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SHORT COMMUNICATION

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 isoforme M1 e M2 della piruvatochinasi* (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 Trottatore 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 di* 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 Trottatore 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-1β/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 glycolitic 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, Hannnover, Holstein, Italian Heavy Draught Horse, Italian Saddler, Murgese, Rheinland, Salernitano and Standarbred) 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 *OLIGO* 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 $MgCl_2$ 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 v.3.1 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

Table 1. Primer pairs used to amplify and sequence *MYH7* and *PKM2* horse gene regions and PCR profile.

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: AATGTAGCAAACCTGGGTGC Reverse: TCCCTGAAGCCCAGACAGATG	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

number AF130771, Caetano *et al.*, 1999) 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

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.

```
AM182988 (allele 1)    GAAGGCGGTGGGTGAGTTGCAGAAGCCCGTGTGACCTCCATGTCTGCAGACCAGAGGCAG 60
AM182990 (allele 2)    GAAGGCGGTGGGTGAGTTGCAGAAGCCCATGTGACCTCCATGTCTGCAGACCAGAGGCAG 60
AF130771 (271-513 nt)  GAAGGCGGTGGGTGAGTTGCAGAA-CCCGAGTGACCTCCATGTCTGCAGACCAGAGGCAG 329
                       ************************ ***  ****************************

AM182988 (allele 1)    GCCCAGGGTCAAAGGGACGCCACAAGAAGGCCCTCTTGGGTGCTGTGGGTCTTCACGTCT 120
AM182990 (allele 2)    GCCCAGGGTCAAAGGGACGCCACAAGAAGGCCCTCTTGGGTGCTGTGGGTCTTCACGTCT 120
AF130771 (271-513 nt)  GCCCAGGGTCAAAGGGACGCCACAAGAAGGCC-TCTTGGCTGCTGTGGGTCTTCACGTCT 388
                       ******************************** ****** ******************

AM182988 (allele 1)    GTCAGGAGACCCTCTGCCCACCTGTCCTCAGGCTTCTCCAACAATGCTTGAGTTTTCAAG 180
AM182990 (allele 2)    GTCAGGAGACCCTCTGCCCACCTGTCCTCAGGCTTCTCCAACAATGCTTGAGTTTTCAAG 180
AF130771 (271-513 nt)  GTCAGGAGACCNTCT-CCCACCTGTCCTCAGGCTTCTCCAACAATGCTTGAGTTTTCAAG 447
                       *********** *** ********************************************

AM182988 (allele 1)    GATTGTTTTCTAGAAGACTCACATGGTTTCCCCTTGTCCAAATCTGCACCTTCCATCCAC 240
AM182990 (allele 2)    GATTGTTTTCTAGAAGACTCACATGGTTTCCCCTTGTCCAAATCTGCACCTTCCATCCAC 240
AF130771 (271-513 nt)  GATTGTTTTCTAGAAGACTCACATGGTTTCCCCTTGTCCAAATCTGCACCTTCCATCCAC 507
                       ************************************************************

AM182988 (allele 1)    CCCACC 246
AM182990 (allele 2)    CCCACC 246
AF130771 (271-513 nt)  CCCACC 513
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Figure 2. PCR-SSCPs at the equine *MYH7* locus in three half-sib families. The genotypes are indicated at the bottom of each lane.

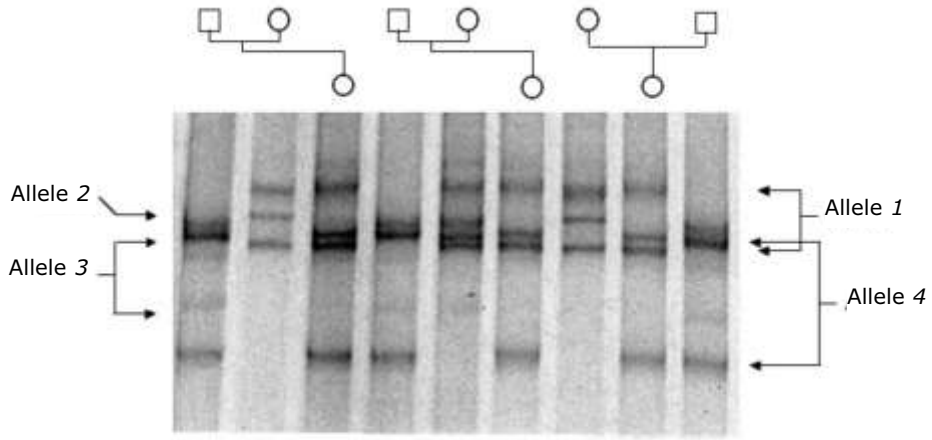

Table 2. Allele frequencies at the *MYH7* locus in three horse breeds.

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

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*=nucleoide 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

Table 3. Allele frequencies at the *PKM2* locus in five horse breeds.

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 news 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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