

Short Communication

Analysis of two single-nucleotide polymorphisms (SNPs) located in exon 1 of kappa-casein gene (CSN3) in Martina Franca donkey breed

Maria Selvaggi* and Cataldo Dario

Department of Animal Health and Welfare, University of Bari, strada prov. le per Casamassima Km 3 – 70010, Valenzano (Ba), Italy.

Accepted 18 January, 2011

The aim of this study is to assess genetic polymorphism at two *loci* in the exon 1 of the kappa-casein gene (CSN3) in Martina Franca donkey breed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Martina Franca donkey was derived from the Catalan donkey brought to Apulia at the time of the Spanish rule. This donkey is tall and well built and has good temperament. Both considered *loci* were found to be monomorphic in the considered population. At CSN3/*Pst*I locus, all the animals were genotyped as AA since no AG and GG animals were found in the population. A similar result was found at CSN3/*Bse*YI locus: all the donkeys were monomorphic and genotyped as AA. As a consequence, only one out of nine possible combined genotype (AAAA) was detected.

Key words: Martina Franca donkey, kappa-casein gene (CSN3), gene polymorphism, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

INTRODUCTION

Kappa-casein is the protein that determines the size and the specific function of milk micelles; its cleavage by chymosin is responsible for milk coagulation (Yahyaoui et al., 2003). The comparison of amino acids (aa) sequences of the equine caseins with the corresponding camel, pig, human, bovine, ovine and goat counterparts revealed sequences identity between 40 and 67% (Lenasi et al., 2003).

Equine κ -casein proves to be the most conserved casein, closely followed by β -casein in agreement with the physiological function of these proteins in micelle formation and with the role of κ -casein in milk coagulation (Holt, 1992). Based on interspecies comparison, five exons were found in equine kappa-casein gene (CSN3)

(Lenasi et al., 2003). CSN3 is not evolutionarily related to the "calcium-sensitive" casein genes, but is physically linked to this gene family, and is functionally important for stabilizing the Ca-sensitive caseins in the micelle. The organization of CSN3 is conserved in all species studied.

Recently, four SNPs were detected in the exon 1 and 4 of the equine CSN3 (Hobor et al., 2006). The SNPs located in exon 1 were investigated with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis using two restriction enzymes. The polymorphisms studied using *Pst*I and *Bse*YI are transition, A→G (c.-66A>G, GenBank AY579426) and a transversion, C→A (c.-36C>A, GenBank AY579426), respectively. Exon 1 is not part of the coding sequence of the gene, therefore, these single-nucleotide polymorphisms (SNPs) do not cause an amino acid substitution. However, due to the closeness between the CSN3 promoter region and the exon 1, this exon may be involved in the regulation of the expression of the gene. The coding region of the CSN3 gene comprises exon 3 and a majority of the exon 4. Two SNPs were localized in exon 4 of the equine CSN3 gene at position c.383A>T and c.517A>G that cause two aa substitutions (Hobor et

*Corresponding author. E-mail: mariaselvaggi@libero.it. Tel: +390805443836. Fax: +390805443925.

Abbreviations: aa, Amino acids; SNPs, single-nucleotide polymorphisms; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; CSN3, kappa-casein gene.

al., 2008).

A comparative analysis of gene sequences among horse, donkey and zebra was done only on a 400 bp long fragment belonging to exon 4 of CSN3 gene (Hobor et al., 2008). No data concerning CSN3/*Pst*I and CSN3/*Bse*YI gene polymorphisms in *Equus asinus* is available in literature yet.

In the past, donkey breeding was a useful economical activity in Italy; in fact a census taken in 1918 counted 949.162 heads, Italy being at the second place in Western Europe. The progressive reduction of demand for working donkeys, caused by the mechanization of agricultural activities, determined the strong decrease of its population. The fifth Italian agricultural census (ISTAT, 2006) reports an ass population of 19.325 heads. The FAO Institution (<http://dad.fao.org/en/Home.htm>) classifies the population with a strong risk of extinction, considering it a critical situation. Originally, from the phylogenetic point of view, Italian donkeys were divided into four ancestral breeds: Apulian, Sicilian, Pantelleria and Sardinian (Marchi and Mascheroni, 1925). The Apulian breed, was in the past the most wide spread in Italy, it includes the Calabrese, Basilicata, Leccese, Martina Franca, Marchigiana and Romagnolo populations. Martina Franca donkey derived from the Catalan donkey, was brought to Apulia at the time of the Spanish domination. This donkey has a dark coat; its mouth and the inside of the paws is grey. Martina Franca donkey is tall and well built and has good temperament. This donkey was used to carry cargoes by the alpine troops and adapted itself well to the territory difficulties. This breed has been greatly appreciated in the past for its elevated stature when compared to other donkey breeds, and it is considered useful for the production of hybrids. The typical breeding area is on Mediterranean woody scrubland at altitudes of more than 400 m s.l., with cold and rainy weather during winter and humid conditions in summer (Pagano et al., 1999). At present, asses are bred for amateur interest using a system of extensive or semi-extensive breeding (D'Alessandro et al., 2007). Nowadays, the Martina Franca donkey breed is included in the list of Italian endangered breeds drawn up by the Department of the Environment, Food and Rural Affairs (MiPAF, 2007).

No previous studies have investigated the polymorphisms in exon 1 of CSN3 gene in donkey population. Therefore, the aim of this study is to identify the variations in the exon 1 of the CSN3 gene in Martina Franca donkey breed.

MATERIALS AND METHODS

SNPs determination

A total of sixty-five (49 female and 16 male) donkeys belonging to the Martina Franca breed were included in this study. Individual blood samples for DNA genotyping were collected on K₂-ethylenediaminetetraacetic acid (EDTA) tubes and stored at -25°C.

Genomic DNA was isolated from whole blood using GFX Genomic Kit (Amersham, Germany). After DNA isolation, the samples were genotyped for the two SNPs in exon 1.

The primers were designed on the basis of the GenBank DNA sequence (EU429803) to amplify a part of the promoter region and a part of the exon 1 of CSN3 gene. The sequences of primers were as follow: CSN3_F (forward): 5'- GAT GAC AAC TCT ATT TCC CCC T -3'; CSN3_R (reverse): 5'- CCA GGG TCA GGT CTT GCT -3'. The 235 bp gene fragment, harboring both the SNPs, was amplified using thirty-six amplification cycles (94°C/1 min, 55°C/30 s and 72°C/45 s). The considered SNPs were genotyped by RFLP using two enzymes: *Pst*I (Fermentas; 2 units/10 µl) and *Bse*YI (NEB; 2 units/20 µl). After digestion (37°C, 4 h), the restriction fragments were analyzed using electrophoresis on a 2% agarose gel. The *Pst*I potentially cuts the 235 bp product into 189 and 46 bp fragments for allele G, while allele A remains uncut. The following restriction fragments were expected: 189 and 46 bp (GG genotype); 235, 189 and 46 bp (AG) and 235 bp (AA). The *Bse*YI cuts the amplicon into 215 and 20 bp fragments for the C allele, while allele A remains uncut. The possible genotype patterns were: 215 and 20 bp (CC); 235, 215 and 20 bp (CA) and 235 bp (AA). The allele frequencies were calculated by a simple allele counting.

RESULTS AND DISCUSSION

In the present study, PCR-RFLP with *Pst*I and *Bse*YI digestion was used to investigate the two polymorphisms in exon 1 of CSN3 gene in donkey. The basic finding of the current study was the absence of polymorphism at both the considered *loci* of CSN3 in Martina Franca donkey. At CSN3/*Pst*I *locus*, all the animals were genotyped as AA and no AG and GG animals were found in the population. A similar result was found at CSN3/*Bse*YI *locus*: all the donkeys were monomorphic and genotyped as AA. As a consequence, only one out of nine possible combined genotype (AAAA) was detected.

Table 1 illustrates the CSN3/*Pst*I and CSN3/*Bse*YI allelic frequencies in Martina Franca donkey breed and in different equine breeds as observed by other authors (Hobor et al., 2008; Selvaggi et al., 2010). Genetic polymorphism at the two considered *loci* has not been previously reported for donkey. Considering the results reported for some equine populations, the absence of polymorphism at CSN3/*Pst*I *locus* was reported only in three breeds: Ljutomer trotter, Lippizan and Slovenian haflinger, while no population was found to be monomorphic at CSN3/*Bse*YI *locus*.

A possible explanation for these findings may be the recent history connected with the strong reduction of number of Martina Franca donkeys (bottleneck effect) and, as a consequence, its restitution on the basis of a very limited number of animals that results in a high inbred and genetic uniformity of the population (founder effect).

The considered SNPs were located in a non-coding region. From this point of view, these two SNPs could be investigated also as useful instruments for population studies. Nevertheless, further studies should be carried out to analyze other donkey breeds in order to better clarify the reasons for polymorphisms absence.

Table 1. Allele frequencies of the two considered SNPs in Martina Franca donkey breed and in different equine breeds observed by other authors.

Breed	CSN3/ <i>Pst</i> I		CSN3/ <i>Bse</i> YI		References
	A	G	C	A	
Slovenian cold-blood	0.60	0.40	0.52	0.47	Hobor et al. (2008)
Ljutomer trotter	1.00	-	0.80	0.20	Hobor et al. (2008)
Slovenian haflinger	1.00	-	0.57	0.43	Hobor et al. (2008)
Posavina horse	0.82	0.18	0.55	0.45	Hobor et al. (2008)
Slovenian warm-blood	0.80	0.20	0.80	0.20	Hobor et al. (2008)
Lippizan	1.00	-	0.97	0.03	Hobor et al. (2008)
Murgese horse	0.80	0.20	0.74	0.26	Selvaggi et al. (2010)
Martina Franca donkey	1.00	-	1.00	-	Present work

REFERENCES

- D'Alessandro AG, Martemucci G, Casamassima D (2007). Behavioural profile during suckling period in Martina Franca breed jennies reared under semi-extensive conditions. *Appl. Anim. Behav. Sci.* 106(1-3): 107-124.
- Hobor S, Kunej T, Lenasi T, Dovc P (2006). Kappa Casein gene (CSN3) in horse. *Acta. Agric. Slov.* 88(2): 83-89.
- Hobor S, Kunej T, Dovc P (2008). Polymorphisms in the kappa casein (CSN3) gene in horse and comparative analysis of its promoter and coding region. *Anim. Genet.* 39(5): 520-530.
- Holt C (1992). Structure and stability of bovine casein micelles. *Adv. Protein Chem.* 43: 63-151.
- ISTAT http://www.census.istat.it/index_agricoltura.htm.
- Lenasi T, Rogelj I, Dovc P (2003). Characterization of equine cDNA sequences for α_{s1} , β and κ -casein. *J. Dairy Res.* 70(1): 29-36.
- Marchi E, Mascheroni E (1925). *Zootecnia Speciale* Ed. UTET, Torino. Italy. MiPAF <http://www.politicheagricole.it/SviluppoRurale/default>
- Pagano MC, Pelosi S, Franchi M, Scialandrone M (1999) Parametri biologici e morfologici in asini di Martina Franca. *Atti SIDI* 3: 47-51.
- Selvaggi M, Pesce Delfino AR, Dario C (2010). Exon 1 Polymorphisms in the Equine CSN3 Gene: SNPs Distribution Analysis in Murgese Horse Breed. *Anim. Biotechnol.* 21(4): 252-256.
- Yahyaoui MH, Angiolillo A, Pilla F, Sanchez A, Folch JM (2003). Characterization and Genotyping of the Caprine κ -Casein Variants. *J. Dairy Sci.* 86(10): 3726-3729.