

Variation in Salivary and Pancreatic Alpha-Amylase Genes in Italian Horse Breeds

BEATRICE COIZET, LETIZIA NICOLOSO, DONATA MARLETTA, ALESSANDRA TAMIOZZO-CALLIGARICH, GIULIO PAGNACCO, AND PAOLA CREPALDI

From the Dipartimento di Scienze Veterinarie e sanità pubblica, Università degli Studi di Milano, via Celoria 10, 20133 Milano, Italy (Coizet, Nicoloso, Pagnacco, and Crepaldi); the Dipartimento di Scienze delle Produzioni Agrarie e Alimentari, Università degli Studi di Catania, Catania, Italy (Marletta); and the Nutritional Equine Consultant, San Bernardino Verbano (VB), Italy (Tamiozzo).

Address correspondence to Dr Beatrice Coizet at the address above, or e-mail: beatrice.coizet@unimi.it.

Data deposited at Dryad: <http://dx.doi.org/doi:10.5061/dryad.m3v72>

The dietary demand of the modern horse relies on high-cereal feeding and limited forage compared with natural grazing conditions, predisposing the horse to several important diseases. Salivary and pancreatic alpha-amylases (coded by *AMY1* and *AMY2* genes, respectively) play a crucial role in carbohydrate digestion in nonruminants, but little is known about these 2 genes in the horse. Aim of this work has been to distinguish genomic sequences of horse *AMY1* and *AMY2* genes and to analyze any polymorphisms in breeds historically characterized by marked differences in nutritional management. A single nucleotide polymorphism detection was performed and 7 novel single nucleotide polymorphisms were found. Three single nucleotide polymorphisms are in exons and were genotyped in 112 horses belonging to 6 breeds. One single nucleotide polymorphism in *AMY1* gene distinguished Haflinger and the Italian native Murgese from the other breeds, whereas both the single nucleotide polymorphisms in *AMY2* gene showed different allelic frequencies in Friesian compared with the other breeds. These differences are confirmed by quite high fixation index (*F_{st}*) values for these 2 nonsynonymous single nucleotide polymorphisms. These preliminary results highlight marked divergences in allele frequencies of *AMY1* and *AMY2* genes, involved in starch digestion, between horse breeds characterized by different histories of selection, thus providing first indications of possible relations between genetics and nutritional management.

Subject areas: *Conservation genetics and biodiversity; Molecular adaptation and selection*

Key words: *genetic diversity, SNP, starch digestion*

The dietary demand of modern competition horse varies considerably compared with natural grazing conditions.

In order to provide enough energy for work and performance, domestic horses are fed diets supplemented with readily hydrolyzable carbohydrates, which differ substantially from feral equid continually browsing for fiber-rich, low-starch pasture grasses (Durham 2009). The equine gastrointestinal tract is not evolutionarily adapted to concentrate diets, which have been identified by epidemiological and clinical studies as important risk factors for several horse diseases. Among the most common are osteochondritis dissecans, a developmental orthopedic disease (Huntington and Pagan 2008); equine metabolic syndrome, which increases the risk of laminitis (Frank and Andrews 2007); recurrent equine rhabdomyolysis, an inherited muscle disorder (Finno et al. 2010); polysaccharide storage myopathy, resulting from a hypersensitivity of the muscle to insulin (Borgia et al. 2011); equine gastric ulcer syndrome, whose prevalence decreases in horses grazing at pasture thanks to the continuous flow of saliva that buffers stomach acid (Buchanan and Andrews 2003). Moreover, excessive hydrolyzable carbohydrates intake leads to gastrointestinal disturbances, which can precede colic, the most frequent cause of emergency treatment and death in horses (Shirazi-Beechey 2008).

The main glycemic carbohydrate component in today's concentrate horse diet is starch, which is mainly hydrolyzed by salivary and pancreatic α -amylases, coded by *AMY1* and *AMY2* genes, respectively. Concentration of α -amylase enzyme in the equine intestine is low compared with other species and its activity and expression is significantly enhanced when horses are fed high-grain diets compared with horses on pasture, with a wide variation between individuals (Kienle et al. 1994; Dyer et al. 2002), the latter reflecting a likely genetic variability in genes coding for these enzymes. The primary rate limiting step in the increased glucose absorptive capacity of equine small intestine is the inability to hydrolyze starch to glucose rapidly (Shirazi-Beechey 2008), which

is largely dependent on the amylase activity. In man, copy number of salivary α -amylase gene has been correlated positively with salivary amylase protein level and individuals from populations with high-starch diets showed, on average, more *AMY1* copies than those with traditionally low-starch diets (Perry et al. 2007). More recent evidences showed that individuals with high level activity of salivary α -amylase, having significantly more *AMY1* gene copies within their genomes, may be better adapted to ingest starches, whereas individuals with lower activity levels of this protein and with less copies of *AMY1* gene may be at greater risk for insulin resistance and diabetes if chronically ingesting starch-rich diets (Mandel and Breslin 2012).

Little is known about horse *AMY1* and *AMY2* genes, which are still not well distinguished at a genomic level. Aim of this work has been to differentiate horse *AMY1* and *AMY2* genes at a genomic level and to analyze their polymorphism in cosmopolite and Italian native horse breeds, historically characterized by marked differences in feeding and management conditions.

Materials and Methods

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total RNA extraction was performed from pancreas and salivary glands tissue samples, where the amylase genes are differentially expressed in mammals. Tissue samples were collected from 1 animal at an authorized, commercial, EU-licensed abattoir located in Italy, following the recommendations of the European Council (1986) concerning animal care. Small sections of pancreas and salivary glands, approximately 1 cm in length, were collected and frozen immediately in liquid nitrogen. Following transportation to the laboratory, frozen tissue samples were stored at -80°C until use. The RNA isolation was performed using a commercially available kit (Promega SV Total RNA Isolation System), according to the manufacturer's instructions. Reverse transcription–polymerase chain reaction (RT–PCR) against total RNA isolated was performed using Fermentas First Strand cDNA Synthesis kit, with M-MuLV Reverse Transcriptase (20U/ μL) and random primers. PCR amplification on complementary DNAs (cDNAs) obtained was carried out using primers (forward primer: 5'-ATTTGGAGGGGTTTCAGGTCT-3' and reverse primer: 5'-CCCAACCAGAGTATGGGACT-3') designed to be specific for the 2 mRNA sequences available in database for horse (National Center for Biotechnology Information), both called *a-amylase 2B-like* (accession numbers XM_001487859 and XM_001491728). PCR products were purified and sequenced. The sequences were aligned using BioEdit software (Hall 1999), allowing the distinction of the 2 genes at cDNA level.

Single Nucleotide Polymorphism Discovery

Blood samples from horses belonging to 3 breeds historically characterized by different systems of breeding and

selection—Thoroughbred, Purebred Arabian, and Friesian—were collected at 3 different farms located in north of Italy (Supplementary Table 2 online). Genomic DNA was extracted from blood, 2 samples per breed, and pooled. Extraction was performed using a commercial kit (Promega Wizard Genomic DNA Purification Kit) following the manufacturer's instructions.

Based on the finding of the previous analysis performed on RNA from tissue samples collected at the abattoir, primer pairs for the amplification of all 10 exons were designed to be specific for the *AMY1* and *AMY2* genes, allowing the amplification of the entire, specific, coding sequence and nearby intron regions for both these genes. All primer pairs with annealing temperatures are reported in Supplementary Information Items online. Single nucleotide polymorphism (SNP) detection was performed on 1 DNA pool per breed. PCR products obtained were purified and sequenced; fragments were aligned for SNP discovery. The analysis was performed also in intron regions as it is well established that noncoding SNPs may have effects on important mechanisms such as transcription, translation, and splicing.

SNP Genotyping and Breeds Studied

Genotyping of 3 out of 7 SNPs found was carried out on genomic DNA extracted from blood samples. The analysis was performed in outsourcing, exploiting the KASPar SNP genotyping system developed and patented by LGC Genomics (www.lgcgenomics.com).

SNP genotyping was performed on 112 total horses from Friesian (FRI), Sanfratellano (SF), Indigeno Siciliano (IND), and Murgese (MUR), with the addition of Haflinger (HAF) and Sella Italiano (SI) breeds. The samples analyzed, reported in Supplementary Table 3 online, were all taken in Italy at different farms and are well representative of the breeds analyzed. Three breeds among those studied are autochthonous populations of the south of Italy. Sanfratellano is a native Sicilian breed, well adapted to harsh conditions. This meso-dolicomorph saddle horse is characterized by a uniform blackish or bay coat and is mainly employed in light draft and equestrian tourism (Zuccaro et al. 2008). The origin of Sanfratellano horse can be traced back to the Middle Ages (700–1200 A.D.), when native Sicilian horses were crossed with North African, Oriental and, later, Iberian populations (Zuccaro et al. 2008). Limited introgression of Thoroughbred and Oriental Stallions was practiced in 1925 to improve the morphological structure of Sanfratellano (Hendricks 2007). More recently, Maremmano stallions, a native horse breed originated in Tuscany and now bred throughout Italy, were used in planned mating to improve withers height and size (Chiofalo et al. 2003).

Sicilian Indigenous is probably derived from a primitive strain of Sicilian horse and from the Borbon Real Casa di Ficuzza breed (related to the Napoletano, Persano, and Arab breeds), and today it can be considered an Anglo-Oriental half-breed (Zuccaro et al. 2008).

The black or rarely roan Murgese is a mesomorph horse, recently selected for the saddle and harness (Silvestrelli 2001). This horse originates in the arid and rocky hills of Apulia and it has a rustic nature necessary for survival in such a difficult environment, characterized by harsh climate (cold in the winter, hot and dry in the summer), poor pastures, and the presence of enzootic pathogens (Pieragostini et al. 2005). The official denomination of the breed and the first “individuals” registration were established in 1926. The existing Murgese horses can be traced back to a founder group constituted by 46 mares and 9 stallions (Buonavolontà and Silvestrelli 1986).

The other breeds analyzed are distributed over a larger geographical area. The Friesian is a horse breed originating in Friesland, the Netherlands. Important characteristics of this breed are its size and endurance, as demonstrated by its historical use. Ancestors of the modern Friesians were used in medieval times as heavy war horses, carrying knights in armor. Later, battle arms changed and Andalusian introgression was observed, lightening their weights and thereby rendering them more suitable (in terms of less food intake and waste output) for work as more urban carriage horses (Hyland 1990). Today, Friesian horses are reared in both Europe and North America. The Haflinger, the most numerous horse breed in Italy, was developed in Austria and northern Italy during the late 19th century (Samore et al. 1997). Haflinger horses are relatively small, always chestnut in color, have distinctive gaits described as energetic but smooth, and are well muscled yet elegant. The breed was developed for use in mountainous terrain, thanks to the hardiness of these animals. Their current conformation and appearance are the result of introgression of Arabian and various European breeds in the original native Tyrolean ponies. In the postwar era, the Haflinger was indiscriminately crossed with other breeds. However, in 1946, breeders focused on producing purebred Haflingers, and a closed stud book was created. Today the Haflinger is found all over the world, although the majority of breeding stock still comes from Austria.

Finally, Sella Italiano is a breed formed in Italy from crosses of Italian native horses with mainly Arabian and Thoroughbred to produce a sport horse with attitude for show jumping and dressage.

In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad.

Results

Through the alignment of cDNA sequences, obtained with the RT-PCR analysis on RNA extracted from tissue samples, we were able to design primers to have sequence mismatches with salivary and pancreatic amylase genes at genomic DNA level. The following PCR and sequencing analyses performed allowed the distinction of the 2 amylase sequences reported in database as “ α -amylase 2B-like,” identifying as *AMY1* gene the sequence reported with accession no. XM_001491728 and *AMY2* gene the sequence with accession no. XM_001487859. The complete coding region of *AMY1* and *AMY2* genes was characterized in 10 horses belonging to different breeds by sequencing little more than 3300 bp, encompassing all 10 exons and nearby intron regions (for primers used and annealing temperatures, see [Supplementary Material](#) online). These sequences have been submitted to GenBank (accession numbers JX418227 for *AMY1* and JX418228 for *AMY2*).

SNP discovery analysis allowed the identification of a total of 7 novel SNPs. Six markers are located in *AMY1* gene and 3 are in exons, of which 1 is nonsynonymous and 1 nonsynonymous SNP was identified in exon 1 of *AMY2* gene (Table 1).

The 3 coding SNPs were genotyped in 112 horses belonging to Friesian, Sanfratellano, Indigeno Siciliano, and Murgese, with the addition of Haflinger and Sella Italiano breeds samples (Table 3). The SNP analyzed are described in Tables 2 and 3. At the *AMY1* g.1963A>G locus, the alanine allele resulted in fixation in Friesian and Haflinger breeds. The guanine allele was rare in the other populations studied, except for Murgese and Sanfratellano, which showed little higher frequencies, equal to 0.11 and 0.18, respectively, for G allele at this locus. Fst level, which describes the amount of inbreeding-like effects within subpopulations, resulted low for this polymorphism, whereas it showed higher values for the other 2 coding SNPs analyzed (Table 2).

In the other polymorphism in *AMY1* gene, the cytosine resulted the major allele in the analyzed samples of Haflinger and Murgese breeds, whereas it was the less frequent in the other populations analyzed. The alanine allele of *AMY2* g.174A>G locus was absent in Sanfratellano and rare in Haflinger, Indigeno Siciliano, and Murgese (minor allele frequency [MAF] < 0.05), whereas Sella Italiano breed showed little higher frequency, equal to 0.12, that is still low. Friesian

Table 1 Characteristics of 7 SNPs found in horse *AMY1* and *AMY2* genes in 3 cosmopolite breeds historically characterized by different systems of breeding and selection (Thoroughbred, Purebred Arabian, and Friesian), 2 samples per breed, collected in Italy

SNP name	Gene	Location	Amino acid change	SNP identification ^a
g.289G>A	<i>AMY1</i>	Intron 1–2	—	ss539238874
g.1924T>C		Exon 3	No	ss539238875
g.1963A>G		Exon 3	No	ss539238876
g.6261A>C		Exon 8	Asn>His	ss539238884
g.6381T>C		Intron 8–9	—	ss539238885
g.6617C>T	<i>AMY2</i>	Intron 9–10	—	ss539238886
g.174G>A		Exon 1	Arg>Glu	ss539238888

^aIdentification numbers deposited in the National Center for Biotechnology Information database.

Table 2 Population genetics parameters: MAFs, expected and observed heterozygosity (Het exp and Het obs), and *F*_{st} at 3 SNPs found in *AMY1* and *AMY2* genes genotyped on 112 total horses from Friesian (FRI), Sanfratellano (SF), Indigeno Siciliano (IND), Murgese (MUR), Haflinger (HAF), and Sella Italiano (SI) breeds

Gene	SNP	Region	Minor allele	MAF	N. observations	Het exp	Het obs	<i>F</i> _{st}
<i>AMY1</i>	g.1963A>G	Exon 3	G	0.059	110	0.111	0.082	0.062
<i>AMY1</i>	g.6261A>C	Exon 8	C	0.400	105	0.480	0.381	0.251
<i>AMY2</i>	g.174A>G	Exon 1	A	0.104	111	0.186	0.171	0.257

The samples analyzed were all taken in Italy at different farms.

showed a completely different trend in allelic frequencies compared with all the other populations analyzed, with MAF equal to 0.42. The analyzed population resulted in Hardy–Weinberg equilibrium ($P = 0.315$) for this SNP with balanced genotypic frequencies.

Discussion

Salivary and pancreatic α -amylases, the major enzymes in starch digestion in nonruminants, are endoglycosidases that catalyze the hydrolysis of α -(1,4)-glycosidic bonds of glucose polymers with retention of the anomeric configuration and belong to the family 13 of glycosidases (Henrissat 1991). In man, the architecture of salivary amylase consists of 3 structural domains, domain A (residues 1–99, 170–404), domain B (residues 100–169), and domain C (residues 405–496). The domain A contains the 3 catalytic residues Asp197, Glu233, and Asp300 and the chloride binding site (Ramasubbu et al. 1996). Also in porcine pancreatic α -amylase, the 3 residues Asp197, Glu233, and Asp300 close together in the active site (Qian et al. 1993). The salivary and pancreatic α -amylase enzymes are highly homologous in mammals, with a sequence identity of more than 99% in man (Brayer et al. 1995) and more than 96% in horse. Also *AMY1* and *AMY2* genes are highly related, with similar intron/exon boundaries. In man, there is 98% nucleotide sequence identity over their coding regions (Hori et al. 1987). In the National Center for Biotechnology Information database, 2 sequences can be found for horse species, both reported as “ α -amylase 2B-like,” showing an identity of more than 97% in coding regions. Through the analysis performed on RNA extracted from tissue samples, we were able to distinguish these 2 sequences. After this distinction, we characterized complete coding region of *AMY1* and *AMY2* genes in 10 horses belonging to different breeds. SNP discovery analysis performed on horse samples belonging to 3 cosmopolite breeds (Thoroughbred, Purebred Arabian, and Friesian) and 3 southern Italian native breeds (Sanfratellano, Indigeno Siciliano, and Murgese) allowed the identification of several SNPs. The noncoding SNPs found in present work, all located in *AMY1*, are not placed in splice donor or splice acceptor sites of the gene. Concerning the nonsynonymous variations, the SNP found in exon 8 of *AMY1* gene implies the amino acid change from histidine to asparagine. Histidines are the most common amino acids in protein active or binding sites and it is not so frequent to see them exchanged for any amino acid (Barnes and Gray 2003).

Asparagine is a polar amino acid, quite frequently involved in protein active or binding sites. This position corresponds to the human Asp373 residue, which is part of the A structural domain of the protein, spanning from 1 to 99 and from 170 to 404 residues (Ramasubbu et al. 2004).

The missense SNP located in *AMY2* gene determines the exchange of an arginine with a glutamine. Both are polar amino acids, frequently involved in protein active or binding sites (Barnes and Gray 2003). The wild-type arginine is included in the A domain of the protein and this position seems to be highly conserved between species. In fact, arginine residue can be also found at the corresponding site in man, bovine, and pig amylases. Moreover, the corresponding human Arg20 is part of 2 salt bridges, contributing stability to the conformation of protein: the first, ranging from residues 20–33, is important for the geometrical regularity of helical and β -strand segment structures, whereas the second, comprising residues 20–23, creates a bulge near the N-terminus of the helical segment in man (Ramasubbu et al. 1996).

Genotyping analysis provided interesting differences between the analyzed breeds. Haflinger, together with Friesian, can be included in the cold-blooded breeds (van de Goor et al. 2011). This similarity can be observed in allelic frequencies for *AMY1* g.1963A>G locus, with the alanine allele fixed in both these 2 breeds, whereas polymorphic in the other. However, although Haflinger was originally selected as a draft horse for agricultural work, it subsequently underwent a selection oriented toward a saddle horse for riding purpose, and the progeny of a few Arabian horses were in the past introduced into the Italian Haflinger (Gandini et al. 1997). This trend has been also confirmed by morphological evidences highlighting the evolution of these horses toward a lighter type, more suitable for saddle (Falaschini et al. 2003). These characteristics should distinguish this breed from Friesian, which have a completely different history of selection. The guanine allele showed very low frequencies also in the other populations studied, with some differences. The divergence observed between the 2 Sicilian breeds, Sanfratellano and Indigeno Siciliano, is notable considering their close relationship, demonstrated in a previous study based on genetic distances and cluster analysis (Guastella et al. 2011) and in accordance with their history. On the other hand, our results showed a low *F*_{st} level for this polymorphism, which means that it is not particularly informative for the distinction of the analyzed breeds. The high prevalence of alanine allele observed at this locus in all the populations studied could be an indication of evolutionary selection of phenotype. In fact,

Table 3 Allele frequencies per breed at 3 SNPs found in *AMY1* and *AMY2* genes genotyped on 112 total horses from Friesian (FR), Sanfratellano (SF), Indigeno Siciliano (IND), Murgese (MUR), Haflinger (HAF), and Sella Italiano (SI) breeds

Breed	Gene	Marker	Friesian (N = 20)		Haflinger (N = 20)		Indigeno Siciliano (N = 15)		Murgese (N = 20)		Sanfratellano (N = 20)		Sella Italiano (N = 17)	
			Allele	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count	Frequency	Count
<i>AMY1</i>	g.1963A>G	A	40	1.00	40	1.00	29	0.97	32	0.89	33	0.83	33	0.97
		G	0	0.00	0	0.00	1	0.03	4	0.11	7	0.18	1	0.03
<i>AMY1</i>	g.6261A>C	A	36	0.90	12	0.33	21	0.70	6	0.20	29	0.73	22	0.65
		C	4	0.10	24	0.67	9	0.30	24	0.80	11	0.28	12	0.35
<i>AMY2</i>	g.174A>G	A	16	0.42	1	0.03	1	0.03	1	0.03	0	0.00	4	0.12
		G	22	0.58	39	0.98	29	0.97	39	0.98	40	1.00	30	0.88

The samples analyzed were all taken in Italy at different farms. Fixed alleles are highlighted in bold.

it is demonstrated that minor allele frequencies tendency to be lower in nonsynonymous SNPs is a strong indication that these replacement polymorphisms are deleterious (Cargill et al. 1999) or at least somewhat undesirable.

In the case of the other *AMY1* polymorphism, the 2 Sicilian breeds were in accordance as expected, whereas Murgese, another native Southern Italian breed, showed an opposite trend. Also Friesian and Haflinger breeds behave differently and did not share the minor allele. Relying on the allelic frequencies of the nonsynonymous SNP located in *AMY2* gene, Haflinger behave as the Southern Italian native breeds, in accordance to the genetic contribution of the Arabian horse they share. Interestingly, Friesian showed a completely different trend compared with all the other populations analyzed, having both allelic and genotypic frequencies balanced at this locus. This does not reflect other results found in literature and showing reduced number of alleles and low levels of heterozygosity for Friesian (Luis et al. 2007). In a phylogenetic study performed on horses belonging to 35 populations (van de Goor et al. 2011), authors found that Friesian, by its genetic isolation, was the most inbred population between those studied. The Friesian has experienced a severe bottleneck in recent times with the number of breeding stallions reduced to just 3 after World War II (Hendricks 2007). The breed was developed in the northern Netherlands, and it is said to have descended from the primitive “forest horse.” This definition belongs to a theory about the origin of horse domestication, postulating 3 primitive horse types, considered subspecies of *Equus caballus*, as ancestors of modern breeds (Edwards 1995). They were the “Forest Horse,” *E. caballus germanicus*, descendant of a “Diluvial Horse,” *E. caballus sylvaticus*; the Asiatic Wild Horse or Przewalski horse, then considered *E. caballus przewalskii* and the Tarpan, then considered *E. caballus gmelini*. In a work published in 2001, authors stated that domestication of the stallion most likely occurred only once, while wild mares of various regions were included in local domesticated herds (Vila et al. 2001). More recent genetic evidence suggested that, unlike other agricultural species such as sheep (Pedrosa et al. 2005) and pigs (Larson et al. 2005), multiple horse domestication events occurred (Cieslak et al. 2010; Lippold et al. 2011; Achilli et al. 2012) in a geographically defined area in the Western Eurasian steppe as suggested by archaeology (Warmuth et al. 2012).

Conclusions

In the current work, horse *AMY1* and *AMY2* genes, involved in digestion of carbohydrates, particularly starch, were characterized at genomic level. Three SNPs identified in coding sequence of the genes were analyzed in horses belonging to breeds characterized by different histories of breeding and selection. Different allelic frequencies and quite high *F_{st}* values were observed/detected. This work provides first indications of the possible relations between genetics and nutritional management, underlying marked differences in allele frequencies of genes playing a fundamental role in

starch digestion, between breeds characterized by different histories of selection. Genomics and nutrigenomics are new fascinating and fundamental aspects, which, with the recent provision of the equine complete genomic sequence and the Equine SNP50 genotyping chip tool, deserve to be deeply investigated also in horse species.

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

Funding

Programma dell'Università per la Ricerca – PUR 2009, Università degli Studi di Milano.

Acknowledgments

We are grateful to Dr A. Carnevali (University of Milan, Italy) for collecting tissue samples and to Dr S. Chessa (IBBA-CNR), Professor A. Caroli (University of Brescia, Italy), Professor E. Pieragostini (University of Bari Aldo Moro, Italy), and Dr E. Tuccia, as well as all the breeders, for providing the blood samples analyzed in the present work. We also acknowledge the ATZ project, supported by the administration of the Apulian Region, for allowing the Murgesse breed samples collection. The tissue samples analyzed in present work were collected at an authorized, commercial, EU-licensed abattoir, following the recommendations of the European Council (1986) concerning animal care. Blood samples were collected by a veterinarian within the routine tests carried out at the farm, with the consent of the breeders and in compliance with relevant national and international regulations and animal welfare requirements.

References

- Achilli A, Olivieri A, Soares P, Lancioni H, Hooshar Kashani B, Perego UA, Nergadze SG, Carossa V, Santagostino M, Capomaccio S, et al. 2012. Mitochondrial genomes from modern horses reveal the major haplogroups that underwent domestication. *Proc Natl Acad Sci USA*. 109:2449–2454.
- Baker CS. 2013. Journal of Heredity adopts joint data archiving policy. *J Hered*. 104:1.
- Barnes MR, Gray IC. 2003. *Bioinformatics for geneticists*. West Sussex (UK): John Wiley & Sons.
- Borgia L, Valberg S, McCue M, Watts K, Pagan J. 2011. Glycaemic and insulinaemic responses to feeding hay with different non-structural carbohydrate content in control and polysaccharide storage myopathy-affected horses. *J Anim Physiol Anim Nutr*. 95:798–807.
- Brayer GD, Luo YG, Withers SG. 1995. The structure of human pancreatic alpha-amylase at 1.8 angstrom resolution and comparisons with related enzymes. *Protein Sci*. 4:1730–1742.
- Buchanan BR, Andrews FM. 2003. Treatment and prevention of equine gastric ulcer syndrome. *Vet Clin North Am Equine Pract*. 19:575–597.
- Buonavolontà G, Silvestrelli M. 1986. *Il Murgesse*. Milano (Italy): Equestri.
- Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Shaw N, Lane CR, Lim EP, Kalyanaraman N, et al. 1999. Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet*. 22:231–238.
- Chiofalo L, Portolano B, Liotta L, Sotera AR, Finocchiaro R. 2003. Demographic characterization, inbreeding and genetic variability within Sanfratellano population horse from genealogical data. *Ital J Anim Sci*. 2:592–594.
- Cieslak M, Pruvost M, Benecke N, Hofreiter M, Morales A, Reissmann M, Ludwig A. 2010. Origin and history of mitochondrial DNA lineages in domestic horses. *PLoS One*. 5:e15311.
- Durham AE. 2009. The role of nutrition in colic. *Vet Clin North Am Equine Pract*. 25:67–78.
- Dyer J, Fernandez-Castaño Merediz E, Salmon KS, Proudman CJ, Edwards GB, Shirazi-Beechey SP. 2002. Molecular characterisation of carbohydrate digestion and absorption in equine small intestine. *Equine Vet J*. 34:349–358.
- Edwards EH. 1995. *The encyclopedia of the horse*. London: Dorling Kindersley.
- Falaschini A, Rizzi S, Pasquini M. 2003. Morphological evolution of the Haflinger horse. *Ital J Anim Sci*. 2:595–597.
- Finno CJ, McKenzie E, Valberg SJ, Pagan J. 2010. Effect of fitness on glucose, insulin and cortisol responses to diets varying in starch and fat content in Thoroughbred horses with recurrent exertional rhabdomyolysis. *Equine Vet J*. 42:323–328.
- Frank N, Andrews FM. 2007. Equine metabolic syndrome. Proceedings of the 29th Bain Fallon Memorial Lectures: Behaviour, Lameness and Medicine; 2007 Jul 1–7; Fremantle, Western Australia, Australia. St Leonards (Australia): Equine Veterinarians Australia.
- Gandini GC, Samore A, Pagnacco G. 1997. Genetic contribution of the Arabian to the Italian Haflinger horse. *J Anim Breed Genet*. 114:457–464.
- Guastella AM, Zuccaro A, Criscione A, Marletta D, Bordonaro S. 2011. Genetic analysis of sicilian autochthonous horse breeds using nuclear and mitochondrial DNA markers. *J Hered*. 102:753–758.
- Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser*. 41:95–98.
- Hendricks BL. 2007. *International encyclopedia of horse breeds*. 1st ed. Norman (OK): University of Oklahoma Press.
- Henrissat B. 1991. A classification of glycosyl hydrolases based on amino-acid-sequence similarities. *Biochem J*. 280:309–316.
- Horii A, Emi M, Tomita N, Nishide T, Ogawa M, Mori T, Matsubara K. 1987. Primary structure of human pancreatic alpha-amylase gene: its comparison with human salivary alpha-amylase gene. *Gene*. 60:57–64.
- Huntington P, Pagan J. 2008. Nutritional management of equine metabolic disorders. *Aust Equine Vet*. 27:40–44.
- Hyland A. 1990. *Equus: the horse in the Roman world*. New Haven (CT): Yale University Press.
- Kienzle E, Radicke S, Landes E, Kleffken D, Illenseer M, Meyer H. 1994. Activity of amylase in the gastrointestinal-tract of the horse. *J Anim Physiol Anim Nutr*. 72:234–241.
- Larson G, Dohney K, Albarella U, Fang M, Matisoo-Smith E, Robins J, Lowden S, Finlayson H, Brand T, Willerslev E, et al. 2005. Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. *Science*. 307:1618–1621.
- Lippold S, Matzke NJ, Reissmann M, Hofreiter M. 2011. Whole mitochondrial genome sequencing of domestic horses reveals incorporation of extensive wild horse diversity during domestication. *BMC Evol Biol*. 11:328.
- Luis C, Juras R, Oom MM, Cothran EG. 2007. Genetic diversity and relationships of Portuguese and other horse breeds based on protein and microsatellite loci variation. *Anim Genet*. 38:20–27.
- Mandel AL, Breslin PAS. 2012. High endogenous salivary amylase activity is associated with improved glycemic homeostasis following starch ingestion in adults. *J Nutr*. 142:853–858.
- Pedrosa S, Uzun M, Arranz JJ, Gutiérrez-Gil B, San Primitivo F, Bayón Y. 2005. Evidence of three maternal lineages in Near Eastern sheep supporting multiple domestication events. *Proc Biol Sci*. 272:2211–2217.
- Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H, Redon R, Werner J, Villanea FA, Mountain JL, Misra R, et al. 2007. Diet and the evolution of human amylase gene copy number variation. *Nat Genet*. 39:1256–1260.

- Pieragostini E, Rizzi R, Bramante G, Rosati A, Perrotta G, Caroli A. 2005. Genetic study of Murghese horse from genealogical data and microsatellites. *Ital J Anim Sci.* 4:197–202.
- Qian MX, Haser R, Payan F. 1993. Structure and molecular-model refinement of pig pancreatic alpha-amylase at 2.1 angstrom resolution. *J Mol Biol.* 231:785–799.
- Ramasubbu N, Paloth V, Luo YG, Brayer GD, Levine MJ. 1996. Structure of human salivary alpha-amylase at 1.6 angstrom resolution: implications for its role in the oral cavity. *Acta Crystallogr Sect D Biol Crystallogr.* 52:435–446.
- Ramasubbu N, Sundar K, Ragunath C, Rafi MM. 2004. Structural studies of a Phe256Trp mutant of human salivary alpha-amylase: implications for the role of a conserved water molecule in enzyme activity. *Arch Biochem Biophys.* 421:115–124.
- Samore AB, Pagnacco G, Miglior F. 1997. Genetic parameters and breeding values for linear type traits in the Haflinger horse. *Livest Prod Sci.* 52:105–111.
- Shirazi-Beechey SP. 2008. Molecular insights into dietary induced colic in the horse. *Equine Vet J.* 40:414–421.
- Silvestrelli M. 2001. The role of the horse in rural policies—from an Italian perspective. *Proc. Eur. Conf. Equus 2001*; Skara, Sweden. p. 1–14.
- van de Goor LHP, van Haeringen WA, Lenstra JA. 2011. Population studies of 17 equine STR for forensic and phylogenetic analysis. *Anim Genet.* 42:627–633.
- Vila C, Leonard JA, Gotherstrom A, Marklund S, Sandberg K, Liden K, Wayne RK, Ellegren H. 2001. Widespread origins of domestic horse lineages. *Science.* 291:474–477.
- Warmuth V, Eriksson A, Bower MA, Barker G, Barrett E, Hanks BK, Li S, Lomitashvili D, Ochir-Goryaeva M, Sizonov GV, et al. 2012. Reconstructing the origin and spread of horse domestication in the Eurasian steppe. *Proc Natl Acad Sci USA.* 109:8202–8206.
- Zuccaro A, Bordonaro S, Criscione A, Guastella AM, Perrotta G, Blasi M, D'Urso G, Marletta D. 2008. Genetic diversity and admixture analysis of Sanfratellano and three other Italian horse breeds assessed by microsatellite markers. *Animal.* 2:991–998.

Received September 20, 2013; First decision December 3, 2013;
Accepted January 14, 2014

Corresponding Editor: Ernest Bailey