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Transcript analysis at *DGAT1* reveals different mRNA profiles in river buffaloes with extreme phenotypes for milk fat

M. Gu,*† G. Cosenza,‡ I. Nicolae,§ A. Bota,# Y. Guo,† L. Di Stasio,* and A. Pauciullo*[1]
*Department of Agricultural, Forest and Food Science, University of Torino, 10095 Grugliasco (TO), Italy
†College of Animal Science and Technology, Beijing University of Agriculture, 102206 Beijing, China
‡Department of Agricultural Sciences, University of Naples "Federico II," 80055 Portici (NA), Italy
§Research and Development Institute for Bovine Breeding, Balotesti, 077015 Bucharest, Romania
#Research and Development Station for Buffalo Breeding, 507195 Şercaia, Romania

ABSTRACT

Buffalo *DGAT1* (diacylglycerol O-acyltransferase 1) was mainly investigated for the characterization of the gene itself and for the identification of the K232A polymorphism, similar to what has been accomplished in cattle, although no information has been reported so far at the mRNA level. The importance of *DGAT1* for lipid metabolism led us to investigate the transcript profiles of lactating buffaloes characterized as high (9.13 ± 0.23) and low (7.94 ± 0.29) for milk fat percentage, and to explore the genetic diversity at the RNA and DNA level. A total of 336 positive clones for the *DGAT1* cDNA were analyzed by PCR and chosen for sequencing according to the differences in length. The clone assembling revealed a very complex mRNA pattern with a total of 21 transcripts differently represented in the 2 groups of animals. Apart from the correct transcript (17 exons long), the skipping of exon 12 is the most significant in terms of distribution of clones with 11.6% difference between the 2 groups, whereas a totally different mRNA profile was found in approximately 12% of clones. The sequencing of genomic DNA allowed the identification of 10 polymorphic sites at the intron level, which clarify, at least partially, the genetic events behind the production of complex mRNA. Genetic diversity was found also at the exon level. The single nucleotide polymorphism c.1053C>T represents the first example of polymorphism in a coding region for the *DGAT1* in the Italian Mediterranean breed. To establish whether this polymorphism is present in other buffalo breeds, a quick method based on PCR-RFLP was set up for allelic discrimination in the Italian Mediterranean and the Romanian Murrah (200 animals in total). The alleles were equally represented in the overall population, whereas the analysis of the 2 breeds showed different frequencies, likely indicating diverse genetic structure of the 2 breeds. The T allele might be considered as the ancestral condition of the *DGAT1* gene, being present in the great part of the sequenced species. These data add knowledge at the transcript and genetic levels for the buffalo *DGAT1* and open the opportunity for further investigation of other genes involved in milk fat metabolism for the river buffalo, including the future possibility of selecting alleles with quantitative or qualitative favorable effects (or both).
Key words: *DGAT1*, transcript analysis, alternative splicing, genetic diversity, river buffalo

INTRODUCTION

The Mediterranean river buffalo represents a fundamental economic resource for Italy, mainly for the milk used for different dairy production. The growing interest at both the national and international level for the most famous buffalo dairy product (Mozzarella Campana PDO, Reg. EC 510/2006; European Council, 2006) led to a great development of the buffalo dairy industry, which, in the last 10 yr, doubled the number of buffalo stock, currently assessed as more than 350,000 (FAO, 2014).

Despite such a high numerical increase, the production level remains insufficient to satisfy the market demand and to meet the economic goals of farmers. Therefore, management, feeding, and breeding improvements are still necessary to achieve these aims.

It is well known that, among ruminants, the buffalo produces milk characterized by a higher level of fat. It varies between 7.5% at the beginning of the lactation (after the colostrum phase) and 12 to 14% at the end of the lactation (Arumughan and Narayanan, 1981; Catillo et al., 2002). As milk fat has a great influence

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[1]Corresponding author: alfredo.pauciullo@unito.it

on cheese-making properties and yield, one of the main goals of the Italian National Association of Buffalo Breeders is the increase of milk fat content, which contributes to the determination of the production in kilograms of mozzarella, the genetic index used for the evaluation of EBV. Therefore, the genetic improvement of buffalos for the fat content represents a fundamental step for the progress of this species.

Many candidate genes for lipid metabolism have been identified so far, including *FASN* (fatty acid synthase), *DGAT1* (diacylglycerol O-acyltransferase 1), *SCD* (stearoyl CoA desaturase), and *ACACA* (acetyl-CoA carboxylase α). However, in the last 15 yr, only the *DGAT1* has been recognized as a strong functional candidate for milk fat content (Winter et al., 2002; Grisart et al., 2002).

The DGAT1 catalyzes the last reaction step in the synthesis of triacylglycerol. In cattle, a nonconservative substitution at exon 8 responsible for the AA change K232A has been associated with high and low milk fat percentage (**FP**; Winter et al., 2002; Thaller et al., 2003; Grisart et al., 2004; Kühn et al., 2004) and, later, also associated with milk fat composition (Schennink et al., 2007, 2008; Conte et al., 2010) and milk fat globule structure (Argov-Argaman et al., 2013). This polymorphism has been deeply investigated worldwide and found in many cattle breeds.

Conversely, the *DGAT1* gene in river buffalo has received less attention so far, with information limited to gene structure (Mishra et al., 2007; Yuan et al., 2007) and polymorphism detection (Mishra et al., 2007; Yuan et al., 2007; Raut et al., 2012; Silva et al., 2016). In this respect, the K232A polymorphism has also been investigated in buffalo breeds (Tantia et al., 2006; Shi et al., 2012), which were monomorphic for the K allele.

Recently, new polymorphic sites have been identified and associated with the fat trait. Cardoso et al. (2015) found that a variable nucleotide repeat in the promoter region of *DGAT1* explained 32% of the additive genetic variance of FP, and de Freitas et al. (2016) reported a SNP in exon 17 significantly associated with fat and protein percentage in Brazilian Murrah buffaloes.

Apart from these studies, no additional information is available and no investigation has been carried out at a transcriptomic level for the buffalo *DGAT1*. Furthermore, no genetic diversity has been reported in *DGAT1* coding regions for the Italian Mediterranean breed.

To contribute to a more detailed knowledge of the river buffalo *DGAT1*, an investigation was undertaken to analyze the transcriptional profiles of buffalo cows characterized by extreme phenotypes (high and low) for milk FP, and to explore the genetic diversity at the RNA and DNA level.

MATERIALS AND METHODS

Sample Collection and Nucleic Acid Isolation

Milk and blood samples were collected from 8 unrelated lactating buffalos reared in the Piedmont region (Northern Italy) and belonging to 1 farm. They were chosen among more than 500 lactating buffaloes ranked for milk FP, and separated in 2 groups at the extreme sides for this trait: 4 buffalo cows (high group) with high FP (9.13 ± 0.23), and 4 buffalo cows (low group) with low FP (7.94 ± 0.29). The milk yield (kg/d) was comparable for the 8 animals (8.74 ± 0.96). The selection was based on their monthly test-day milk FP records for the current and previous lactations, which were provided by the Italian National Association of Buffalo Breeders. The animals were comparable for age (approximately 6 yr old), feeding system, number of lactation (third), and lactation stage (fourth month).

An additional 200 blood samples were collected for DNA genotyping, 100 samples (Italian Mediterranean breed) from 8 buffalo farms in the Campania region (Southern Italy) and 100 samples (Murrah breed) from Şercaia research station (Romania).

Total RNA was isolated from milk somatic cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines (https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf), whereas the remaining traces of DNA were removed with DNase I (Qiagen). The genomic DNA was isolated from blood samples according to the procedure described by Sambrook et al. (1989) and then resuspended in 100 µL of TE buffer pH 7.6 (10 m*M* Tris, 1 m*M* EDTA).

The RNA and DNA concentrations and optical density at 260/280 nm ratios were measured with the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Average concentrations were 50 ng/µL for both RNA and DNA samples. A ratio higher than 1.8 was recorded for all the DNA samples, whereas a ratio higher than 2.0 was detected for RNA samples. These values are generally accepted as pure for DNA and RNA, respectively, and therefore they indicated the absence of protein, phenol, or other contaminants.

RT-PCR, Cloning, and Sequencing

The reverse transcription of total RNA was performed by using an oligo dT_{18}. The mix was set up in a final volume of 20 µL using ImProm-II Reverse Transcriptase (Promega) according to the standard protocol recommended by the firm. The PCR reaction was performed by using the following primers (for-

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