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Occurrence of the beta-casein allele A1 in *Bovidae* species

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SUMMARY

In the last two decades, the bovine A1 variant of beta-casein has attracted attention in the dairy industry as a source of beta-casomorphin-7, an opioid-like peptide with potentially harmful effects on human health. Because the origin of this variant is unknown, the aim of the study was to investigate its occurrence in different species of the *Bovidae* family. Blood samples were taken from 137 animals belonging to 17 *Bovidae* species. All animals were genotyped for the beta-casein locus using the Illumina Bovine MDv2 Chip. The results showed that the A1 allele was present in *Bos taurus* cattle, including primitive cattle (Watussi and Ukrainian Grey) and a modern dairy breed (Holstein). In Dahomey cattle, two heterozygous A1A2 individuals were found. In all remaining species, no animal carrying the A1 allele of beta-casein was identified. It can be concluded that the A2 allele is ancestral to the A1 allele and was spread especially among modern dairy cattle.

KEY WORDS: **Bovidae, beta-casein, A2 milk**

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INTRODUCTION

Cattle are the most important livestock species owing to their products and role in human culture (Felius et al., 2014). Cow milk, together with other dairy products, has been recognized as nature's perfect food, providing all essential nutrients for both infants and adults since ancient times (Mukesh et al., 2022). Caseins, as milk-specific proteins, are not only a source of protein but also contain active peptides which have a beneficial influence on human metabolism and health (Gobbetti et al., 2007). The second most abundant casein – beta-casein – is a source of various bioactive peptides (expressing opioid-like activity), depending on the genetic variant of the gene (Cieślińska et al., 2022). A total of 15 genetic variants have been discovered in the beta-casein gene (Caroli et al., 2009). Beta-casein polymorphism did not attract attention until its benefits in comparison to milk containing the A1 variant were discovered in the late 1990s, as described by Truswell (2005) in 'The A2 milk case'. Briefly, at amino acid position 67, proline in variant A2 is substituted with histidine in variant A1. This change allows for cleavage of variant A1 by digestive enzymes, releasing the opioid peptide known as β-casomorphin-7 (BCM7), corresponding to the 60–66 sequence of bovine beta-casein (Tyr-Pro-Phe-Pro-Gly-Pro-Ile). The potential presence of BCM7 in dairy products was allegedly associated with a higher risk of an array of human diseases (Thiruvengadam et al., 2021; Cieślińska et al., 2022). A2 milk is produced by Asian and African native cattle, buffalo, goats, sheep, yaks and camels (Mukesh et al., 2022). Therefore, it is hypothesized that all species belonging to the genus *Bos* were initially type A2A2, and due to a single nucleotide mutation some animals became A1. It was further postulated that selection for high milk production unintentionally increased the prevalence of the A1 allele in European dairy cattle breeds derived from *Bos taurus* several thousands of years ago. In contrast, cattle derived from *Bos indicus* evolved naturally, and therefore have a much higher frequency of the A2 allele (Mishra et al., 2009; Mukesh et al., 2022; Khan et al., 2023).

The unknown origin of the beta-casein A1 allele prompted the authors to investigate various species of the *Bovidae* family to determine which species have acquired the A1 allele.

MATERIALS AND METHODS

Blood samples were collected from 137 animals belonging to 17 *Bovidae* species: *Bubalus depressicornis* (4), *Hippotragus equinus* (5), *Hippotragus niger* (5), *Syncerus caffer caffer* (5), *Syncerus caffer nanus* (5), *Bos taurus* (Holstein breed, 12), *Bos taurus* (Ukrainian Grey breed, 3), *Bos taurus* Watussi (5), *Bos indicus* Dahomey (5), *Oryx gazella* (3), *Connochaetes taurinus* (5), *Aepyceros melampus* (5), *Kobus leche* (5), *Kobus ellipsiprymnus* (5), *Tragelaphus strepsiceros* (5), *Tragelaphus imberbis* (3), *Tragelaphus angasii* (3), *Tragelaphus spekei gratus* (4), and *Bison bonasus* (48). The animals were kept at the Dvur Kralove Zoo (Czechia), except for the European bison (wisent). In the case of wisents, genomic DNA was provided by the Mammal Research Institute in Białowieża, Polish Academy of Sciences.

Blood samples were collected during routine veterinary inspections, in accordance with legal and ethical regulations provided in Directive 2010/63/EU, and in the case of the wisent, during annual culling approved by the General Directorate for Environmental Protection (Warsaw, Poland) and the Regional Directorate for Environmental Protection (Białystok, Poland). Samples were collected between 2016 and 2020 and frozen at −20°C until DNA preparation. Genomic DNA was isolated using a NucleoSpin Tissue Mini kit (Macherey-Nagel). All animals were genotyped for the

beta-casein locus using the Illumina Bovine MDv2 Chip (Illumina, San Diego, USA), according to the manufacturer's protocol. The quality of the SNP cluster was evaluated using Illumina GenomeStudio software (v. 2011.1, Illumina, San Diego, USA). To ensure that the genotyping by microarray was correct, a subset of samples was genotyped by the Polymerase Chain Reaction–Allele Created Restriction Site (PCR-ACRS) method as previously described (Cieślińska et al., 2012). Briefly, a pair of primers (forward: 5' GCAGAATTCTAGTCTATCCCTTCCCTGGACCCATGC 3' and reverse: 5' ACGGACTGAGGAGGAAACATGACAGTTGGAGGAAG 3') was used to amplify a 320 bp fragment of the beta-casein exon 7 sequence. The PCR thermal profile consisted of 35 cycles of 94°C for 30 s, 62.5°C for 60 s, 72°C for 30 s, and a final extension at 72°C for 5 min. PCR was performed in a reaction mixture containing 20–40 ng of genomic DNA, 0.3 μL (50 μM) of each primer, 2.5 μL KAPA Taq PCR Buffer A (10x) (KAPA Biosystems), 2 μL dNTPs mix (2.5mM each, KAPA Biosystem), 2.5 units of KAPA Taq DNA polymerase, and deionized water to reach a volume of 25 μl. The PCR products were digested with FastDigest Mph1103I (Thermo Scientific). Restriction fragments were separated by electrophoresis in a standard 2.5% agarose gel stained with ethidium bromide and analysed with Fluor-S^{TM} MultiImager (Bio-Rad). To confirm the specificity of PCR products and the correctness of beta-casein genotypes, PCR products from four animals were sequenced. In addition, ancient DNA (aDNA) from aurochs *Bos primigenius* was analysed to find sequences homologous to beta-casein polymorphic sites determining A1/A2 alleles. DNA has been sequenced from eight *B. primigenius* specimens: two from Belgium (SAMEA112360719; SRA: ERS14471074; SAMEA112360720; SRA: ERS14471075), two from Israel (SAMEA5577344; SRA: ERS3381579; SAMEA5577345; SRA: ERS3381580), and single specimens from Armenia (SAMEA5577362; SRA: ERS3381597), Turkey (SAMEA5577358; SRA: ERS3381593), Morocco (SAMEA5577395; SRA: ERS3381630) and Greece (SAMEA5577391; ERS338162). The *B. taurus* casein beta cDNA (XM_010806178.3) sequence was aligned against those present in the Sequence Read Archive (SRA) database for *B. primigenius* using BLASTn software. A consensus sequence was created by BioEdit 7.7.1 (Hall, 1999).

RESULTS

The genotyping of beta-casein polymorphisms using the Illumina Bovine MDv2 Chip for 137 animals was fully successful. In the Illumina Bovine MDv2 Chip, the beta-casein locus is represented by marker CSN2_X14711_8101 and is capable of differentiating between the A1 and A2 alleles. As shown in Figure 1, the quality of the genotype cluster is very good: dots representing animals are located in clearly separated areas.

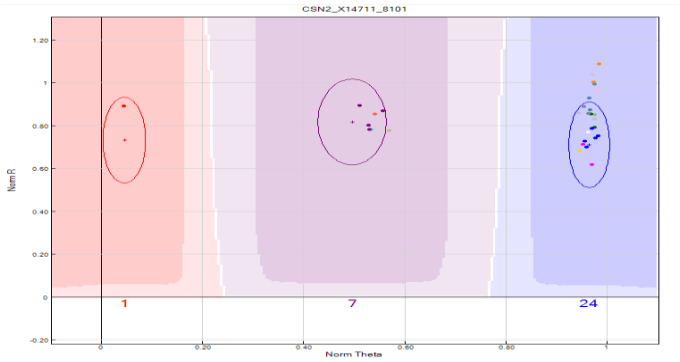

Figure 1. Example of cluster quality of beta-casein genotyping using the Illumina Bovine EuroG_MDv2 Chip. In a group of 32 individuals, one, seven and 24 were genotyped as A1A1, A1A2 and A2A2, respectively. Each dot represents a single individual. Among seven A1A2 heterozygotes, there were two Watussi and two Dahomey cattle.

This is also confirmed by results shown in Figure 2, where the beta-casein A2A2 genotype was identified for all European bison (*Bison bonasus*) bulls. The results of the study (Table 1) show that the A1 allele is present only in *Bos taurus* cattle, including primitive cattle (Watussi and Ukrainian Grey) and a modern dairy breed (Holstein).

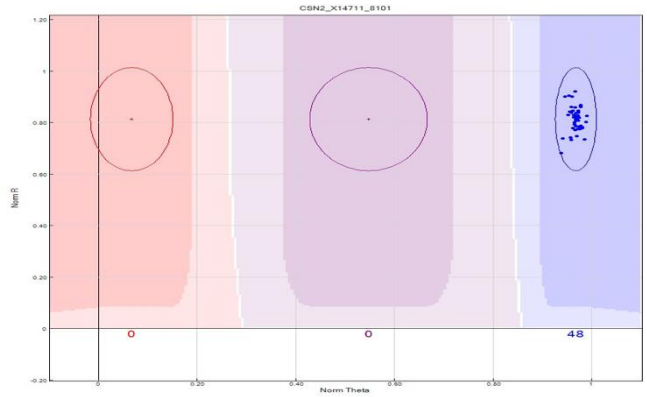

Figure 2. Example of cluster quality of beta-casein genotyping using the Illumina Bovine EuroG_MDv2 Chip. In a group of 48 *Bison bonasus* bulls, all were genotyped as A2A2. Each dot represents a single individual.

Table 1.
Genotype of the beta-casein locus (A1 and A1 allele) in *Bovidae* species

Species name	Number of animals	Genotype		
		A1A1	A1A2	A2A2
Bubalus depressicornis	4	0	0	4
Hippotragus equinus	5	0	0	5
Hippotragus niger	5	0	0	5
Syncerus caffer caffer	5	0	0	5
Syncerus caffer nanus	5	0	0	5
Bos taurus (Holstein)	12	1	5	6
Bos taurus (Ukrainian Grey)	3	2	1	0
Bos taurus Watussi	5	1	2	2
Bos indicus Dahomey	5	0	2	3
Oryx gazella	3	0	0	3
Connochaetes taurinus	5	0	0	5
Aepyceros melampus	5	0	0	5
Kobus leche	5	0	0	5
Kobus ellipsiprymnus	5	0	0	5
Tragelaphus strepsiceros	5	0	0	5
Tragelaphus imberbis	3	0	0	3
Tragelaphus angasii	5	0	0	5
Tragelaphus spekii gratus	4	0	0	4
Bison bonasus	48	0	0	48

In Dahomey cattle, two heterozygous A1A2 individuals were found. No animals carrying the A1 allele of beta-casein were found in any of the remaining species. The genotypes of Watussi and Dahomey cattle were confirmed by the PCR-ACRS method (Figure 3). Complete agreement between genotypes was obtained by the microarray and PCR-ACRS methods.

In addition, the alignment of aDNA reads to the *B. taurus* sequence enabled the reconstruction of CSN2 cDNA in *B. primigenius.* A 957 bp sequence overlapped with 87.5% of the *B. taurus* sequence. The sequence obtained differs by 47 SNPs (4.91%) from *B. taurus*. These SNPs are mainly located in two regions: 530–550 (14 SNPs) and 1009–1059 (14 SNPs). An SNP determining the A1 and A2 alleles was clearly identified as genotype A2A2 (Figure 3).

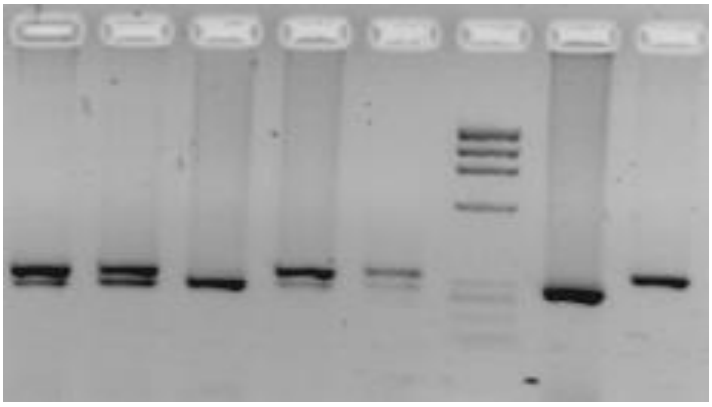

Figure 3. Electropherogram showing genotyping of A1 and A2 alleles in beta-casein exon 7 using the PCR-ACRS method. An A1 allele is cut by the Mph1103I enzyme into 283 bp and 37 bp fragments; the A2 allele is uncut and retains its size of 320 bp. A1A2 heterozygotes have three bands: 320, 283 and 37 bp. From left: lanes 1–3 – Watussi cattle, A1A2, A1A2, A1A1; lanes 4–5 – Dahomey cattle, A1A2, A1A2; lane 6 – DNA size marker ΦX174/HaeIII; lanes 7–8 – Holstein cattle (*Bos taurus*), A1A1, A2A2. Due to its small size of 37 bp, a restriction fragment migrates rapidly and may not be visible on the gel.

DISCUSSION

For over 20 years there has been discussion regarding the potential negative influence of the beta-casein A1 variant on human health. According to this hypothesis, the digestion of cows' milk with A1 beta-casein releases the opioid peptide β-casomorphin-7 (BCM-7), which impairs gastrointestinal function and is a risk factor for various diseases (McLachlan, 2001; Cieślińska et al., 2022). The frequency of the A1 allele in the most popular dairy breed – Holstein cattle – is still relatively high, at about 30% (Sanchez et al., 2020; Sebastiani et al., 2020; Kamiński et al., 2023), due to the high proportion of A1A2 heterozygotes, amounting to about 50% of the Holstein cow population. Attempts to eliminate the A1 beta-casein allele were initiated in New Zealand, which is a leader in promoting milk with the A2 variant in the dairy market.

In the present study, several species of the *Bovidae* family were genotyped in an attempt to obtain more information on the origin of the A1 variant.

The A1 allele was found only in the species *Bos taurus*, which confirms the hypothesis presented by Formaggioni et al. (1999) that the A2 allele is ancestral to the A1 allele. Indeed, the results of the present study clearly demonstrated the absence of the A1 allele in all investigated species which are evolutionarily older than *Bos taurus*. The occurrence of the A1 allele in Watussi and Dahomey cattle indicated that it may have appeared during the evolution of *Bos taurus* itself.

A.

-6121 CTGTGAAGAA AGTGGGTTAA TGAGAAATCC TTCAGTGAGC ATTTTACTCA TTAGTCTTCA
-6181 TATGACCCCA ATTTCTTAAC CAAACCAAAT GGAAGATTTT CTTTCTCTCT CTTCACTGAA
-6241 TTATGTTTTA AAAAGAGGAG GATAATTCAT CATGAATAAC AATTATAACT GGATTATGGA
-6301 CTCAAAGATT TGTTTTCCTT CTTTCCAGGA TGAACTCCAG GATAAAATCC ACCCCTTTGC
-6361 CCAGACACAG TCTCTAGTCT ATCCCTTCCC TGGGCCCATC C**A**TAACAGCC TCCCACAAAA
-6421 CATCCCTCCT CTTACTCAAA CCCCTGTGGT GGTGCCGCCT TTCCTTCAGC CTGAAGTAAT
-6481 GGGAGTCTCC AAAGTGAAGG AGGCTATGGC TCCTAAGCAC AAAGAAATGC CCTTCCCTAA
-6541 ATATCCAGTT GAGCCCTTTA CTGAAAGGCA GAGCCTGACT CTCACTGATG TTGAAAATCT
-6601 GCACCTTCCT CTGCCTCTGC TCCAGTCTTG GATGCACCAG CCTCACCAGC CTCTTCCTCC
-6661 AACTGTCATG TTTCCTCCTC AGTCCGTGCT GTCCCTTTCT CAGTCCAAAG TCCTGCCTGT
-6721 TCCCCAGAAA GCAGTGCCCT ATCCCCAGAG AGATATGCCC ATTCAGGCCT TTCTGCTGTA
-6781 CCAGGAGCCT GTACTCGGTC CTGTCCGGGG ACCCTTCCCT ATTATTGTAA GTCTAAATTT
-6841 ACTAACTGTG CTGTTTAACT TCTGATGTTT GTATGATATT CGAGTAATTA AGAGTCCTAT

B.

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   1.   ERX3342820:  SRA:ERR3317392.399      ----*-----  --CTCCCACA
      AAACATCCCT CCTCTTACTC AAACCCCTGT
   2.   ERX3342824:  SRA:ERR3317396.347      ATCCCTAACA  GCCTCCCACA
      AAACATCCC- ---------- ----------
   3.   ERX3342824:  SRA:ERR3317396.266      ATCCCTAACA  GCCTCCCACA
      AAACATCCC- ---------- ----------
   4.   ERX3342825:  SRA:ERR3317397.921      ATCCCTAACA  GCCTCCCACA
      AAACATCCCT CCTCTTACTC AAA-------
   5.   ERX3342826: SRA:ERR3317398.316    ---------- ---------- ---
      ------- ---------- ------CTGT
   6.   ERX3342826:  SRA:ERR3317398.324      ATCCCTAACA  GCCTCCCACA
      AAACATCC-- ---------- ----------
   7.   ERX3342897: SRA:ERR3317469.673    ---------- ---------- ---
      ------T CTTCTTACTC AAACCCCTGT
Consensus:  ATCCCTAACA GCCTCCCACA AAACATCCCT CYTCTTACTC AAACCCCTGT
```

Figure 4. A: Fragment of cDNA encoding beta-casein (XM_010806178.3). Variant A1 at position 6402 is highlighted by the A in bold representing adenine; B: Alignment of seven aDNA sequences from *Bos primigenius* available in the Sequence Read Archive with cDNA of bovine beta-casein revealed a C variant (in red and with an asterisk) determining beta-casein protein variant A2 in four of these sequences.

This assumption is supported by an analysis of a population of 24 yaks (*Bos grunniens*) which all revealed only the A2A2 genotype (Chen et al., 2021). The ancestral nature of the A2 allele is further confirmed by archaeological DNA of *Bos primigenius*, in which only sequences encoding

the A2 beta-casein variant were detected (Figure 4). The data support the hypothesis that the A2 allele occurred primarily in aurochs and mutated into the A1 allele following the division into *Bos taurus* and *Bos indicus*. The results of the present study do not reveal exactly when and in which species the A1 allele first appeared, but they indicate the presence of the A1 allele in Dahomey cattle and Watussi cattle. It is anticipated that in the future, genome sequencing of all three aurochs subspecies (*B. p. primigenius*, *B. p. namadicus*, and *B. p. africanus*) will provide data for a more precise estimation of when the A1 mutation occurred. Paleontological remains found in the Western Desert in Egypt suggested that this was an independent African centre of cattle domestication (Felius et al., 2014).

The origin of Watussi cattle was influenced by zebu cattle. According to the World Watussi Association (https://www.thecattlesite.com/breeds/beef/85/watussi), long-horned, 'humpless' domestic cattle were well established in the Nile Valley by 4000 BCE. These cattle, known as the Egyptian or Hamitic Longhorn, appear in pictographs in Egyptian pyramids. Over the next 2000 years, the Egyptian Longhorn migrated with its owners from the Nile to Ethiopia, and then to southern Africa. By 2000 BCE, humped cattle (Longhorn Zebu) from Pakistan and India reached Africa. Therefore, the Watussi is a descendant of two ancient breeds of cattle, the Egyptian Longhorn and the Zebu longhorn, which migrated from Asia.

A study of native zebu-based breeds (Kosali, Tharparkar, Gangatiri, Sahiwal, Gir, Khariar, and Motu) found that the A1A1 genotype was absent, although 19% of cows had the A1A2 genotype (Khan et al., 2023), which means that the A1 allele is segregating within indigenous zebu breeds, but with minor frequency.

The A2 allele is probably linked to loci involved in milk performance traits, as its high frequency in modern dairy cattle breeds is well documented (Oleński et al., 2012; Cieślińska et al., 2022). Its high frequency may also be due to the effect of breeding on isolated islands, as in the case of Jersey and Guernsey cattle (Huson et al., 2020).

Another advantage of the A2 allele over A1 is its association with the growth of calves. Hohmann et al. (2020) observed that the composite BB|A2A2|AA|AB casein genotype (order of genes on bovine chromosome 6: α s1-|β-|α s2-|κ-CN) was associated with greater average daily weight gains and heavier age-adjusted weaning weights of calves.

In conclusion, the A1 beta-casein allele appeared in the evolution of the Bovidae family after *Bos primigenius* had split into *Bos taurus* and *Bos indicus* and survived to the present in the taurine breeds Watussi and Dahomey. This long period of time has enabled the spread of the A1 allele to other dairy cattle breeds. The positive associations of the A2 variant with milk yield and the weaning weight of calves and the growing demand for A2 milk will lead to a steady decrease in the occurrence of the A1 variant among modern dairy breeds.

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