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Analysis of single nucleotide polymorphisms in major and candidate genes for production traits in Nero Siciliano pig breed

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ABSTRACT

Nero Siciliano (NS; Sicilian Black) is a local pig breed reared on the island of Sicily mainly under extensive management. The breed is well adapted to marginal conditions and is appreciated for its reproductive performance, disease resistance and production of tasty meat. For a genetic characterization of this breed we analyzed the allele frequencies of single nucleotide polymorphisms (SNPs) in eight major or candidate genes (ryanodine receptor 1, *RYR1*; Na^+, K^+ ATPase subunit α 2, *ATP1A2*; myosin heavy chain 2B, *MYH4;* sarcolipin, *SLN*; cathepsin B, *CTSB*; cystatin B, *CSTB*; estrogen receptor, *ESR*; melanocortin receptor 1, *MC1R*) for performance and phenotypic traits. The animals that were sampled and analyzed represent about 6-8% of the total NS pig population. PCR-RFLP or PCR-SSCP techniques were used to type the DNA markers in the selected loci. Exact test of Hardy-Weinberg equilibrium was computed for each locus, F_{is} statistics and heterozygosity were calculated for each locus and over all loci. Allele frequencies obtained in NS breed were compared to the frequencies already available in literature for the Large White, Landrace, Duroc, Belgian Landrace, Piétrain, Hampshire and Meishan breeds. For the *ESR* locus, as no information on the distribution of the two alleles were available, we typed a sample of unrelated pigs from the considered breeds.
Even if only eight loci were studied in NS breed, important elements were obtained from the data. The *1843T* (*n*) allele at the *RYR1* locus is present in NS breed, thus the molecular test to identify the carriers of this allele should be adopted to avoid its spreading in the population. Moreover, other studies are needed to clarify the allelic structure of the *MC1R* gene, which affects coat color, in order to evaluate if this gene could be used in genetic tests for the traceability of the meat products of this breed. Finally, the present work represents an attempt to evaluate data on mutations within major and candidate genes with the final aim to provide information that could be useful for the conservation and valorization of local farm animal genetic resources.

Key words: Nero Siciliano, Pig breeds, SNP, Allele frequency, Genetic diversity.

RIASSUNTO

ANALISI DI ALCUNE MUTAZIONI PUNTIFORMI IN GENI MAGGIORI E GENI CANDIDATI PER CARATTERI PRODUTTIVI NELLA RAZZA SUINA NERO SICILIANO.

Il Suino Nero Siciliano rappresenta una razza allevata allo stato brado in Sicilia prevalentemente nella zona dei Monti Nebrodi e delle Madonie nelle province di Messina e Palermo. La razza costituisce un'interessante risorsa genetica in

quanto è ben adattata all'ambiente in cui viene allevata. Con l'obiettivo di caratterizzare dal punto di vista genetico questa razza, per un campione di 119 suini abbiamo studiato il polimorfismo di otto geni (recettore della rianodina 1, RYR1; Na^+, K^+ ATPasi subunità α 2, ATP1A2*; miosina catena pesante 2B,* MYH4*; sarcolipina,* SLN*; catepsina B,* CTSB*; cistatina B,* CSTB*; recettore degli estrogeni,* ESR*; recettore 1 della melanocortina,* MC1R*). Questi geni sono stati scelti perché alcune mutazioni influenzano direttamente caratteristiche produttive o fenotipiche quali qualità della carne e della carcassa e il colore del mantello (rispettivamente* RYR1 *e* MC1R*) oppure perché segnalate in letteratura come associate a caratteri legati alla qualità della carcassa (*ATP1A2 *e* CTSB*), all'accrescimento (*MYH4 *e* CSTB*) e a caratteristiche riproduttive (*ESR*). Il gene* SLN *è stato selezionato perché, in base alla sua funzione e alla sua posizione di mappa può essere considerato come gene candidato per l'accrescimento e l'adiposità della carcassa. L'analisi di queste mutazioni è stata effettuata utilizzando le tecniche di PCR-RFLP e PCR-SSCP. Per ogni locus è stato analizzato l'equilibrio di Hardy-Weinberg, calcolati F_{is} ed eterozigosità (H). F_{is} e H sono stati calcolati anche considerando l'insieme dei loci. Fra i diversi geni studiati, il gene SLN è risultato significativamente non in equilibrio a causa dell'eccesso di omozigoti (F_{is} = + 0,554). L'analisi di tutti i loci ha mostrato che la popolazione presenta, in generale, un eccesso di omozigoti, sebbene il dato non sia statisticamente significativo. Per il* locus MC1R *sono state analizzate le mutazioni in due codoni, mettendo in evidenza il completo* linkage disequilibrium *tra i due siti polimorfi. Le frequenze alleliche nel Suino Nero ai diversi* loci *analizzati sono state confrontate con quelle disponibili in letteratura per le principali razze allevate in Italia e per la razza cinese Meishan. Per il* locus ESR*, non essendo disponibili in letteratura dati sulle razze allevate in Italia, è stato analizzato un campione di suini appartenenti alle razze Large White, Landrace, Duroc, Landrace Belga, Piétrain e Hampshire. Nel complesso, sebbene siano stati analizzati solo otto geni, è stato possibile dedurre alcune informazioni che potrebbero risultare importanti per la conservazione del Nero Siciliano. In particolare, la presenza dell'allele* 1843T *al* locus RYR1 *indica la necessità di utilizzare il test PCR-RFLP per identificare i portatori di questo allele negativo al fine di evitare la sua diffusione nella razza. Per quanto riguarda il* locus MC1R *ulteriori studi sono necessari per il suo eventuale utilizzo per la tracciabilità della carne e dei salumi che derivano dal Suino Nero. In conclusione, il presente lavoro rappresenta un primo contributo sull'analisi della struttura genetica di questa razza suina effettuata utilizzando marcatori non anonimi, che, se ulteriormente approfondita con lo studio di altri* loci*, potrebbe fornire importanti elementi per la conservazione e la valorizzazione delle risorse genetiche animali autoctone.*

Parole chiave: *Nero Siciliano, Razze suine, SNP, Frequenze alleliche, Diversità genetica.*

Introduction

Nero Siciliano (NS) breed (Sicilian Black), also known as "Nero di Sicilia", "Nero dei Nebrodi", "Nero delle Madonie" or "Suino Nero" is one of the few Italian local pig breeds that has survived despite the introduction of higher performing breeds. This breed is under a national conservation program and recent estimates indicate that NS population consists of about 1500-2000 animals (Chiofalo and Liotta, 2003).

Today, NS is reared mainly under extensive management in the Nebrodi and Madonie mountains of the provinces of Messina and Palermo on the island of Sicily. Its origin dates back to ancient times. Archaeological remains and indication from ancient writers attest the presence of a pig in the island since the pre-Roman period (8^{th} - 7^{th} century B.C.) and even in Rome the production of a Sicilian pig was well known as early as the 2^{nd} century B.C. (Pino, 1947). However, the genetic pool of NS breed seems to have been influenced and formed mainly during the last few centuries. Chicoli (1870) described the presence of several populations with Neapolitan blood in Sicily. Furthermore, the Casertana breed seems to be used in the constitution of some nucleus present in the districts of Calascibetta (province of Enna) and Mistretta (province of Messina) (Faelli, 1928; Chiofalo and Liotta, 2003). Montanaro (1939) indicated that Iberian blood was introduced in the breed and Porter (1993) suggested that NS originated also by crossing with Large Black and Large White animals. Recently, some crossings with more productive white pigs were attempted in order to improve the performance.

The breed is well adapted to the local environment and marginal conditions in which it is appreciated for reproductive performance (2 litters per year with about 7-8 piglets each), disease resistance and production of tasty meat suitable for the production of typical salami and other cured products. The animals grow slowly under the usual extensive management that is based on the uti-

Table 1. List of the studied genes, their chromosome localization (Chr.), their associations (or supposed role on the basis of its biochemical functions) with production or phenotypic traits, PCR conditions and method of analysis of the polymorphisms.

Gene symbol	Gene name	Chr.	Associated or supposed quantitative trait effects	PCR primers[3]	PCR conditions[4]	Method of analysis[5]	References
RYR1 (CRC)	Ryanodine Receptor 1 (Calcium Release Channel)	6	Higher lean meat yield; Lower meat quality; Stress susceptibility; Halothane sensitivity	GTGCTGGATGTCCTGTGTTCCCT *CTGGTGACATAGTTGATGAGGTTTG*	134/61/1.5/P/R	PCR-RFLP (*Cfo*I, *Asp*HI)	Fuji *et al.* (1991), Patent no. WO9211387, Russo *et al.* (1993)
ATP1A2	Na^+, K^+ ATPase subunit α 2	4	Meat and carcass traits	ACCCTAAGGGAAAATGGAAGAC *CACGGTCAATAAATCTCAAATG*	219/57/1.5/P/R	PCR-SSCP	Russo *et al.* (1999)
MYH4	Myosin Heavy Chain 2B	12	Average daily gain; Meat and carcass traits[2]	AGTGAAGAGTAATTCATCTAAA *GATTGCAAAATTCTCTGTAGA*	124/57/1.5/P/R	PCR-RFLP (*Hpy*F44III)	Davoli *et al.* (2003)
SLN	Sarcolipin	9	Meat and carcass traits[2]	ATATGGCTTCTCGTGAGGTC *TTGAGAGCAGCATCGTTAGA*	218/57/1.5/M/R	PCR-RFLP (*Taq*I)	Fontanesi *et al.* (2001)
CTSB	Cathepsin B	14	Back fat thickness; Cathepsin B activity[2]	GTGGCCGGGTGGGTTTTA *GGCAAGTTCCCCTCAAGTCTGT*	173/56/2.0/P/G	PCR-SSCP	Russo *et al.* (2002)
CSTB	Cystatin B	13	Average daily gain; Cathepsin B activity[2]	GAAGCTGGGCGTGTCATC *GGGGTCAAGGGCTTGTTCTCGTG*	229/60/1.5/P/R	PCR-RFLP (*Pvu*II)	Russo *et al.* (2002)
ESR	Estrogen Receptor	1	Litter size	CCTGTTTTTACAGTGACTTTTACAGAG *CACTTCGAGGGTCAGTCCAATTAG*	120/61/1.5/M/R	PCR-RFLP (*Pvu*II)	Rothschild *et al.* (1996), Short *et al.* (1997), Patent no. US5550024
MC1R [1]	Melanocortin Receptor 1	6	Coat color	CTGCACTCGCCCATGTACTA *AGCAGAGGCTGGACACCAT* (codon 124)	196/58/1.5/M/S	PCR-RFLP (*Bsp*HI)	Kijas *et al.* (1998), Patent no. WO9854360
				GCGGGTACTGTACGTCCACAT *CCCAGCAGAGGAGGAAGAC* (codon 243)	154/60/1.5/M/S	PCR-RFLP (*Mvn*I)	

[1] *The numbers of the codons analyzed are according to Gustafsson* et al. *(2001) and are based on the full coding sequence of the porcine* MC1R *gene. Codon 124 and codon 243 of Gustafsson* et al. *(2001) correspond to codon 121 and 240 of Kijas* et al. *(1998), respectively. Two primer pairs were designed to analyze the mutations at these two codons. The polymorphism at codon 243 of this locus was genotyped using endonuclease* MvnI *instead of AccII as was originally described by Kijas* et al. *(1998). These two enzymes are isoschizomers.*

[2] *Traits that the candidate genes are supposed to affect.*

[3] *Primers are written from 5' to 3'. The reverse primers are indicated in italics.*

[4] *Length in bp of the amplified fragment / annealing temperature in °C / $MgCl_2$ concentration / Thermal cycler: M = PT100 (MJ Research); P = Perkin Elmer 9600 / DNA Taq polymerase: G = Taq Gold (Applied Biosystems, Foster City, CA, USA); R = Taq polymerase (Roche Molecular Diagnostics, Mannheim, Germany); S = Taq polymerase (Sigma Aldrich, St. Louis, MO, USA).*

[5] *The restriction enzymes are indicated in parenthesis.*

lization of woodland feed resources and they can reach 50-70 kg of weight at 1 year of age with some integration to the natural alimentary resources. The adults are 60-65 cm of height on average, the head is long with a straight profile and there are often two goatlike wattles ("tettole") hanging down behind the jaw. The neck is of medium length, the body is not very long with flat sides, the back is slightly rounded and the legs are long and robust. The animals are usually completely black (skin and hair) with a dorsal stripe ("cresta cinghialina") but a few present a white face or a face with white portions ("suino facciolo") or a white belt that can involve the fore legs as well.

In order to help with the conservation and valorization of this breed several projects are currently underway to phenotypically characterize NS breed and its derived products (i.e: Chiofalo and Liotta, 2003; Liotta *et al.*, 2002; Zumbo *et al.*, 2002). Genetic preservation of the breed has been the aim of a European project with *ex situ* conservation of semen by means of cryoconservation

Table 2. Allele frequencies, genotype frequencies, exact test of Hardy-Weinberg equilibrium (HWE), F_{is} (*, P<0.05) and heterozygosity (*H*) for each locus in the Nero Siciliano population studied.

Loci	Alleles	Allele frequencies	Genotypes	Genotype frequencies	HWE P-value	F_{is}	*H* (SE)
RYR1	*C*	0.996	*CC*	0.992	-	- 0.000	0.008
	T	0.004	*CT*	0.008			(0.008)
			TT	-			
ATP1A2	*1*	0.412	*11*	0.151	0.4550	- 0.071	0.486
	2	0.588	*12*	0.521			(0.012)
			22	0.328			
MYH4	*1*	0.160	*11*	0.042	0.1746	+ 0.127	0.269
	2	0.840	*12*	0.235			(0.032)
			22	0.723			
SLN	*1*	0.769	*11*	0.689	<0.0001	+ 0.554*	0.357
	2	0.231	*12*	0.160			(0.029)
			22	0.151			
CTSB	*1*	0.063	*11*	0.017	0.0531	+ 0.185	0.165
	2	0.912	*12*	0.084			(0.032)
	3	0.025	*22*	0.849			
			13	0.008			
			23	0.042			
			33	-			
CSTB	*1*	0.282	*11*	0.075	1.0000	- 0.014	0.406
	2	0.718	*12*	0.412			(0.026)
			22	0.513			
ESR	*A*	0.908	*AA*	0.840	0.0576	+ 0.203	0.168
	B	0.092	*AB*	0.134			(0.031)
			BB	0.026			
MC1R^{124}	*1*	0.164	*11*	0.008	0.1935	- 0.131	0.275
	2	0.836	*12*	0.311			(0.032)
			22	0.681			
MC1R^{243}	1	0.164	*11*	0.008	0.1935	-0.131	0.275
	2	0.836	*12*	0.311			(0.032)
			22	0.681			

(Labroue *et al.*, 2000). Moreover, a preliminary genetic characterization of the breed has been obtained in the context of the European Pig Biodiversity Project that, using a set of anonymous DNA markers (microsatellites), produced data of genetic distances between several other local and commercial European breeds/populations (Ollivier *et al.*, 2001; SanCristobal *et al.*, 2002). These preliminary microsatellite data showed that NS seems genetically closer to the French Créole, the German Angler Sattelschwein, the Czech Presticke and the Spanish Negro Iberico and Retinto breeds (Ollivier *et al.*, 2001).

However, criticism has been raised in the use of anonymous DNA markers for the conservation of genetic resources (Milligan *et al.*, 1994; Burstin and Charcosset, 1997; Ruane, 1999). Using neutral marker loci, it is not usually possible to obtain direct information on the molecular events that concur in the typical production, reproduction and adaptation performance that are characteristics of a particular breed. Thus, it could be interesting to consider mutations within major genes and candidate genes for some production or phenotypic traits as an alternative for the measurement of genetic diversity (Davoli *et al.*, 1996; Ciobanu *et al.*, 2001).

As an attempt towards this aim, this study reports on the analysis in NS breed of allele frequencies of single nucleotide polymorphisms (SNPs) of eight major or candidate genes for performance and phenotypic traits: ryanodine receptor 1 (*RYR1*), Na^+, K^+ ATPase subunit α 2 (*ATP1A2*), myosin heavy chain 2B (*MYH4*), sarcolipin (*SLN*), cathepsin B *(CTSB)*, cystatin B (*CSTB*), estrogen receptor (*ESR*) and melanocortin receptor 1 (*MC1R*).

Material and methods

A total of 119 NS pigs reared in 13 different farms were used in this study. On the basis of the estimates on the consistency of NS pig population,

Table 3. Comparison of allele frequencies at the loci investigated among different pig breeds including the data obtained in the present work for NS pig breed. Data about the two *MC1R* polymorphic sites investigated have been obtained only for NS pig breed.

Breeds[1] (N. of pigs)[2]	*RYR1*		*ATP1A2*		*MYH4*		*SLN*		*CTSB*[4]			*CSTB*		*ESR*[3]	
	C	*T*	*1*	*2*	*1*	*2*	*1*	*2*	*1*	*2*	*3*	*1*	*2*	*A*	*B*
NS (119)[3]	**0.99**	**0.01**	**0.41**	**0.59**	**0.16**	**0.84**	**0.77**	**0.23**	**0.04**	**0.94**	**0.02**	**0.28**	**0.72**	**0.91**	**0.09**
LW (30-257)	0.97	0.03	0.31	0.69	0.23	0.77	0.90	0.10	0.21	0.70	0.09	0.06	0.94	**0.65**	**0.35**
L (21-150)	0.90	0.10	0.70	0.30	0.17	0.83	0.91	0.09	0.24	0.70	0.06	0.12	0.88	**0.98**	**0.02**
D (25-154)	0.93	0.07	0.52	0.48	0.02	0.98	0.58	0.42	0.26	0.62	0.12	0.05	0.95	**1.00**	**0.00**
BL (16-44)	0.02	0.98	0.62	0.38	0.34	0.66	0.71	0.29	0.27	0.64	0.09	0.26	0.74	**0.97**	**0.03**
P (14-48)	0.03	0.97	0.50	0.50	0.24	0.76	0.87	0.13	0.14	0.81	0.05	0.13	0.87	**1.00**	**0.00**
H (10-27)	1.00	0.00	0.10	0.90	0.09	0.91	0.85	0.15	0.64	0.27	0.09	0.22	0.78	**1.00**	**0.00**
M (9-14)	-	-	0.93	0.07	0.79	0.21	0.00	1.00	0.82	0.18	0.00	1.00	0.00	0.22	0.78

[1] *NS = Nero Siciliano; LW = Large White; L = Landrace; D = Duroc; BL = Belgian Landrace; P = Piétrain; H = Hampshire; M = Meishan.*

[2] *The number of animals genotyped for each breed/locus is different and the range of pigs tested (minimum-maximum) is reported between the brackets. The references cited below report the exact number of animals genotyped for each breed/locus.*

[3] *Data obtained in the present work are indicated in bold.*

[4] Allele 4 *at the* CTSB *locus has been identified in the LW, L and D breeds with a frequency < 0.01.*
Data are approximated to two decimals. References for allele frequencies in the other breeds are the following: RYR1*, Russo* et al. *(1996);* ATP1A2*, Russo* et al. *(1999 ; 2000);* MYH4*, Davoli* et al. *(2003); SLN, Fontanesi* et al. *(2001);* CTSB *and* CSTB*, Russo* et al. *(2002);* ESR*, Rothschild* et al. *(1996) for M.*

the animals sampled represent about 6-8% of the total number of NS pigs.

Blood was collected with Vacutainer™ tubes (BD, Franklin Lakes, NJ, USA) containing EDTA. Total genomic DNA was extracted from frozen blood following a standard protocol (Sambrook *et al.*, 1989). Polymerase chain reactions were carried out in a Perkin Elmer 9600 (Perkin Elmer, Roche Molecular System, Branehburg, NJ, USA) or in a PT100 (MJ Research, Watertown, MA, USA) thermal cycler. Primers and PCR conditions for the genes that have been analyzed are reported in Table 1. The polymorphisms were analyzed by means of PCR-RFLP or PCR-SSCP as indicated in Table 1.

For each polymorphism analyzed in NS breed, allele and genotype frequencies were calculated. Hardy-Weinberg equilibrium (HWE) was analyzed for each locus using the exact test of Guo and Thompson (1992) as implemented in GENEPOP software version 3.3 (Raimond and Rousset, 1995). F_{is} statistics (Weir and Cockerham, 1984) for each locus and over all loci were calculated using FSTAT program version 2.9.3 (Goudet, 1995). Significance of the F_{is} statistics was determined from permutation tests implemented in the FSTAT software with the sequential Bonferroni procedure (Rice, 1988). Heterozygosity (H) and its standard error was calculated for each locus using the HET program (Ott, 1997) and over all loci using the software DISPAN (Ota, 1993).

Data reported in the literature about allele frequencies at the selected loci in other breeds (Russo *et al.*, 1993; 1999; 2002; Fontanesi *et al.*, 2001; Davoli *et al.*, 2003) were used to compare the results of allele frequencies obtained for NS breed. For this comparison, as no information about allele frequencies at the *ESR* locus were available in porcine breeds reared in Italy, 30 Large White, 21 Landrace, 25 Duroc, 16 Belgian Landrace, 14 Piétrain and 10 Hampshire unrelated pigs were genotyped at this locus using the same protocol reported in Table 1. For the Chinese Meishan breed information at this locus were obtained from Rothschild *et al.* (1996).

Results and discussion

In this study we have analyzed in NS breed the allele frequencies of SNPs in eight candidate genes for meat quality and production traits, reproduction traits and coat color (Table 1). Allele and genotype frequencies, including exact test of Hardy-Weinberg equilibrium, F_{is} and H for each locus in NS breed are shown in Table 2. A comparison between the allele frequencies identified in NS breed with the allele frequencies at the same loci obtained by other studies in commercial and exotic pig breeds is reported in Table 3.

Analysis of single loci

RYR1, also indicated as *CRC* or Halothane gene, has been the target of the first application of a DNA test in pig breeding. A single nucleotide mutation (C→T) at position 1843, in homozygous condition, causes the Halothane sensitivity, determines increased muscling and low quality meat. The negative and recessive allele (T or n) has been identified only in one NS pig in heterozygous condition. This finding is important and may suggest that this mutation was introduced in NS breed by means of crossings with more productive animals of other breeds. The presence of the T allele, even if with low frequency, supports the utility of testing NS pigs to exclude the carriers from breeding programs to preserve the quality characteristics of the meat of NS breed.

Three genes (*ATP1A2*, *MYH4* and *SLN*) that have been studied in this work have been isolated from an adult porcine skeletal muscle cDNA library (Davoli *et al.*, 1999; 2002) and represent genes highly expressed in the fast twitch skeletal muscle fibers.

ATP1A2 codes for a subunit of the transmembrane complex that maintains the Na^+-K^+ electrochemical gradient across the sarcolemma. It maps on porcine chromosome 4 (Fontanesi *et al.*, 1999; Russo *et al.*, 1999; Davoli *et al.*, 2002), in a region where QTLs for back-fat thickness, meat production and average daily gain have been localized (Andersson *et al.*, 1994; Walling *et al.*, 1998; Walling *et al.*, 2000). An SNP (C→G) has been identified in the 3'-untranslated region (3'-UTR) of the porcine *ATP1A2* gene (Russo *et al.*, 1999) and association analysis with production traits indicated that this locus seems associated ($P<0.05$) with ham and neck weights in commercial pig pop-

ulations (Russo *et al.*, 2000). Allele *1* (C), that seems the negative allele, is the less frequent in NS breed (0.41). At this locus NS breed showed a high level of heterozygosity, as was also reported for other pig breeds (Table 3).

MYH4 codes for the myosin heavy chain 2B isoform that is mainly expressed in IIBw muscle fibers. The heavy chains are components of the functional myosin complex that is the main protein of the skeletal muscle. Allen *et al.* (2001) suggested that *MYH4* may be important for growth and body mass in mice and Davoli *et al.* (2003) identified an SNP (T→A) in the 3'-UTR of the porcine gene, whose allele frequencies differed significantly in extreme divergent groups of pigs for growth rate. Allele *1* (*T*), which is the less frequent in NS breed (0.16) as well as in all the other Euro-American pig breeds compared (Table 3), was regarded as the negative allele in that study.

SLN is a small amino acid proteolipid that regulates the activity of the fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+} ATPase (ATP2A1). *SLN* maps on porcine chromosome 9 (Fontanesi *et al.*, 2001), close to the region in which QTLs or suggestive QTLs for average daily gain (Wada *et al.*, 2000; Malek *et al.*, 2001) and fat deposition (Rohrer *et al.*, 1998) have been detected. The polymorphism (A→G) in this candidate gene is on the 3'-UTR and in NS breed, allele *1* was the most frequent, as it was in all the Euro-American breeds for which this information is available (Table 3). An excess of homozygous animals has been observed in NS breed (F_{is} = + 0.554) and thus this locus was not in Hardy-Weinberg equilibrium (Table 2). The significant F_{is} value observed for this locus could be attributed to the result of inbreeding or presence of a null allele in this population.

CTSB codes for a lysosomal proteinase whose high activity level in fresh pork muscles has been associated with the defect of excessive softness of dry-cured hams (Parolari *et al.*, 1994). Russo *et al.* (2002) indicated that a polymorphism in the sixth intron of this gene may be associated to back-fat thickness in commercial pig breeds. A preliminary study of allele frequencies at this locus using a different sample of NS pigs (28 animals; Russo *et al.*, 2002) showed almost the same distribution of the three *CTSB* alleles (allele *1* = 0.05, allele *2* = 0.90, allele *3* = 0.05) as obtained in the present work (Table 2). HWE exact test at this locus was close to the significance (P = 0.0531) and a slight excess of homozygous animals, even if not significant, was observed (F_{is} = + 0.185).

CSTB is a cysteine proteinase inhibitor that was first described as an inhibitor of cathepsin B. This candidate gene was investigated because it could affect the level of proteinase activity and, in turn, the quality of dry-cured hams. Furthermore, a missense mutation in the third exon of this porcine gene has been associated with average daily gain (Russo *et al.*, 2002; 2003). Allele *1*, which was identified with a frequency of 0.282 in NS breed, seems the negative allele that may confer a lower growth rate. It is worth noting that NS breed presents the highest frequency of this allele, if we exclude the Meishan breed in which it was the only one identified (Table 3). A first evaluation of allele frequency distribution at this locus obtained with a different sample of NS pigs (32 animals; Russo *et al.*, 2002) is in agreement with the results of the present study. Therefore, as the same results have been obtained in two different samples for this locus and for the *CTSB* gene, the animals analyzed can be considered a good representation of NS breed genetic structure.

A PCR-RFLP in the *ESR* gene, that has been indicated to have a significant effect on reproductive performance (Rothschild *et al.*, 1996; Short *et al.*, 1997), was analyzed in this study. Allele *B* that, according to these investigations, may confer a higher litter size and is assumed to be originated from Chinese pigs, has been identified in NS breed with a frequency of 0.092. HWE exact test at this locus was close to the significance (*P* = 0.0576) and this result could be attributed to a defect of heterozygous animals (F_{is} = +0.203), even if this data is not statistically significant. To compare these results obtained for the Sicilian breed, we studied for the first time the allelic distribution at this locus in samples of Large White, Landrace, Duroc, Belgian Landrace, Piétrain and Hampshire animals reared in Italy. Excluding the Meishan breed, only the Large White breed showed a higher allele *B* frequency than the Sicilian pigs (Table 3). These data may indicate that NS breed might have experienced a migration of Chinese genes

when Neapolitan blood was introduced in the breed and/or crosses with Large White pigs in more recent times. The high frequency of allele *B* might positively affect the good reproductive performance attributed to NS breed, despite the environment in which is reared, but further studies are needed to confirm the favorable effect of this allele in the Sicilian breed.

The *Extension* locus (*E*) that influences coat colors in mammals has been recently characterized at the molecular level and was identified to encode the melanocortin receptor 1 (MC1R; Robbins *et al.*, 1993). Dominant mutations at this locus act to produce a black color, while other mutations that inactivate the function of this gene produce a red/yellow coat color in different mammals (i.e: Klungland *et al.*, 1995; Jackson, 1997; Newton *et al.*, 2000), including the pig (Kijas *et al.*, 1998). To date, ten mutations have been identified in the coding region of porcine *MC1R* gene (Kijas *et al.*, 1998; Giuffra *et al.*, 2000; Gustafsson *et al.*, 2001; Kijas *et al.*, 2001). These mutations constitute seven haplotypes (*MC1R*1* to *MC1R*7*). In this work we analyzed only the mutations at codon 124 and codon 243 in order to test if in the NS breed this locus is fixed or more haplotypes are present. Both point mutations analyzed presented two alleles (allele *1*: fragments not cut by the endonucleases; allele *2:* fragments cut by the restriction enzymes) and complete linkage disequibrium was observed between these two polymorphic sites (Table 2). These data indicate that at least two haplotypes exist in the NS breed. As only two mutations have been analyzed at this locus, it is not possible to correctly name the two haplotypes observed according to the reported nomenclature (Gustafsson *et al.*, 2001). However, on the basis of the molecular data obtained, it is possible to speculate that the most frequent haplotype (0.836) could be *MC1R*3* (observed also in Hampshire pigs) or *MC1R*6* (detected in Landrace, Large White and Piétrain), while the less frequent haplotype could be *MC1R*2* identified also in Meishan and Large Black pigs. This hypothesis is consistent with the black coat color present in the NS breed and may consider also the presence of few animals with white areas. Nevertheless, further studies are needed to better characterize this locus and its phenotypic effects in NS breed. A complete characterization of this locus in the NS breed could provide information for the traceability of meat products obtained from this breed as also proposed for other pig breeds (Kijas *et al.*, 1998).

Analysis over all loci

F_{is} statistics over all loci (+ 0.077) may indicate a small excess of homozygosity in NS analyzed sample, even if not significant. However, the presence in the breed of some alleles that may have entered by migration from other populations (like the *1843T* allele at the *RYR1* locus and the *B* allele at the *ESR* gene) may have contributed to maintain a discrete level of variation. This might be confirmed by the fact that this breed is not fixed at the *MC1R* locus, where at least two haplotypes are present. Actually, considering all the analyzed loci average *H* was 0.267 ± 0.054. If we exclude *RYR1* in this analysis, that is the less polymorphic locus, average *H* increases to 0.304 ± 0.045. However, as the breed has not undergone any selection program, genetic drift seems to have played an important role in shaping the genetic structure of NS breed as the comparison with the distribution of the alleles in other breeds might indicate. However, more loci should be analyzed to deduce more complete conclusions on the structure, evolution and phylogenesis of NS breed based on SNPs.

Conclusions

The analysis of genetic variability in farm animal breeds using DNA markers in genes of known functions could provide additional information that may be considered together with the data obtained with the usual microsatellite approach. This study is the first investigation that proposes the use of type I markers to analyze the genetic structure of a local Italian pig breed.

Considering the structure of the NS breed at the eight loci indicated above, important elements were obtained from the data. A genetic flow from other populations/breeds, as deduced from the *RYR1*, *ESR* and *MC1R* analyses, may

have contributed to the structure of NS breed, confirming what was supposed from historical information. The *1843T* allele at the *RYR1* locus is present in NS breed, thus the molecular test to identify the carriers of this allele could be adopted to avoid its spreading in the population. Furthermore, other studies are needed to clarify the allelic structure of the *MC1R* gene in order to evaluate if this gene could be the basis of genetic tests useful for the traceability of meat products derived from the Sicilian breed. Allele frequencies of several other candidate genes were obtained for the first time in NS and if their putative effects are confirmed it could be interesting to use this information in the selection and conservation programs of local genetic resources.

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