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Comparative analysis of A¹ and A² allele detection efficiency for bovine *CSN2* gene by AS-PCR methods

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This study was aimed at conducting a comparative analysis of the efficiency in genotyping cattle by beta-casein locus using the allele-specific PCR methods. The results of the study have demonstrated the necessity to optimize the protocol for the use of AS-PCR to detect alleles A¹ and A². It was found that the use of non-optimized PCR protocols led to the genotyping errors, manifested regarding beta-casein locus (*CSN2*). The impossibility of using the touchdown PCR as an optimization instrument for AS-PCR was proven. The elaborated typing protocols were used to study the genetic structure of the cattle populations of different breeds, reared in Ukraine – Ukrainian Red-and-White dairy, Ukrainian Black-and-White dairy (two populations), and Charolais. It was found that locus *CSN2* was polymorphic in all the cattle populations. The frequencies of allele A² varied within 0.34–0.91 depending on the population of the animals, which may be conditioned by the specificities in the selection work. No deviation from the Hardy-Weinberg equilibrium was found in any investigated population of cattle.

Key words: β-casein, allele, gene, polymorphism, cattle, population

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Abbreviations: ACRS-PCR, artificially created restriction site polymerase chain reaction; AS-PCR, allele-specific polymerase chain reaction; bp, base pair; BW, Ukrainian Black-and-White dairy breed; DNA, deoxyribonucleic acid; Fis, Wright's fixation index; He, expected heterozygosity; Ho, observed heterozygosity; PCR, polymerase chain reaction; Pop, population; RW, Ukrainian Red-and-White dairy breed

INTRODUCTION

The genetic structure analysis for the experimental populations of cattle is among the routine tasks of genetics and marker-associated selection in cattle breeding (Wakchaure *et al.*, 2015). However, along with such widely known qualitative trait loci as kappa casein, prolactin, beta-lactoglobulin, leptin, etc., related to the expression of productive traits of animals, in a few recent years, there has been a steady interest in the beta-casein gene, the allelic variants of which are related to the parameters of milk quality (Cieślińska *et al.*, 2019; Sebastiani *et al.*, 2022; Jiménez-Montenegro *et al.*, 2022). A¹ and A² belong to one of the best-studied and promising allele systems of beta-casein from the standpoint of applied genetics of cattle (Antonopoulos *et al.*, 2021). As shown in numerous studies, various forms of beta-casein are associated with a number of human pathological states which makes the *CSN2* gene a relevant research object in the context of the production performance of different dairy cattle breeds (Summer *et al.*, 2020; Bisutti *et al.*, 2022; De Vitte *et al.*, 2022). Alleles A¹ and A² differ by the presence of a specific amino acid in position 67 of the beta-casein molecule (Sebastiani *et al.*, 2020). In the case of form A¹, it is histidine, and as for A² – proline (Thiruvengadam *et al.*, 2020). Different forms of protein (beta-casein) are determined by the mutation in the *CSN2* gene, in position 304 (GenBank, NM_181008) – by the presence of adenine for allele A¹ and cytosine for allele A² (Dai *et al.*, 2016; Kay *et al.*, 2021). The replacement of nitrogenous bases in DNA does not relate to the restriction site for endonucleases which necessitated the elaboration of alternative typing methods for alleles at the *CSN2* locus. One of the most common methods is ACRS-PCR (Artificially Created Restriction Site), using TaqI and DdeI as restriction endonucleases (Lien *et al.*, 1992; McLachlan, 2006). The alternative elaborated variant was found in the methods of allele-specific PCR (AS-PCR), real-time probes for PCR, etc. (Manga *et al.*, 2010; Rahimi *et al.*, 2015; Giglioti *et al.*, 2021; Ristanic *et al.*, 2022). Sequencing is conducted in some cases, but regardless of its efficiency, at this stage, it is not always reasonable for routine large-scale population studies (Dai *et al.*, 2016). The genotyping of cattle by alleles A¹ and A² at the beta-casein locus is conducted in different regions of the world which demonstrates the increasing interest in this problem (Zepeda-Batista *et al.*, 2015; Vougiouklaki *et al.*, 2020; Ivanković *et al.*, 2021). In recent years, the interest in genotyping cattle from different breeds has been noted in Ukraine, which is confirmed by a number of publications (Ladyka *et al.*, 2020; Ladyka *et al.*, 2021).

The availability of rather a large spectrum of different typing methods for beta-casein alleles leads to the need for a comparative analysis of their efficiency, which is especially relevant if similar methodological approaches are applied. Compared to other methods of differentiating beta-casein alleles, allele-specific PCR is one of the most promising technologies due to its low cost, speed, and accuracy (Pabitra *et al.*, 2022). However, this matter has a number of additional complications, related to the specificities of conducting the amplification and analysis of genotyping results, which, considering the promising nature of using AS-PCR as the main instrument in large-scale studies, substantiates the need for the analysis of different critical constituents of the method.

The analysis of a number of publications demonstrated that in many instances of using the AS-PCR method for typing of *CSN2* alleles, the issues of PCR protocol optimizations are ignored during routine analyses (Ramkaran *et al.*, 2017; Raja *et al.*, 2021; Adoligbe *et al.*, 2022). However, the analysis shows that there is rather a high risk of inaccurate genotyping which, first of all, is caused

Table 1. The methods, primer nucleotide structure, and melting temperature.

Method	Primer	Sequence	Tm, °C	Source
ACRS-PCR	DdeI F	ccttctttccaggatgaactccagg	64.6	McLachlan (2006)
	DdeI R	gagtaagaggagggatgttttgtgggaggctct	69.6	
AS-PCR 854 bp	854 F	gcccagatgagagaagtgagg	62.2	Keating *et al.* (2008)
	854 R (A^1)	gatgttttgtgggaggctgttat	59.8	
	854 R (A^2)	gatgttttgtgggaggctgttag	60.8	
AS-PCR 244 bp	IGBR	agactggagcagaggcagag	60.1	Ganguly *et al.* (2013)
	IGBhF (A^1)	cttccctgggcccatcca	63.1	
	IGBpF (A^2)	cttccctgggcccatccc	64.6	

by the simple replication of the methods, described in the scientific literature. First and foremost, it is related to the specificities of amplification programs for different AS-PCR methods (Gaudet *et al.*, 2009). Inaccurate genotyping may lead to a false interpretation of the obtained genetic structure parameters of the experimental groups of animals which distorts the results of the population studies completely. In addition, the genotyping of the cattle individuals is one of the demanded commercial services for the tasks of further selection and for the purpose of obtaining A2 milk, which is an additional indication of the need for maximization of the accuracy and reproducibility of the typing procedure (Park *et al.*, 2021).

Therefore, the aim of our studies is to conduct a comparative analysis of the efficiency of cattle genotyping by beta-casein gene using the methods of allele-specific PCR.

MATERIAL AND METHODS

The studies were conducted in the Laboratory of Molecular and Genetic Studies at the Department of Animal Biology, the National University of Life and Environmental Sciences of Ukraine. To study the efficiency of genotyping by *CSN2* locus, the cows of the Ukrainian Black-and-White dairy breed were used as a model object.

DNA was extracted using the commercial set of reagents "DNA-sorb-B" (Amplisense, RF) according to the manufacturer's recommendations. Hair follicles were used as a source of biological material.

The genotyping of cattle was conducted using the methods of AS-PCR (as the methods under investigation) and ACRS-PCR (as a control method of genotyping).

The primers, presented in Table 1, were used for the amplification of exon 7 of the *CSN2* gene.

The following amplification program was used for the ACRS-PCR method: 1 cycle – denaturation at 94°C; 35 cycles – denaturation at 94°C, 30 s, annealing at 60°C, 30 s, elongation – 72°C, 30 s. The size of restriction fragments was 121 bp for allele A^1; 86 and 35 bp for allele A^2.

Tm Calculator (Thermo Fisher Scientific) based on Allawi & SantaLucia's thermodynamics method (Allawi & SantaLucia, 1997) was used to calculate the temperatures of annealing primers.

In both cases of applying the AS-PCR method, the amplification protocol was optimized according to the following schemes:

The variations of the annealing temperature of primers (from 55°C to 68°C with 1°C interval).

The number of intermediate steps of the second stage of PCR (from two to ten with one step interval).

The duration of the intermediate steps of the second stage of PCR (from 15 to 120 s with 15 s. interval).

Touchdown PCR protocol (Korbie & Mattick, 2008).

PCR was conducted using MiniAmp™ Thermal Cycler (Thermo Fisher Scientific) and the commercial reagent kit "DreamTaq PCR Master Mix" (Thermo Scientific). The volume of the final reaction mixture was 10 μl. The final concentration of primers in the reaction mixture was 0.2 μM.

The electrophoretic separation of the amplification/restriction products involved the use of 3% agarose gel for the ACRS-PCR method and 2% – while using the allele-specific PCR.

The visualization of DNA fragments in the gel was conducted using ethidium bromide in the ultraviolet spectrum (312 nm). The molecular mass marker GeneRuler 50 bp (Thermo Scientific) was used to determine the size of the amplified/restriction fragments.

The object of the study was the populations of cows of different breeds, reared in Ukraine: Ukrainian Red-and-White dairy breed (n=38), Ukrainian Black-and-White dairy breed (two populations from different farms and regions of Ukraine – n=31 and n=100, respectively), Charolais (n=29).

The results of the individual genotyping of the animals were used to estimate the main genetic structure parameters: the frequencies of genotypes and alleles, observed (H_o) and expected (H_e) heterozygosity, the Wright's fixation index (F_{is}), the correspondence to the Hardy-Weinberg genetic equilibrium by the method of χ^2. The estimation of genetic structure parameters was conducted using Popgen32 Version 1.32 (Yeh *et al.*, 2000).

RESULTS AND DISCUSSIONS

The use of standard amplification programs for AS-PCR, described in the scientific literature, allowed for differentiating between alleles A^1 and A^2 of the beta-casein locus of cattle. To conduct the comparative analysis of the efficiency of genotyping the cattle by the beta-casein locus for the purpose of the amplification of the experimental fragment of *CSN2,* only the animals, homozygous by allele A^2 (A^2A^2) were used, whose genotype had previously been determined using the ACRS-PCR (DdeI) method (McLachlan, 2006). The variation in the temperature regimes (PCR parameters) led to a considerable change in the genotyping results. Figure 1 presents the electrophoregram for the amplification products of

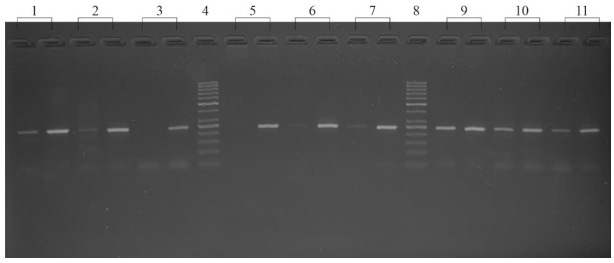

Figure 1. Different PCR protocols for the amplification of *CSN2* fragment using the primers of AS-PCR 244 bp.
1–11, numbers of the samples; 4, 8, molecular mass marker GeneRuler 50 bp; 1–3, annealing temperature for oligonucleotides, 58°C; 5–7, annealing temperature for oligonucleotides, 62°C; 9–10, Touchdown PCR.

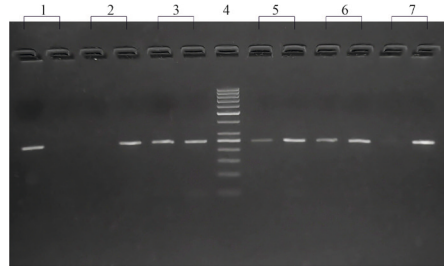

Figure 2. The results of typing the cattle individuals by AS-PCR 244 bp method using the optimized amplification protocol.
1, A^1A^1; 2, 7, A^2A^2; 3, 5, 6, A^1A^2, 4, molecular mass marker GeneRuler 50 bp.

the fragment of the *CSN2* gene using the AS-PCR 244 bp.

In the case of genotype A^1A^1, the electrophoregram shows the DNA fragment of 244 bp only in the well, corresponding to allele A^1, for genotype A^2A^2 – only in the well, corresponding to allele A^2; as for the heterozygous genotype, the amplified fragments are found in both wells. The use of different amplification programs led to significant variations in PCR efficiency. In this case, a critical moment is the selection of optimal parameters for amplification protocols of the experimental fragments. This variation can be implemented into the general program both *via* temperature and the introduction of the intermediate cycles. In our studies, we have used a wide spectrum of annealing temperatures – from 58 to 69°C (with one-degree interval), the Touchdown PCR method, and two additional cycles for the second stage of PCR, one of which was optimized for the annealing temperature for the first (forward) primer, and the other – for the second (reverse) one. The theoretical analysis of the annealing temperatures demonstrated the differences of several degrees between the used primers (under 4°C). The study results were used to set optimal temperatures for both inner cycles.

Figure 1 shows the results of applying three different PCR protocols for the amplification of *CSN2* fragment using primers for AS-PCR 244 bp. All the samples in the presented electrophoregram are related to genotype A^2A^2, but the results of typing differ considerably.

The decrease in the annealing temperature below the minimal theoretically estimated temperature for primers (Table 1) leads to the reduction in the specificity of the method – allele A^1 is amplified in the samples (samples 1, 2, 6, 7). The maximal decrease in the annealing temperature values practically leads to the coincidence in the staining intensity for the amplified fragments of alleles A^1 and A^2. In its turn, the use of the Touchdown PCR method results in the amplification of all the possible variants of alleles in each sample (samples 9–11), which is a reason for their false interpretation as heterozygous individuals A^1A^2.

The use of the optimal values of the annealing temperature and the number of cycles allowed for a maximal decrease in the concentration of "unused" primers (to increase the amplification efficiency to the maximum), in case of preserving the high specificity of the reaction and in case of complete absence of non-specific PCR products. In this case, two cycles with different annealing temperatures are used: 67°C – 10 cycles; 65°C – 25 cycles. The final optimal protocol, which ensured the successful genotyping of the cattle, is as follows: 1 cycle – denaturation at 94°C for 5 min; 10 cycles – denaturation at 94°C for 30 s, annealing at 67°C for 30 s, elongation at 72°C for 30 s; 25 cycles – denaturation at 94°C for 30 s, annealing at 65°C for 30 s, elongation at 72°C for 30 s.

However, even in the case of optimal parameters, some samples may still have the fragment, remarkable for the second allele, which may be explained by a close value of the annealing temperatures for allele-specific primers (IGBhF A^1 and IGBpF A^2), as well as a number of additional potential factors – the differences in the values of the initial concentration of the DNA under analysis, the type of the used enzyme (different modified forms of Taq-polymerase), the concentrations of primers, etc. In this case, we used the primers in the concentration of 0.2 μM, which allowed obtaining the maximally vivid and unambiguous picture in each case of genotyping the animals of *Bos taurus* by *CSN2* locus. In general, when the method of allele-specific PCR is used, there is still a possibility for the amplification of the non-specific allele in a sufficiently wide range. If the luminosity intensity is insufficient regarding the second fragment, one can make a conclusion about the amplification of the non-specific allele (Fig. 1, samples 2 and 7). In the ideal system (under maximally possible amplification efficiency), the luminosity intensity of different fragments (A^1 and A^2) in the case of heterozygous samples will be the same, as both variants are present in the initial DNA in the equivalent number. In case of the insufficient luminosity of one of the specific fragments, two assumptions are possible: either the phenomenon of non-specific amplification or the presence of inhibitors in a specific sample. Then the algorithm of targeted testing of "problematic" samples should be applied based on the alternative method (ACRS-PCR), which is actually ignored by different researchers. In addition to the above, it is reasonable to have regular "blind testing" of the samples using the alternative method (ACRS-PCR).

Regardless of the present difficulties, the application of the optimized (described above) PCR protocol ensured the successful genotyping for the animals of *Bos taurus* by alleles A^1 and A^2 (Fig. 2).

The results of the typing were entirely confirmed by the ACRS-PCR method (DdeI), the coincidence of geno-types, found by both methods, was 100%.

The application of the described methodological approaches allowed for obtaining similar results while using the AS-PCR 854 bp method as well. In this case, the optimal results, which ensure unambiguous identification of *CSN2* alleles, were also obtained using the two-stage PCR algorithm. The values of the annealing temperature for primers were considerably different from the ones, presented in other scientific works (Keating *et al.*, 2008; Rahimi *et al.*, 2015).

The best result was obtained using the following amplification program – the use of two cycles on the second stage

Table 2. The genetic structure parameters of different cattle populations.

Breed	Genotype frequency			Allele frequency		H_e	F_{is}	χ^2 test
	A^1A^1	A^1A^2	A^2A^2	A^1	A^2			
RW	0.13	0.61	0.26	0.43	0.57	0.49	–0.24	2.06
BW (pop 1)	0.42	0.48	0.1	0.66	0.34	0.45	–0.07	0.201
BW (pop 2)	0.12	0.49	0.39	0.37	0.63	0.47	–0.04	0.34
Charolais	0	0.17	0.83	0.09	0.91	0.16	–0.06	0.24

RW, Ukrainian Red-and-White dairy breed; BW (pop 1), Ukrainian Black-and-White dairy breed (Population 1); BW (pop 2), Ukrainian Black-and-White dairy breed (Population 2); He, expected heterozygosity; Fis, Wright's fixation index

of PCR with different annealing temperatures: 66°C – 10 cycles; 64°C – 25 cycles (Fig. 3). The final optimal protocol, which ensured the successful genotyping of the cattle, is as follows: 1 cycle – denaturation at 94°C for 5 min; 10 cycles – denaturation at 94°C for 30 s, annealing at 66°C for 30 s, elongation at 72°C for 30 s; 25 cycles – denaturation at 94°C for 30 s, annealing at 64°C for 30 s, elongation at 72°C for 30 s. The amplification was conducted without the final elongation stage (stage 3). The obtained results of typing are completely confirmed by the ACRS-PCR (DdeI) method and are in complete correlation with the results of using AS-PCR 244 bp. The coincidence of genotypes between all the used methods of typing was 100 %.

Therefore, the study results directly demonstrate the need for a thorough selection of amplification parameters while using the allele-specific PCR for the genotyping of cattle by beta-casein locus. The identity of the typing results for two different primer systems for AS-PCR demonstrates the commonality of the methodological approaches in solving the task of enhancing the specificity, reproducibility, and efficiency of amplifying alleles A^1 and A^2. The obtained results can be used for large-scale routine genotyping of individuals of different cattle breeds by allelic variants of the beta-casein gene.

The above-mentioned methodological approaches were used to determine the genetic structure parameters of the cattle population of different breeds, reared in Ukraine (Table 2).

The study results demonstrated that the beta-casein locus is related to polymorphous ones in all the experimental populations. The animals with all the possible genotypes were found in each experimental population except for Charolais.

The maximal value for allele A^2 frequency was found in the Charolais population, which may be determined by the fact of its origin. Charolais are meat cattle, so, taking into consideration the descent of allele A^1 from the European dairy cattle breeds, the minimal number of heterozygotes A^1A^2 in the population (5 out of 29) is absolutely reasonable. Homozygotes A^1A^1 are entirely absent in this population.

The analysis of the genetic structure of the populations of dairy cows shows the following picture: two populations of Ukrainian Black-and-White breed from different farms demonstrate significant differences regarding the values of allele frequencies. For instance, in population 2 there is a prevalence of allele A^2 frequency, while in population 1, the situation is diametrically opposite – there is a significant prevalence of allele A^1 frequency (practically two-fold). At the same time, the values of the observed (H_o) and expected (H_e) heterozygosity in both populations are very similar. The obtained study results allow for the assumption that the observed picture is related to the specificities of the selection work with this breed at each farm and to the degree of "Holsteinization", used in breeding to enhance the parameters of dairy performance of cattle. The study results confirm the data for the distribution of allelic frequencies obtained in the analysis of the Holsteinized Black-and-White cattle, reared in Latvia (Smiltina *et al.*, 2018). This assumption is also indirectly confirmed by the actual presence of allele A^1 (with different frequency) in the populations of Holstein cattle in different regions of the world (Yamada *et al.,* 2021; Ivankovi´c *et al.*, 2021; Bisutti *et al.*, 2022).

In its turn, the insignificant domination of allele A^2 frequency was noted for the population of the Ukrainian Red-and-White breed, with the two-fold prevalence of the number of homozygotes A^2A^2 over A^1A^1. The excess of heterozygous animals was also noted in this population of cows (24%). The data about the distribution of allelic frequencies in the populations of Black-and-White and Red-and-White dairy breeds are confirmed by the results of other authors obtained for these breeds in other countries (Ehrmann *et al.*, 1997; Kamiński *et al.*, 2006).

It should be noted that no deviation from the Hardy-Weinberg genetic equilibrium was found in any cattle population under study.

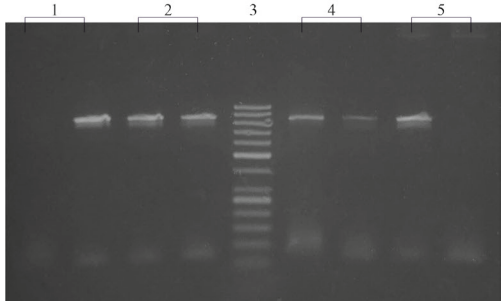

Figure 3. The results of typing the cattle individuals by AS-PCR 854 bp method using the optimized amplification protocol.
1, A^2A^2, 2, 4, A^1A^2, 5, A^1A^1, 3, molecular mass marker GeneRuler 50 bp.

CONCLUSIONS

The application of the above-described methodological approaches to the optimization of PCR methods allows for efficient genotyping of the animals (A^1 and A^2 allele detection) on the background of current advantages of AS-PCR before the alternative variants – low material-technical and time expenditures under completely comparable efficiency.

To achieve the maximal efficiency of the amplification, it is recommended to use the modified PCR protocol, including the two-step second stage with the following values of the annealing temperatures for primers: for AS-PCR 244 bp – 67°C (10 cycles) and 65°C (25 cycles); for AS-PCR 854 bp – 66°C (10 cycles) and 64°C (25 cycles).

The use of the touchdown PCR method as the instrument for optimization of AS-PCR protocols is not efficient for the primer systems under investigation and leads to genotyping mistakes.

To control the specificity of the amplification of the experimental *CSN2* gene fragment, it is recommended to use the alternative method (ACRS-PCR) via both so-called analyses of "problematic samples" and blind typing from the sampling frame.

Declarations

Acknowledgments. None.

Conflicts of Interest. The authors declare no conflict of interest.

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