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Caprine and ovine Greek dairy products: The official German method generates false-positive results due to κ-casein gene polymorphism

V. Tsartsianidou,* D. Triantafillidou,† N. Karaiskou,* P. Tarantili,† G. Triantafillidis,‡ E. Georgakis,‡ and A. Triantafyllidis*[1]
*School of Biology, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece
†Thessaloniki Subdivision of General Chemical State Laboratory, Thessaloniki 54625, Greece
‡MEVGAL SA Dairy Company, Thessaloniki 51100, Greece

ABSTRACT

Caseins are widely used for species identification of dairy products. Isoelectric focusing (IEF) of *para*-κ-casein peptide is used as the official German method for the differentiation between caprine (isoform A) and ovine (isoform B) dairy products, based on their different isoelectric points. The discrimination between Greek goat and ewe dairy products using IEF has, however, been shown to be problematic because of the existence of the ewe isoform in milk from Greek indigenous dairy goats. This could be due to nucleotide polymorphisms within the goat κ-casein gene of Greek indigenous breeds, which alter the isoelectric point of the *para*-κ-casein peptide and lead to false positive results. Previous DNA analysis of the goat κ-casein gene has shown high levels of polymorphism; however, no such information is available for Greek indigenous dairy goats. Therefore, 87 indigenous dairy goats were sequenced at exon IV of κ-casein gene. In total, 9 polymorphic sites were detected. Three nonsynonymous point mutations were identified, which change the isoelectric point of the goat *para*-κ-casein peptide so that it appears identical to that of the ewe peptide. Ten composite genotypes were reconstructed and 6 of them included the problematic point mutations. For the verification of genetic results, IEF was carried out. Both goat and ewe patterns appeared in the problematic genotypes. The frequency of these genotypes could be characterized as moderate (0.23) to high (0.60) within Greek indigenous breeds. However, this is not an issue restricted to Greece, as such genotypes have been detected in various non-Greek goat breeds. In conclusion, IEF based on the official German method is certainly inappropriate for ovine and caprine discrimination concerning Greek dairy goat products, and consequently a new method should be established.

Key words: goat, κ-casein polymorphism, isoelectric focusing, species identification

INTRODUCTION

The importance of milk and dairy products for the human diet has led to the development of methods to detect possible adulterations in the dairy industry. In particular, the production of sheep and goat milk is of major economic importance, resulting from the widespread acceptance of traditional cheeses, especially those made from one pure species with Protected Designation of Origin (PDO) such as pure sheep or pure goat cheeses (Borkova and Snaselova, 2005; Zachar et al., 2011). The most common fraudulent practice is that of substituting high-value (ewe and goat) milk during technological processing with cow milk, which is lower in price and available in larger and constant quantities. The presence of goat milk in ewe milk is less common. However, substitution of ewe milk with goat milk often occurs in Mediterranean cheese manufacturing (Haza et al., 1999). Lately, an increase in pure goat's milk products has occurred, and this has prompted an interest in authentication methods for such products. The replacement of one type of milk with another could also create problems related to frequent adverse reactions in humans toward some milk proteins (allergies or intolerance) or religious, ethical, and legal issues (Zachar et al., 2011).

The identification of dairy products' authenticity is often carried out by analysis of major milk proteins (Strange et al., 1992; Mayer, 2005). The principal methods are immunological (ELISA; Haza et al., 1999), chromatographic [hydrophobic interaction column (HIC), HPLC; Romero et al., 1996; Bramanti et al., 2003], and electrophoretic techniques [isoelectric focusing (**IEF**), capillary electrophoresis; Mayer et al., 1997). Caseins (α_{S1}, α_{S2}, β, κ), which constitute 80% of

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[1]Corresponding author: atriant@bio.auth.gr

milk protein content, are used widely to evaluate and differentiate dairy products (Kaiser and Krause, 1985). Many studies based on electromigration techniques have been carried out, such as IEF of γ- and *para*-κ-CN (Kaiser and Krause, 1985; Mayer et al., 1997), because protein separation according to isoelectric point (**pI**) is suitable for the analysis of caseins that form many genetic variants among and within species (Strange et al., 1992). Isoelectric focusing is the official German method [L01.00–39 (§64 LFGB); German Central Food and Feed Law Book, 2005] for the differentiation of ewe and goat milk based on charge difference of *para*-κ-CN (Kaiser and Krause, 1985). The chymosin proteolysis of κ-CN results into a caseinomacropeptide and a *para*-κ-CN peptide (Mercier et al., 1976), where the latter is the analyte used (Mayer et al., 1997). The 2 goat κ-CN protein variants, named A and B isoforms, were described by Di Luccia et al. (1990); Caroli et al. (2001) confirmed them via IEF. However, when 100% goat cheese samples from Greece were analyzed across Europe using the official German method, they were misidentified as containing ewe milk. This substitution is, of course, unlikely, because ewe milk is much more expensive than goat milk. Research has shown that the discrimination, based on IEF, between Greek caprine and ovine dairy products is problematic, possibly because the goat κ-CN gene polymorphism leads to *para*-κ-CN peptides identical to the ewe *para*-κ-CN peptide (isoform B, pI = 5.66). As a result, the abundance of isoform B instead of A (pI = 5.29) in Greek goat cheese samples leads to false positive results, falsely identifying ewe milk (Triantafillidou et al., 2011, Supplemental Figure S1; https://doi.org/10.3168/jds.2016-11677).

The goat κ-CN gene has been studied and analyzed widely due to the important role of κ-CN in micelle formation, stabilization, and aggregation, which determine the technological and nutritional properties of milk (Moioli et al., 1998; Wedholm et al., 2006). Previous research has shown that the goat κ-CN gene, and specifically exon IV, is highly polymorphic. The segment which corresponds to the *para*-κ-CN fraction revealed the majority of polymorphic sites (e.g., Coll et al., 1993; Caroli et al., 2001; Yahyaoui et al., 2001; Angiolillo et al., 2002; Prinzenberg et al., 2005; Kiplagat et al., 2010; Di Gerlando et al., 2015). Genetic analysis of this gene has not been carried out, until now, within Greek indigenous goats to identify possible point mutations. On the other hand, ewe κ-CN gene seems to be monomorphic (Moioli et al., 1998; Othman et al., 2013).

The aim of our study concerns the sequence determination and analysis of exon IV of κ-CN gene from Greek indigenous goats, including the detection of (1) the variability found within the gene, (2) the nonsynonymous mutations that alter the isoelectric point of the *para*-κ-CN peptide and complicate the differentiation between caprine and ovine milk by IEF, as well as (3) their allelic frequencies. Discussion of species identification methods also constitutes part of this work.

MATERIALS AND METHODS

Animal Samples

Eighty-seven goats (*Capra hircus*), belonging to the Greek indigenous breed, from 4 herds in northern Greece were analyzed. The selection of these specific herds is related to the high protein and fat content of their milk according to the breeders. Goat DNA was extracted from blood samples using a standard phenol-chloroform procedure (Sambrook et al., 1989). Thirty-two blood samples were collected from 2 herds in the prefecture of Thessaloniki, 29 blood samples from the area of Kilkis, and 26 blood samples from the area of Serres.

Amplification of the Caprine κ-CN Gene

A 645-bp fragment, including a part of intron 3 and the full sequence of exon IV (523 bp), of the goat κ-CN gene was amplified by PCR using a set of primers: I3F (5′-TCCCAATGTTGTACTTTCTTAACATC-3′; Angiolillo et al., 2002) and Kb2 (5′-GCGTTGTCCTCTTTGATGTCTCCTTAG-3′; Yahyaoui et al., 2001). The PCR reaction was performed in a 25-μL final volume containing 1 U of Taq DNA polymerase (Qiagen GmbH, Hilden, Germany), 1× PCR buffer, 0.25 μ*M* each deoxynucleotide triphosphate (dNTP; Life Technologies, Rockville, MD), 50 pmol of each primer, and approximately 100 ng of goat genomic DNA. Eppendorf 5331 (GE Healthcare Life Sciences, Pittsburgh, PA) thermal cycler conditions were adjusted to 95°C for 4 min, followed by 34 cycles of 94°C for 50 s, 56°C for 50 s, and a final extension at 72°C for 1 min.

Sequencing and Sequence Analysis

The PCR products were purified with PureLink PCR purification kit (Life Technologies) and sequenced by Macrogen (Tokyo, Japan) and Beckman Coulter Genomics (Danvers, MA) using the forward primer (I3F). The nucleotide sequences were analyzed by Geneious 8.0 (Kearse et al., 2012; http://www.geneious.com) and BioEdit v7.1.3 (Hall, 1999; http://www.mbio.ncsu.edu/bioedit/bioedit.html) software. In addition, DnaSP software v5 (Librado and Rozas, 2009; http://www.ub.edu/dnasp) was used to reconstruct the nucleotide