ANALYSIS OF CANDIDATE GENES FOR MEAT PRODUCTION TRAITS IN DOMESTIC RABBIT BREEDS

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ABSTRACT

A candidate gene approach has been already successfully applied to identify several DNA markers associated with production traits in livestock. The principle is based on the fact that variability within genes coding for protein products involved in key physiological mechanisms and metabolic pathways directly or indirectly involved in determining an economic trait (e.g. feed efficiency, muscle mass accretion, reproduction efficiency, disease resistance, etc.) might probably explain a fraction of the genetic variability for the production trait itself. Growth hormone (*GH*) and myostatin (*MSTN*) genes play important roles in animal growth, development and muscle mass accretion. For their functions, these two genes can be considered candidate genes for meat production traits. Here we resequenced parts of these two genes in four rabbit breeds (Belgian Hare, Burgundy Fawn, Checkered Giant and Giant Grey) in order to identify DNA markers useful for association studies with economic traits. On the whole, resequencing of the *GH* and *MSTN* genes generated sequence information for 9988 bp. No mutation was detected in the sequenced regions of the *GH* gene suggesting the absence of common polymorphisms in this rabbit gene. Resequencing of the rabbit *MSTN* gene identified only a single nucleotide polymorphism (C>T) in intron 2. A PCR-RFLP protocol was designed to investigate this mutation in a larger number of rabbits (15 Checkered Giant, 9 Giant Grey, 6 Dwarf, 4 Burgundy Fawn, 3 Giant White, 3 Lop, 2 Belgian Hare, 1 New Zealand White). Allele frequencies across breeds were 0.51 for allele *C* and 0.49 for allele *T*. Considering the breeds for which at least 5 animals were analysed, allele *C* frequency was 0.56 in Checkered Giant, 0.60 in Burgundy Fawn and Giant Grey and 0.83 in Dwarf. For its allele distribution the identified polymorphism seems an useful gene marker for association studies with production traits in rabbits.

Key words: Candidate genes, Growth hormone, Myostatin, Resequencing, SNP.

INTRODUCTION

A candidate gene approach has been already successfully applied to identify several DNA markers associated with production traits in livestock (Rothschild and Soller, 1997). The principle is based on the fact that variability within genes coding for protein products involved in key physiological mechanisms and metabolic pathways directly or indirectly involved in determining an economic trait (e.g. feed efficiency, muscle mass accretion, reproduction efficiency, disease resistance, etc.) might probably explain a fraction of the genetic variability for the production trait itself. The first step is the identification of mutations in candidate genes that can be analysed in association studies in specific designed experiments. For the roles they play, growth hormone (*GH*) and myostatin (*MSTN*) genes can be considered candidate genes for meat production traits.

GH plays important functions in animal growth and development and several studies have analysed the *GH* gene as a candidate for economic traits in livestock species. Mutations of this gene have been described in dairy cattle (Lagziel *et al*., 1996; Yao *et al*., 1996), beef cattle (Taylor *et al*., 1998; Barendse *et al*., 2006), sheep (Marques Mdo *et al*., 2006), goats (Malveiro *et al*., 2001), pigs (Knorr *et al*., 1998) and poultry (Feng *et al*., 1997; Kuhnlein *et al*., 1997) to affect important production traits. The rabbit *GH* gene was isolated and sequenced by Wallis and Wallis (1995). It comprises four introns and five exons that code for a deduced protein of 216 amino acids with 26 amino acids signal peptide

and 190 amino acid mature peptide. Southern blotting analysis revealed that the *GH* gene is present in the rabbit genome as a single copy gene without *GH*-like genes that, instead, are reported for other species (Wallis and Wallis, 1995).

MSTN, also known as GDF8, is a member of the transformic growth factor (TGF)-β superfamily that actively represses skeletal muscle growth (Lee, 2004). Myostatin null mice generated by gene targeting showed a 2- to 3-fold increase of muscle mass compared to wild type mice, primary due to an increased number of muscle fibers, followed my muscle cell hypertrophy and suppression of body fat accumulation (McPherron *et al*., 1997; McPherron and Lee, 2002). Several mutations disrupting the myostatin function cause double-muscle phenotypes in cattle with the same mechanism described in mice (Grobet *et al.*, 1997; 1998; Kambadur *et al*., 1997; Marchitelli *et al*., 2003; McPherron and Lee, 1997). In dogs, a disrupting mutation in exon 3 of this gene identified in the whippet breed resulted in a double-muscled phenotype linked to enhanced athletic performance of the animals (Mosher *et al*., 2007). The rabbit *MSTN* gene sequence has been recently assembled after the initiative of the Broad Institute that shot gun sequenced the rabbit genome at 2X level (Ensembl Gene ID: ENSOCUG00000012663, http://www.ensembl.org/Oryctolagus_cuniculus/index.html**)**. It comprise three coding exons and two introns as observed in other species.

As no mutation has been reported for the rabbit *GH* and *MSTN* genes yet, here we resequenced these two genes in rabbits of different breeds with the aim to identify mutations useful for association studies with meat production traits.

MATERIALS AND METHODS

Animals and DNA Isolation

Four rabbits (one Belgian Hare, one Burgundy Fawn, one Checkered Giant and one Giant Grey) were used for resequencing of the *GH* and *MSTN* genes. Additional 43 rabbits of different breeds (15 Checkered Giant, 9 Giant Grey, 6 Dwarf, 4 Burgundy Fawn, 3 Giant White, 3 Lop, 2 Belgian Hare, 1 New Zealand White) were genotyped for the single nucleotide polymorphism (SNP) identified in the *MSTN* gene. DNA was isolated from blood and/or hair roots as previously described (Fontanesi *et al*., 2006; 2007).

Polymerase Chain Reactions (PCR) and Sequencing

Two PCR primer pairs were designed on the rabbit *GH* gene sequence (GenBank accession number: Z38127; Wallis and Wallis, 1995) to amplify a fragment of 345 bp encompassing part of intron 1, exon 2 and part of intron 2 (forward: 5'-CTAGCCTAGGGGAGGACTGG-3'; reverse: 5'-CTCATCCGACAGCATCTTCA-3') and a fragment of 560 bp encompassing part of intron 2, exon 3, intron 3, exon 4 and part of intron 4 (forward: 5'-GTTCTGATCAGCAGCCATGA-3'; reverse: 5'-TGGCTGCTAGGAGACAGGAC-3'). Three PCR primer pairs were designed on the rabbit *MSTN* gene sequence (Ensembl Gene ID: ENSOCUG00000012663) to amplify a fragment of 499 bp including part of the 5'-untranslated region and the coding sequence of exon 1 and part of intron 1 (forward: 5'-AATTTTGCTTGCCATTACTGA-3'; reverse: 5'-TCAGCAGAACTGTTGACATACAC-3'), a fragment of 570 bp including part of intron 1, exon 2 and part of intron 2 (forward: 5'-TGCATGCATTATCCCAATAGA-3'; reverse: 5'-TCGGTAGTTGTTTCCCACTTT-3') and a fragment of 523 bp encompassing part of intron 2, the coding sequence of exon 3 and part of the 3'-untranslated region (forward: 5'-AAAGGTATTCCAAGCAAAATGA-3'; reverse: 5'-GGGGAAGACCTTCCATGTTT-3'). PCR was carried out in 20 µl containing 1 U EuroTaq DNA polymerase (EuroClone Ltd.), 1X PCR Buffer, 2.5 mM dNTPs, 10 pmol of each primer and 1.0 mM of $MgCl_2$. PCR profile was as follows: 5 min at 95°C; 35 amplification cycles of 30 sec at 95°C, 30 sec at 57-62°C, 30 sec at 72°C; 10 min at 72°C. PCR was performed using a PT-100 (MJ Research) or a Perkin Elmer 9600 thermal cycler. PCR products obtained from four rabbits were sequenced on both strands after treatment with 1 µl of

ExoSAP-IT® (USB Corporation) for 15 min at 37°C. Cycle sequencing of the treated PCR products was produced using the same PCR primers and the Big Dye v3.1 kit (Applied Biosystems). Sequencing reactions, after a few purification steps using EDTA, Ethanol 100% and Ethanol 70%, were loaded on an ABI3100 Avant sequencer (Applied Biosystem). Sequences were edited and aligned with the help of the CodonCode Aligner software (CodonCode Corporation).

Analysis of Mutation

A PCR-restriction fragment length polymorphism (RFLP) protocol was set up to analyse the SNP identified in the *MSTN* gene. A mutant forward PCR primer (5'-TAACTGAAAAGAACCCTCTAGTAGC-3', mismatch created by the underlined base) was designed to insert an artificial restriction site for *Alu*I when T is present in the polymorphic position. This primer was coupled with reverse primer designed in intron 2 (5'-TCGGTAGTTGTTTCCCACTTT-3') to amplify a fragment of 80 bp using the PCR protocol and profile reported above. Then, 5 µl of PCR product was digested overnight at 37°C with 2 U of *Alu*I (MBI Fermentas) in a final volume of 25 µl containing 1X enzyme reaction buffer. The resulting DNA fragments were separated by electrophoresis in 10% polyacrylamide:bis-acrylamide 29:1 gels with TBE 1X buffer. DNA products were visualized with ethidium bromide on a UV apparatus.

RESULTS AND DISCUSSION

On the whole, resequencing of the *GH* and *MSTN* genes in four different rabbits generated sequence information for 9988 bp.

The rabbit *GH* gene contains five exons (Wallis and Wallis, 1995), three of which (exons 2, 3 and 4) were resequenced in four rabbits of different breeds. No mutations were identified comparing the sequences obtained in the analysed rabbits. Moreover, no difference was observed between the obtained sequences and the sequence reported by Wallis and Wallis (1995) produced from a New Zealand White rabbit genomic library. Thus, based on the information obtained for this gene for rabbits belonging to five different breeds (four from our study and one from the cited reference) it seems that the sequenced regions are highly conserved across breeds and no common mutation might be present.

All three coding *MSTN* exons were resequenced including portions of the 5'- and 3'-untranslated regions as well as parts of introns 1 and 2. No mutation was observed in the transcripted sequence, namely coding sequence and 5- and 3'-untranslated regions. Nevertheless, an SNP was identified in intron 2. The polymorphic site is caused by a C>T transition at position 34 of this intron. As this SNP does not create/eliminate restriction sites for any common endonuclease, an artificial restriction site for *Alu*I was inserted in the amplified product by means of a mismatched forward primer. This PCR-RFLP protocol (Figure 1) was used to genotype the four rabbits utilized for sequencing providing a confirmation of the genotyping results. In addition, other 43 rabbits were genotyped obtaining a first evaluation of the frequency of the two alleles. Considering the 47 analysed rabbits, the frequency of alleles *C* and *T* across breeds was 0.51 and 0.49, respectively, with a high level of heterozygosity (0.50) and PIC (0.37). Considering only the breeds for which at least 5 animals were analysed, allele *C* frequency was 0.56 in Checkered Giant, 0.60 in Burgundy Fawn and Giant Grey and 0.83 in Dwarf. For its allele distribution the identified polymorphism, even if it does not disrupt or affect the coding sequence because is localized in an intronic region without any putative functional role, seems an useful gene marker for association studies.

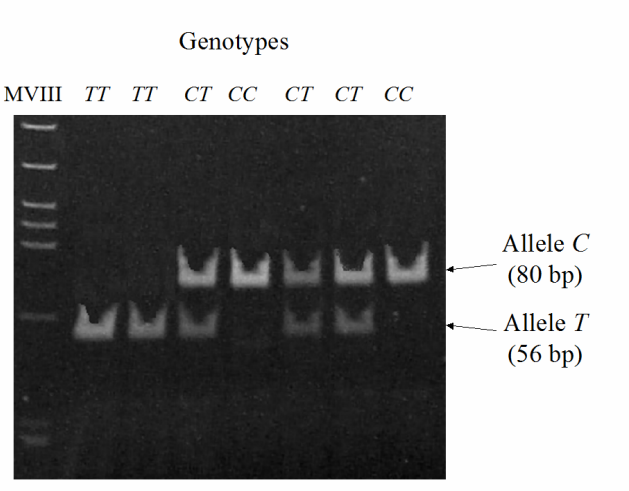

Figure 1: Gel electrophoresis showing the PCR-RFLP products of the SNP identified in intron 2 of the rabbit *MSTN* gene. The genotypes are indicated at the top of each lane. MVIII: DNA molecular weight VIII (Roche Diagnostics). The fragment of 24 bp resulted from the digestion of allele *T* is not shown in the gel

CONCLUSIONS

Partial resequencing of two rabbit genes (*GH* and *MSTN*) resulted in only one detected SNP. Resequencing activities that are under way for a larger number of animals and for additional regions of the considered genes might provide more DNA markers. However, the balanced allele frequency for the intronic *MSTN* polymorphic site makes this SNP an useful tool for association studies To this aim meat production traits will be collected in commercial populations.

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