SNPs within the beta myosin heavy chain (MYH7) and the pyruvate kinase muscle (PKM2) genes in horse

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ABSTRACT

Two highly expressed skeletal muscle genes (the MYH7 gene encoding the myosin heavy chain slow/β-cardiac isoform and the PKM2 gene encoding the pyruvate kinase muscle isoforms) were investigated with the objective to identify DNA markers in horses. A panel of DNA samples from different horse breeds was analysed using a PCR-single strand conformation polymorphism (SSCP) approach. Four and two alleles were identified for the MYH7 and PKM2 loci, respectively. Mendelian inheritance of alleles of the two investigated genes was confirmed analysing horse families. Sequencing of PCR products obtained from the MYH7 and PKM2 genes made it possible to characterise two SSCP alleles for each gene. The polymorphisms found in the MYH7 and PKM2 genes were further studied in 61 and 68 horses of three (Italian Heavy Draught Horse, Italian Saddler and Murgese) and five (Franches-Montagnes, Haflinger, Italian Heavy Draught Horse, Murgese and Standardbred) breeds, respectively. Allele frequencies of the two loci varied among the considered breeds. The SNPs discovery in MYH7 and PKM2 genes makes it possible to locate new molecular markers to ECA1. The identified markers could be used in association analysis with performance traits in horses.

Key words: Horse breeds, MYH7, PKM2, SNPs, Skeletal muscle.

RIASSUNTO

SNPS NEL GENE CODIFICANTE PER LA ISOFORMA BETA DI MIOSINA CATENA PESANTE (MYH7) E NEL GENE PER LA PIRUVATOCHINASI (PKM2) NEL CAVALLO

In questo studio sono stati identificati polimorfismi nei geni codificanti per la isoforma beta di miosina catena pesante (MYH7) e per le isomero M1 e M2 della piruvatochinas (PKM2). Alcuni campioni di DNA estratti da cavalli appartenenti a 13 razze e popolazioni (Anglo-Normanna, Avelignese, Belga, Bretone, Cavallo Agricolo Italiano da Tiro Pesante Rapido, Franches-Montagnes, Hannover, Holstein, Murgese, Rheinland, Salernitano, Sella Italiano e Trottaor Americano) sono stati inizialmente analizzati mediante PCR-SSCP. Le analisi hanno messo in evidenza quattro alleli per il gene MYH7 e due alleli per PKM2. La segregazione degli alleli MYH7 e di PKM2 è stata verificata da analisi in famiglie. Il sequenziamento bidirezionale di alcuni prodotti PCR del gene MYH7 ha permesso di individuare la sostituzione nucleotidica di due alleli SSCP. Il
polimorfismo individuato nel gene PKM2 è originato dalla mutazione puntiforme G>A. La diffusione degli alleli dei geni MYH7 e PKM2 è stata studiata in campioni di rispettivamente tre (Cavallo Agricolo Italiano da Tiro Pesante Rapido, Murgese e Sella Italiano) e cinque (Avelignese, Cavallo Agricolo Italiano da Tiro Pesante Rapido, Franches-Montagnes, Murgese e Trotatore Americano) razze. Nei campioni delle diverse razze sono state osservate differenze nei valori di frequenza genica per entrambi i geni. I polimorfismi identificati costituiscono nuovi marcatori di geni importanti dal punto di vista funzionale e potranno essere utilizzati in analisi di associazione con le prestazioni produttive dei cavalli.

Parole chiave: Cavalli, MYH7, PKM2, SNPs, Muscolo scheletrico.

Introduction

In addition to being a food source, the horse is one of the few livestock animals that is bred for athletic performance and for pleasure. Genetic improvement in horses is greatly reduced by the long generation interval, so the application of genetic markers in selection schemes to improve performance traits could be highly desirable, even if at present this can only be envisaged. Except coat colour genes, just a few genetic markers have been identified in horses compared to others species (Penedo et al., 2005; Swinburne et al., 2006). Skeletal muscle can be considered the most important tissue type in horses because it is directly related to the performances of the animals as well as to meat production traits. Myosins are the fundamental functional units of striated muscles. Each myosin unit is a heterohexamer protein composed of two myosin heavy chains (MyHC) and two non-identical pairs of myosin light chains. At least ten myosin heavy chain isoforms are known in mammals (Schiaffino and Reggiani, 1996; Eizema et al., 2005) and each isoform is encoded by a different gene (Weiss and Leinwand, 1996; Weiss et al., 1999). The MYH7 gene comprises 40 exons and encodes for the MyHC slow/β-cardiac isoform (referred to as MyHC 1 or MyHC-1p/slow) that gives to the muscle resistance to fatigue and is expressed in slow, oxidative, type 1 fibres of adult skeletal muscle as well as in the prenatal ventricular muscle. The equine MYH7 gene was physically mapped on horse chromosome 1 (ECA1) within region 1q25-q26 (Milenkovic et al., 2002). PCR analysis of a horse-hamster radiation hybrid (RH) panel confirmed the physical localisation making it possible to place the gene at 47.9 cR from microsatellite COR006 (Chowdhary et al., 2003; Horse Map Viewer, 2007).

The pyruvate kinase (PK) or ATP:pyruvate phosphotransferase (EC 2.7.1.40) is a glycolytic enzyme involved in the final step of glycolysis, namely the conversion of phosphoenolpyruvate to pyruvate with concomitant phosphorylation of ADP to ATP. In mammals, PK exists as four isoenzymes (referred to as L-, R-, M1- and M2-PK types) which are encoded by two different genes, PKLR and PKM2. The first gene encodes the R- and L- isoenzymes while the PKM2 encodes the M1- and M2-PK isoforms. The human PKM2 gene comprises 12 exons and the M1 and M2 isoenzymes are produced by tissue-specific mutually alternative splicing of exons 9 and 10, respectively (Noguchi et al., 1986; Takenaka et al., 1989, 1991). The M1-isoform is expressed in skeletal muscle, heart and brain and the M2- or pyruvate kinase muscle type 2 is expressed in kidney, adipose tissue and lungs (Yamada and Noguchi, 1999; Munoz and Ponce, 2003). The horse PKM2 gene was mapped to 1q21 by FISH (Lear et al., 2000) and then was located at 55.8 cR from microsatellite CA25.
typing a horse RH panel (Chowdhary et al., 2003; Horse Map Viewer, 2007). In this study we investigated these two highly expressed skeletal muscle genes (MYH7 and PKM2), with the objective to identify equine DNA markers useful in association studies with performance and production traits.

**Material and methods**

**Samples**

A DNA panel of 13 horse breeds (Anglo-Norman, Belgian, Breton, Franches-Montagnes, Haflinger, Hannover, Holstein, Italian Heavy Draught Horse, Italian Saddler, Murgese, Rheinland, Salernitano and Standardbred) was initially used for PCR-single strand conformation polymorphism (SSCP) analysis.

Additional 61 and 68 horses of three breeds (29 Italian Heavy Draught Horse, 8 Italian Saddler and 24 Murgese) and of five breeds (14 Franches-Montagnes, 10 Haflinger, 6 Italian Heavy Draught Horse, 23 Murgese and 15 Standardbred) were sampled and analysed for the polymorphisms identified in the MYH7 and PKM2 genes, respectively. Three half sibs families obtained crossing one Franches-Montagnes stallion with three Murgese mares were used to verify the Mendelian inheritance of the MYH7 and PKM2 polymorphisms. Genomic DNA was isolated from hair roots of horses using proteinase K protocol (Healy et al., 1995).

**PCR, SSCP and sequencing analyses**

The choice of gene regions which we analysed was determined by the limited number of available sequences in GeneBank for MYH7 and PKM2 horse genes, while the sizes of PCR products (no more than 300 bp) were determined on the basis of optimal size of the fragments for SSCP technique. Two PCR primer pairs, designed using OLG0 version 5.0 (MedProbe, Oslo, Norway) on sequence AF130771 (partial sequence of the equine MYH gene, Caetano et al., 1999) and on AY008802 (partial sequence of the equine PKM gene, Shubitowski et al., 2001), were used to amplify genomic DNA. Primer pair sequences and PCR conditions are listed in Table 1.

The PCR mix (final volume of 20 µl) contained about 100 ng horse genomic DNA, 250 µM of each dNTP, 10 pmol of each primer, 1 U of Taq DNA Polymerase (Roche Molecular Diagnostics, Mannheim, Germany) and 1X PCR buffer with 1.0 and 1.5 mM MgCl₂ for MYH7 and PKM2, respectively.

The PCR fragments obtained from the two genes were analysed to search for single strand conformation polymorphisms. Several variables were tested in order to optimise the efficiency of mutation detection and to achieve a separation of the putative alleles: acrylamide or MDE (FMC, Bioproducts), glycerol level (0%, 8% or 10%) and electrophoresis temperature (from 4 to 20°C). A Dcode™ Universal Mutation Detection System (Bio-Rad, Laboratories, Hercules, CA, USA) was used and the gels were run at 35 W constant power. Single strand DNA was revealed using a silver staining procedure (Peng et al., 1995). The PCR products of the two genes obtained from putative homozygous animals were purified by QIAquick PCR Purification Kit (Qiagen, Italy) and then sequenced using the BigDye v3.1 kit (Applied Biosystems, Foster City, CA, USA) and the same forward and reverse PCR primers.

The obtained sequences were aligned using ClustalW software program (http://www.ebi.ac.uk/clustalw/).

**Results and discussion**

A PCR primer pair was designed on equine MYH gene sequence (GenBank
that a BLAST search of the GenBank database (http://ncbi.nih.gov/BLAST/) indicated to correspond to a portion of MYH7 gene. Based on the intron size and organization of the human MYH7 gene (GenBank number M57965), the equine sequence should correspond to a portion of exon 30, part of exon 31 and the intervening intron. The PCR primers were then designed to amplify a putative fragment of 243 bp of intron 30 of the equine corresponding gene. SSCP analysis was applied to identify polymorphisms from a DNA panel. The best SSCP-electrophoresis conditions were obtained using a 10% 39:1 acrylamide-bis acrylamide ratio, 10%, glycerol and 20°C constant temperature for 8 h. Different conformational patterns corresponding to four putative alleles (referred as 1, 2, 3 and 4) were observed.

Sequencing was performed both to confirm that the amplicons obtained corresponded to the expected equine MYH7 gene fragment as well as to characterise the mutations that originated the identified polymorphism. The comparison of sequences obtained from two putative homozygous 1/1 and two putative homozygous 2/2 horses revealed a G>A single nucleotide polymorphism at position 29 of the obtained sequences that were submitted to EMBL Nucleotide Sequence Database under accession number AM182988 (allele 1 carries G at position 29) and AM182990 (allele 2 carries A at position 29). Furthermore, the obtained sequences showed two other nucleotide differences (T>A and G>C at position 30 and 100, respectively) and three additional bases (2 G and 1 C) compared to the sequence (GenBank accession number AF130771) used for primer design (Figure 1). Thus, the size of equine MYH7 amplified fragment actually resulted as 246 bp instead of the expected 243 bp.

In order to exclude artefacts in the PCR-SSCP analysis for the 3 and 4 alleles, found only in heterozygous genotypes, their Mendelian segregation was verified in three half sib horse families (Figure 2). Under our experimental conditions, there

| Locus symbol | Primer sequence (5’- 3’) | Size (bp) | PCR profile |
|--------------|--------------------------|----------|-------------|
| MYH7         | Forward: GAAGGCGGTGGGTGAGTTGC  
               Reverse: GGTGGGGTGGATGGAAGGTG | 246      | 95°C for 5 min-  
               35 cycles: 95°C for  
               30 s/68°C for 20 s/72°C for 30 s -  
               final extension at 72°C for 5 min |
| PKM2         | Forward: AATGTAGCAACCTGGGTGC  
               Reverse: TCCCTGAAGCCAGACAGATG | 231      | 95°C for 5 min-  
               35 cycles: 95°C for  
               30 s/60°C for 30 s/72 °C for 30 s -  
               final extension at 72°C for 5 min |
NEW SNPs IN HORSE GENES

Figure 1. Alignment of the sequences generated in the current study (AM182988 and AM182990) and of the sequence available in GeneBank (AF130771) for equine MYH7 gene region. The bases of the identified G>A mutation are indicated by bold-faced letters.

| Sequence    | Length (nt) |
|-------------|-------------|
| AM182988    | 60          |
| AM182990    | 60          |
| AF130771 (271-513 nt) | 329         |
| AM182988    | 120         |
| AM182990    | 120         |
| AF130771 (271-513 nt) | 388         |
| AM182988    | 180         |
| AM182990    | 180         |
| AF130771 (271-513 nt) | 447         |
| AM182988    | 240         |
| AM182990    | 240         |
| AF130771 (271-513 nt) | 507         |
| AM182988    | 246         |
| AM182990    | 246         |
| AF130771 (271-513 nt) | 513         |

Figure 2. PCR-SSCPs at the equine MYH7 locus in three half-sib families. The genotypes are indicated at the bottom of each lane.

| Allele 2 | Allele 3 |
|----------|----------|
| 3/4      | 1/2      |
| 1/4      | 3/4      |
| 1/3      | 1/4      |
| 1/2      | 1/4      |
| 3/4      |          |

 Allele 1

 Allele 4
were two distinct bands for each allele, except for the allele 2 that gives only one fragment as shown in the Figure 2.

Allele frequencies of the four confirmed MYH7 alleles were studied in a sample of 61 horses belonging to three different breeds (Table 2). The frequency of allele 2 (A allele) was 0.61 in Murgese and 0.36 in Italian Heavy Draught breeds, whereas this allele was not identified in the Italian Saddler samples for which allele 1 (G allele) showed the highest frequency (0.63). The alleles 3 and 4 were the less frequent, allele 3 ranged from 0.08 to 0.25 and allele 4 ranged from 0.03 to 0.12.

For PKM2 gene, a PCR primer pair was designed on the partial horse PKM gene sequence (AY008802) that, according to the exon-intron organisation of the human PKM2 gene (Weiss et al., 1999), should correspond to exon 2 and intron 2 portions. The two PCR primers were designed on the intron sequence to amplify a 231 bp fragment of the equine PKM2 gene. Optimal SSCP-electrophoresis conditions of the denaturated products of the PKM2 gene were obtained using a 0.5 X MDE (FMC) gel, 8% glycerol and 4°C for 7 h. PCR-SSCP analysis showed three different patterns according to the segregation of two putative alleles and their Mendelian inheritance was confirmed.

Sequencing of the PKM2 gene products from two homozygous 1/1 and two homozygous 2/2 horses revealed a SNP (G>A) at base 37 of the sequences deposited in the EMBL database with accession numbers AM182984 (allele 1 = nucleotide G) and AM182983 (allele 2 = nucleotide A), respectively. PCR-SSCP analysis of 68 horses from 5 different breeds (Table 3) showed that G allele was always the most frequent. A allele was not identified in the Standardbred and Haflinger analysed

| Breeds                     | N. animals | Allele frequencies |
|---------------------------|------------|--------------------|
|                           |            | Allele 1 | Allele 2 | Allele 3 | Allele 4 |
| Murgese                   | 24         | 0.25     | 0.61     | 0.08     | 0.06     |
| Italian Heavy Draught Horse | 29       | 0.40     | 0.36     | 0.21     | 0.03     |
| Italian Saddler           | 8          | 0.63     | 0.00     | 0.25     | 0.12     |

| Breeds                      | N. animals | Allele frequencies |
|-----------------------------|------------|--------------------|
|                            |            | Allele G  | Allele A   |
| Murgese                     | 23         | 0.74      | 0.26      |
| Standardbred                | 15         | 1.00      | 0.00      |
| Franches-Montagnes          | 14         | 0.61      | 0.39      |
| Haflinger                   | 10         | 1.00      | 0.00      |
| Italian Heavy Draught Horse | 6          | 0.92      | 0.08      |
horses and its highest frequency (0.39) was observed in the Franches-Montagnes samples.

Conclusions

The SNPs discovery in the equine MYH7 and PKM2 genes made it possible to add two new type I markers to ECA1. A preliminary analysis of the polymorphisms of the two loci showed differences of allele frequencies among breeds that differ in performance and morphological traits. The identification of new polymorphic loci is useful to improve the horse genetic map that although in progress, is still logging in information compared to genome maps of other livestock species. Furthermore, the detection of polymorphisms within genes with important roles in skeletal muscle function and metabolism represents the starting point for association analyses between SNPs and performance traits.

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