciences of Belarus, Agrarian Series*, 59(3), 361-365.

[12] Pal, S., Woodford K., Kukuljan, S., & Ho, S. (2015). Milk Intolerance, Beta-Casein and Lactose. *Nutrients*, 7(9), 7285-7297. doi: 10.3390/nu7095339.

[13] Pandey, A., Thakur, M.S., & Pandey, Y. (2019). Polymorphism of beta (β) casein gene and their association with milk production traits in Malvi and Nimari breeds of cattle. *Indian Journal of Animal Research*, 54(5), 647-650.

[14] Pauciullo, A., Martorello, S., Carku, K., Versace, C., Coletta, A., & Cosenza, G. (2021). A novel duplex ACRS-PCR for composite CSN1S1–CSN3 genotype discrimination in domestic buffalo. *Italian Journal of Animal Science*, 20(1), 1264-1269. doi: 10.1080/1828051X.2021.1952912.

[15] Rahimi, Z., Gholami, M., Rahimi, Z., & Yari, K. (2015). Evaluation of beta-casein locus for detection of A1 and A2 alleles frequency using allele specific PCR in native cattle of Kermanshah, Iran. *Biharean Biologist*, 9, 85-87.

[16] Sebastiani, C., Arcangeli, C., Ciullo, M., Torricelli, M., Cinti, G., Fisichella, S., & Biagetti, M. (2020). Frequencies Evaluation of β-Casein Gene Polymorphisms in Dairy Cows Reared in Central Italy. *Animals*, 10(2), article number 252. doi: 10.3390/ani10020252.

[17] Sebastiani, C., Arcangeli, C., Torricelli, M., Ciullo, M., D'avino, N., Cinti, G., Fisichella, S., & Biagetti, M. (2022). Marker-assisted selection of dairy cows for β-casein gene A2 variant. *Italian Journal of Food Science*, 34(2), 21-27. doi: 10.15586/ijfs.v34i2.2178.

[18] Smiltina, D., & Grislis, Z. (2018). Molecular genetics analysis of milk protein gene polymorphism of dairy cows and breeding bulls in Latvia. *Agronomy Research*, 16(3), 900-909. doi: 10.15159/AR.18.084.

[19] Sodhi, M., Kataria, R.S., Niranjan, S.K., Parvesh, K., Verma, P., Swami, S.K., Sharma, A., Bharti, V.K., Kumar, B., Iqbal, M., Rabgais, S., Kumar, P., Giri, A., Kalia, S., Gagoi, S., Sarangi, P.P., & Mukesh, M. (2021). Sequence Characterisation and Genotyping of Allelic Variants of Beta Casein Gene Establishes Native Cattle of Ladakh to be a Natural Resource for A2 Milk. *Defence Life Science Journal*, 3(2), 177-181. doi:10.14429/dlsj.3.12574.

[20] Summer, A., Di Frangia, F., Marsan, A.P., De Noni, I., & Malacarne, M. (2020). Occurrence, biological properties and potential effects on human health of β-casomorphin 7: Current knowledge and concerns. *Critical Reviews in Food Science and Nutrition*, 60, 3705-3723. doi: 10.1080/10408398.2019.1707157.

[21] Thiruvengadam, M., Venkidasamy, B., Thirupathi, P., Chung, I.M., & Subramanian, U. (2021). β-Casomorphin: A complete health perspective. *Food Chemistry*, 337, article number 127765. doi: 10.1016/j.foodchem.2020.127765.

[22] Vougiouklaki, D., Antonopoulos, D., Allexeli, S., & Houhoula, D. (2020). Identification of Polymorphisms of Gene CSN2 of β Casein in Greek Cow Breeds (Holstein) by Restriction Fragment Length Polymorphism. *Journal of Agricultural Science*, 12(11), 32-39. doi: 10.5539/jas.v12n11p32.

[23] Yamada, A., Sugimura, M., & Kuramoto, T. (2021). Genetic polymorphism of bovine beta-casein gene in Japanese dairy farm herds. *Animal Science Journal*, 92(1), article number e13644. doi: 10.1111/asj.13644.

Аналіз особливостей генотипування особин великої рогатої худоби за локусом *CSN2* за використання методів ACRS-PCR

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Анотація. У контексті вирішення проблеми одержання високої якості молочної продукції скотарства питання визначення типу бета-казеїну (А1 та А2) у білковій фракції молока набуває суттєвого значення. Мета роботи – проведення аналізу використання методів ACRS-PCR для диференціювання алелів A^1 та A^2 локусу бета-казеїну великої рогатої худоби. Аналіз особливостей генотипування проводили за використання методу ACRS-PCR (Artificially Created Restriction Site Polymerase Chain Reaction) із застосуванням ендонуклеаз рестрикції TaqI та DdeI. Для аналізу рестрикційних патернів використовували електрофоретичний розподіл фрагментів ДНК в агарозних гелях різних концентрацій. За результатами біоінформаційного аналізу нуклеотидних еталонних послідовностей дослідного фрагменту гену бета-казеїну з'ясовано, що праймерна система для методу ACRS-PCR DdeI характеризується більш високими параметрами ефективності фланкування таргетної ділянки ДНК у порівнянні з системою ACRS-PCR TaqI внаслідок значно більшої результативності гібридизації олігонуклеотидів на ДНК-мішені. За результатами лабораторних випробувань обох методів запропоновано використання додаткової процедури аналізу інтенсивності флуоресценції окремих елементів рестрикційних патернів, що дає можливість зменшити кількість помилкових генотипувань, що виникають в обох випадках (за результатами використання обох методів) за рахунок утворення неспецифічних ампліфікаційних/рестрикційних фрагментів у межах розміру цільових рестриктів. Застосування методу ACRS-PCR DdeI дає можливість отримати більш

диференційовані патерни відповідних генотипів в агарозному гелі у порівнянні з методом ACRS-PCR TaqI, але при цьому призводить й до більших матеріальних витрат на проведення досліджень. Зазначені недоліки використання праймерних систем для ACRS-PCR локусу бета-казеїну, визначають актуальність розробки альтернативних методів типування алелів A^1 та A^2 до яких належить алель-специфічна ПЛР. Використання результатів досліджень є перспективним для вирішення проблем генотипування особин великої рогатої худоби різних порід за алелями A^1 та A^2 локусу бета-казеїну

Ключові слова: поліморфізм; рестрикція; електрофорез; алель; ампліфікація