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Genetic differentiation between Cinta Senese and commercial pig breeds using microsatellite

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Abstract

Background: Cinta Senese (CS) is an autochthonous Tuscan breed, which risked extinction since the '60s. **Results:** Monitoring the genetic variability of the actual population by use of DNA molecular markers is essential to address a correct breeding policy, finalized to obtain the race preservation and its fitness in the future. 17 SSRs autosomal markers and 1 associated to the X chromosome were used to genotype 86 individuals belonging to the CS and 12 belonging to two main white races Landrace (L), Large White (LW) and crosses between LW and L and L and CS widespread in Tuscany and used in the recent past to obtain hybrids with the CS. **Conclusions:** A dendrogram of similarity measures the relative genetic distance between individuals in the population. Data show that CS pigs have a distinct genotype from L, LW, LW x L and L x CS.

Keywords: Cinta Senese, genetic diversity, genotyping, microsatellites, *Sus scrofa*

INTRODUCTION

Domestic swine (*Sus scrofa domestica*) is one of the most widely widespread domestic animal species in the world. It is supposed that in the history of pig domestication, complex factors such as men-deriving forces and natural selection have influenced the actual diversity and population structure of the species (Amaral et al. 2011).

The CS has always been considered among the best Italian pig breeds and one of the most important in Tuscany. Its origin is the cradle of Montagnola Siena, a wooded area at 250-300 meters above sea level, which includes the communes of Casole d'Elsa, Monteriggioni, Poggibonsi and Siena.

The oldest traces are documented in the Sala della Pace of the Palazzo Pubblico in Siena, the fresco of Good Government (1338-1340), a work by Ambrogio Lorenzetti, where a pig is shown that the morphological features can be ascribed to CS.

By the mid-50s it was estimated that the leaders of the CS had about 160,000 while around the '60s, following the profound transformations undergone by the national agro-livestock and the massive introduction of improved breeds of pigs, the predominant northern European derivation, the abundance

of the race was reduced to the point of risking extinction. In 1986 only 81 sows and 3 boars were recorded (Gandini et al. 2001).

The need, perceived internationally to conserve biodiversity, in parallel with the increased public sensitivity to environmental problems caused by intensive pig farms, has encouraged the policy of recovery and preservation of native species. All this, in Tuscany, has created starting in 1979 with the payment of premiums for the breeding and maintenance of breeding breeds at risk of extinction. It was the beginning of the expansion of the CS, one of the few Italian pig breeds which now seems recovered and started to gain a slice of the market independently from international trends.

A common practice in breeding during the '70s and '80s in order to preserve the CS race was crossing white breeds, namely Landrace (L) and Large White (LW). This has risen the question on actual CS genotype identity. Furthermore, for consumer protection is essential to establish if DNA-based techniques can distinguish meat deriving from CS from that deriving from L and LW. Fraudulent labelling of CS claimed products may be an issue, due to the addition of less expensive ingredients. Since 2004 the CS products (salami, sausages, ham, etc.) must respond to European Union regulatory system on typical food products and only products composed by 100% CS meat from animals grown in Tuscany according to the strict product regulation, may be labelled and priced as "CS-product".

DNA fingerprinting techniques have recently become essential tools for species and varietal identification in the Sus genre (Dun et al. 2007; Fajardo et al. 2008).

Comparative genomic analysis of different domestic animal breeds can explain the genetic basis of phenotypic variation of several domestic animal breeds, including the Sus genre (Andersson and Georges, 2004).

The pig genome sequencing is now at an advanced stage and the information available (http://piggenome.org/) is contributing toward revealing the molecular mechanisms controlling phenotypes and plays significant role in the pork meat production. There is extensive conserved homology with the human genome and the pig genome is considered an important model for human health particularly for understanding complex traits such as obesity and cardiovascular disease.

In the light of such an extensive information deriving from the *Sus scrofa* genome sequencing, the number of DNA molecular markers available for QTLs characterization and genotyping has raised dramatically since 2003 (Chen et al. 2007).

The identification of molecular polymorphisms useful for breed identification could contribute to the valorisation of local genetic resources. Recently, the phylogenetic relationships among eight pig breeds from Shandong province of China and to what extent they were affected by the modern commercial breeds (Duroc, Yorkshire and Landrace) were determined in maternal lineage by using direct sequencing of mitochondrial Cytb and partial control region (CR) DNA sequence (Wang et al. 2010), two molecular markers widely used to analyze the phylogenetic relationships of closely related groups (Jiang et al. 2008).

In pigs, SSRs have been widely used in genetic diversity studies for the conservation and management of genetic diversity of populations. The adoption of these molecular markers is especially important when associated with the conservation programmes of autochthonous breeds (Nidup and Moran, 2011).

Due to the importance of pig breeds colour coat standardization, porcine genes involved in pigmentation are useful candidates for breed pig products traceability. In many cases even if the colour-related gene polymorphic is associated to specific pig breeds, this could not be taken as a unique diagnostic method for pig breed discrimination. A recent study carried out using PCR-RFLP and PCR-SSCP in white breeds Landrace, Yorkshire and a red breed, the Duroc, revealed the presence of five polymorphic sites in the MC1R gene, which might be used to genotyping the recessive trait of red coat respect to the dominant white (Dun et al. 2007). From the sequence analysis of a 13 kb upstream region of the dominant white/kit locus in the pig genome, it seems that the Belt allele (I^{be}) causing a white belt across an otherwise pigmented body could not be associated with the duplication or splice

mutation, but rather might be caused by differences in the regulatory elements of the upstream region of the white/kit locus (Johansson et al. 2006).

The CS genome can be traced by a polymorphism in the MCR1 gene, since it shows a G124A mutation and the absence of the insertion of two nucleotides at codon 22, denoted the MC1R*3 allele, also known as dominant black (Fontanesi et al. 2005; Nicoloso et al. 2006). Even though the genomic information on these genes controlling the coat colour might be used to develop consistent functional markers, current genotyping by use of these tools might result laborious respect to SSRs typing. It could be hypothesized that the inheritance of pig coat colour could result from a complex multi allelic interaction, implicating also coordinated mechanisms of transcriptional regulation factors. For this main reason it seems that genotyping pig breeds with neutral markers such as a good polymorphic SSR panel could result in more informative measurement of genetic diversity and can be more effective in identifying univocally local European breeds, such as the CS.

Conservation programmes both *in-situ* and *ex-situ* can be greatly improved by the adoption of MAS actions. The analysis of the SSRs polymorphism observed in the European pig breeds showed that the allelic diversity pattern among breeds is quasi-independent from that evaluated based on allele frequency and local breeds do not generally cluster with international breeds, confirming the uniqueness of the European local breeds compared to main international breeds (Ollivier et al. 2005; Ollivier, 2009). The European pig industry relies on a limited number of breeds, and is mainly focused on the LW genotype asset. Europe thus needs sources of novel genetic variation in order to improve even the commercial lines. Also for this reason, in addition to typical food traceability perspective, the autochthonous breeds must be correctly preserved.

In order to rationalize the management of pig breeding and preserve pig diversity, it is essential that a simple assay for genetic identification could be quickly developed and applied, taking advantage of the molecular genetic tools available to the scientific community.

This work lays the basis for the development of a traceability control system of CS along the entire production chain. For consumers, traceability is a guarantee for food security and transparency, and it's desirable that the genetic traceability will be an added value for producers who decide to adopt in this type of voluntary certification.

The CS products are considered of high quality even if only locally distributed, they were recently awarded by the EU regulation as DOP (Denominazione di Origine Protetta) products, thus addressing the request of a very demanding market. In addition, the CS products are characterized by tasty meat and fats with dietetic characteristics associated with the type of farming, which are positively influenced by the natural diet and the high quality of environment in which the animals are raised.

MATERIALS AND METHODS

Sampling

In order to analyse the genetic diversity within and between white pig breeds widespread on the Tuscan territory (L and LW) and individuals belonging to the traditional breed CS, biological samples have been collected from 86 males of CS and 12 white pigs belonging to the breeds L (1 to 4), LW (5-8) and cross LW x L (9-11) and L x CS (12). CS pigs were numbered from 1A to 86A.

The biological samples (hair follicles for the CS pigs and blood for the white breeds) have been kept at 4ºC until use.

Genotype determination

Genomic DNA was extracted from hair follicles and blood using GenElute Mammalian genomic Kit (Sigma-Aldrich, MO, USA).

PCR reactions were carried out in an Eppendorf Mastercycler Gradient PCR in a total volume of 12.5 µl, containing 30 ng of genomic DNA, 0.25 mM dNTPs (Promega), 0.25 µM each primer (one of them

being fluorescein labeled), 1X Green GoTaq ® Reaction Buffer containing 1.5 mM MgCl2 (Promega), 0.1U Go Taq ® DNA Polymerase (Promega).

PCR conditions included an initial denaturation step of 5' at 95ºC, 39 cycles of 30" at 95ºC, 30" at 55ºC, 1' at 72ºC and a final extension of 10' at 72ºC.

All the individuals have been analysed in 18 microsatellite loci. The sequences of oligonucleotide primers flanking STR motifs and producing variably-sized DNA fragments depending on the number of repeats were described previously and recommended by International Society for Animal Genetics (ISAG) and Food and Agriculture Organization (FAO) (http://www-lgc.toulouse.inra.fr/pig/panel/panel2004.htm).

2 µl of each PCR product were mixed with 0.25 µl Et400-R size standard (GE Healthcare, USA), and 4.9 µl deionised H_2O, centrifuged, denatured at 95ºC for 2 min, cooled in ice and separated on a MegaBACE 500 capillary sequencer (GE Healthcare, USA).

Dye-labelled amplicons were automatically sized using internal standards and the Fragment Profiler v 1.2 software (GE Healthcare, USA) and then visually inspected.

Sequencing of the PCR product

The PCR amplification products obtained from the amplification of the genomic DNA of four different individuals of CS at the S0217 and five at SW2476 respectively were sequenced (BMR Genomics). The sequences have been analyzed by blast in GeneBank. All the sequences showed a significant sequence similarity with the corresponding *Sus scrofa* microsatellite sequences.

Clustal W analysis showed evident polymorphisms among the individuals in the microsatellite sequences (Figure 1).

The sequence analysis allowed to confirm the identity of the CS alleles as belonging to the genre Sus and to highlight the high degree of genetic variability of those regions of DNA among the different pig breeds.

Statistical evaluation

The allele sizes characterizing the genotype of each individual have been filed and elaborated in a database (MSDBase, Serge-Genomics and SienaBioGrafix) in order to obtain populations and allelic frequences.

The similarity dendogram has been obtained using NTSyS 2.1 software, the clustering algorithm is SAHN and among the different options, UPGMA was selected as hierarchical method for the tree construction.

In the analysis of the within breed genetic variability of the 96 animals the MS Tools software was used to calculate allelic frequencies and population genetic parameters such as: expected Heterozygosity (He), observed Heterozygosity (Ho) and allelic diversity for each locus.

The He is defined as the estimated fraction of individuals who would be heterozygous for any randomly chosen locus. The He differs from the Ho because it is a prediction based on the known allele frequency from a sample of individuals. Deviation of the observed from the expected can be used as an indicator of deviation from the theoretical Hardy-Weinberg equilibrium.

The F-statistic calculations such as Hardy-Weinberg equilibrium, the PIC value of SSRs loci, the Fixation indexes-F_{ST}, F_{IT}, F_{IS}, were obtained by use of GenePop (4.0.10) (Raymond and Rousset, 1995).

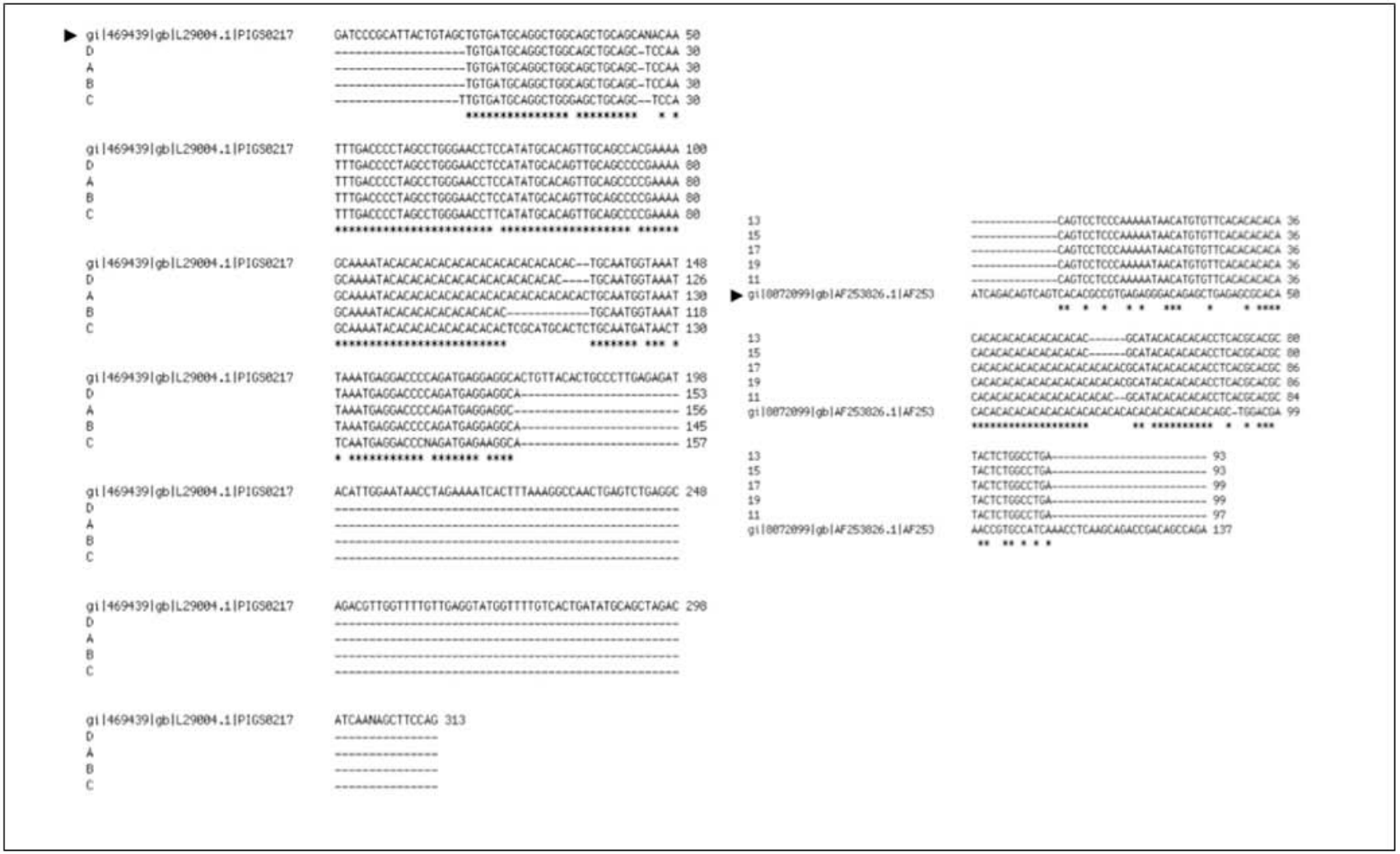

Fig. 1 Sequence comparison of amplified alleles. Amplified products obtained at locus S0217 (left) and SW2476 (right) from CS breed and the corresponding regions in the pig genome sequences in GenBank (bold arrow) were compared. Four CS individuals (A, B, C, D) at locus S0217 and five (11, 13, 15, 17, 19) at locus SW2476 were sequenced and several polymorphisms were detected among individuals. Multiple sequence alignement was obtained by Clustal 2.0.10.

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RESULTS

Heterozygosity and breed differentiation

The Ho and their unbiased estimates, which take into account of sample sizes differences, were computed per 5 subpopulations (L, LW, LW x L, L x CS, CS) (Table 1). Also the populations analyzed vary relatively more in size than in heterozygosity.

Table 1. Sampling information and within population genetic diversity of CS, L, LW and LW x L, L x CS crosses.

Population	Region	Sample size	Loci typed	Unbiased Hz	Obs Hz	No. Alleles
L	Tuscany	4	18	0,3869	0,4583	2,06
LW	Tuscany	4	18	0,5206	0,537	2,44
LWxL	Tuscany	3	18	0,5278	0,5833	2,06
LxCS	Tuscany	1	17	0,4118	0,4118	1,41
CS	Tuscany	86	18	0,4915	0,4507	4,94

L: Landrace; LW: Large White; CS: Cinta Senese; LW x L: Large White x Landrace; L x CS: Landrace x Cinta Senese.

An exact test of Hardy-Weinberg equilibrium was also performed for each subpopulation and the estimation of the P-value was calculated by the Markov chain method. The degree of differentiation between breeds was estimated by calculation of the F_{ST} value, ranging from -0,2249 to 0,4780 and the proportion of the variance in the subpopulation contained in each individual expressed with the F_{IS} (inbreeding coefficient) index ranged from -0,2249 to 0,9541 (Table 2).

Table 2. *F*-Statistic values per locus calculated in all pig breeds considered as one population.

Locus	Fwc(is)	Fwc(st)	Fwc(it)
S0026	-0.2249	-0.2249	0.4315
S0155	0.3412	0.0222	0.3558
S0226	0.0002	0.4371	0.4372
SW240	0.2705	0.1539	0.3828
S0068	-0.0971	0.2822	0.2125
SW857	-0.0138	0.3784	0.3698
S0218	0.9541	0.3617	0.9707
S0228	-0.0849	0.4249	0.3761
SW122	-0.0431	0.478	0.4555
S0005	0.1488	0.3287	0.4286
SW632	0.0257	0.3921	0.4078
IGF1	-0.0836	0.121	0.0475
SW2406	0.0828	0.1047	0.1789
SW72	-0.0421	0.2035	0.17
S0090	-0.0898	0.2669	0.2011
SW1828	-0.0034	0.1932	0.1905
SW1067	-0.0023	0.3573	0.3558
SW936	-0.2789	0.2506	0.0416
All:	0.0618	0.2858	0.3299

Clustering and phylogenetic trees

Genetic distance between individuals were calculated on the basis of allele frequence and by comparing the genotypes as evaluated using a multi-locus estimation of the kinship coefficients. Multilocus allele counting done with GenePop shows that 8 of the 18 loci studied have at least 12 rare alleles that can be biunivocally associated to the CS breed (data not shown).

The degree of polymorphism, which is strictly related to the informativeness of the SSRs panel used, is reported in Table 3 (PIC). The intraspecific and inter-specific diversity of the population, measured by comparing the genotypes assets of individuals, produced a dendrogram of similarity.

Table 3. Source, map and origin of the SSRs markers used. D1: markers from the set of ISAG-FAO originally set up in the frame of PiGMaP II project. D2: markers from the PigBiodiv EU project. Currently, the markers adopted respond to a minimum treshhold of heterozygosity (64%) and a sufficient degree of polymorphism (average number of alleles = 9).

Locus	Chromosome arm	Origin	Na	He	Ho	PIC
S0155	1q	D1	7	0.696	0.457	0.648
S0005	1q	D1	9	0.753	0.577	0.711
SW1828	1q	D2	7	0.709	0.677	0.66
S0026	2q	D1	2	0.276	0.268	0.237
S0226	2q	D1	4	0.245	0.208	0.219
SW240	2p	D1	7	0.615	0.431	0.56
SW72	3p	D1	3	0.53	0.521	0.418
IGF1	5q	D1	4	0.59	0.623	0.521
S0218	6q	D1	5	0.527	0.022	0.426
S0228	6q	D1	4	0.153	0.142	0.146
SW122	6q	D1	7	0.558	0.478	0.523
SW1067	6q	D2	7	0.637	0.58	0.603
SW2406	6p	D2	6	0.624	0.557	0.571
SW632	7q	D1	8	0.567	0.477	0.488
S0090	12q	D1	4	0.422	0.427	0.392
S0068	13q	D1	4	0.727	0.739	0.675
SW857	14q	D1	3	0.266	0.237	0.248
SW936	15q	D1	8	0.674	0.804	0.611
mean				0.532	0.457	0.481
STD				0.183	0.211	0.173

Genotyping data were produced from the 18 loci to construct the dendrogram. No null alleles or technical difficulty were encountered, that could have determined a reduction in the number of loci to be used. Interestingly, the dendrogram evidentiates a clear distinction between the CS breed and the main white breeds analyzed (Figure 2). Red and blue squares show the individuals of white breeds and crosses between white breeds and CS respectively, while all the CS individuals group together in the remaining part of the dendrogram. On average the CS individuals share up to 53% genetic similarity with L, LW. In the red square individuals of L, LW and LW x L hybrids are evidentiated, in the blue square one LW and L x CS hybrid, the remaining belonging to the CS population. The fingerprinting definition of the male individuals during the reproductive age is finalized to obtain both the genetic mapping of the population and in a near future to delineate the bases for the realization of an effective analytical traceability of the CS meats at the level of products.

Distribution and amount of diversity

For each locus Table 3 shows the Ho, the He, the number of allele (Na) and the PIC value averaged across the 17 autosomal loci and 1 associated to the X chromosome. Ho ranged from 0,022 (S0218) to 0,804 (SW936) and the average number of observed alleles for the CS reached 4,94 compared to 1,99 calculated as average value of number of observed alleles for L, LW, LW x L and L x CS (Table 1). The heterozygosities observed are close to their expectations in all loci tested except in S0218, which shows a significant reduced heterozygosity (Table 3).

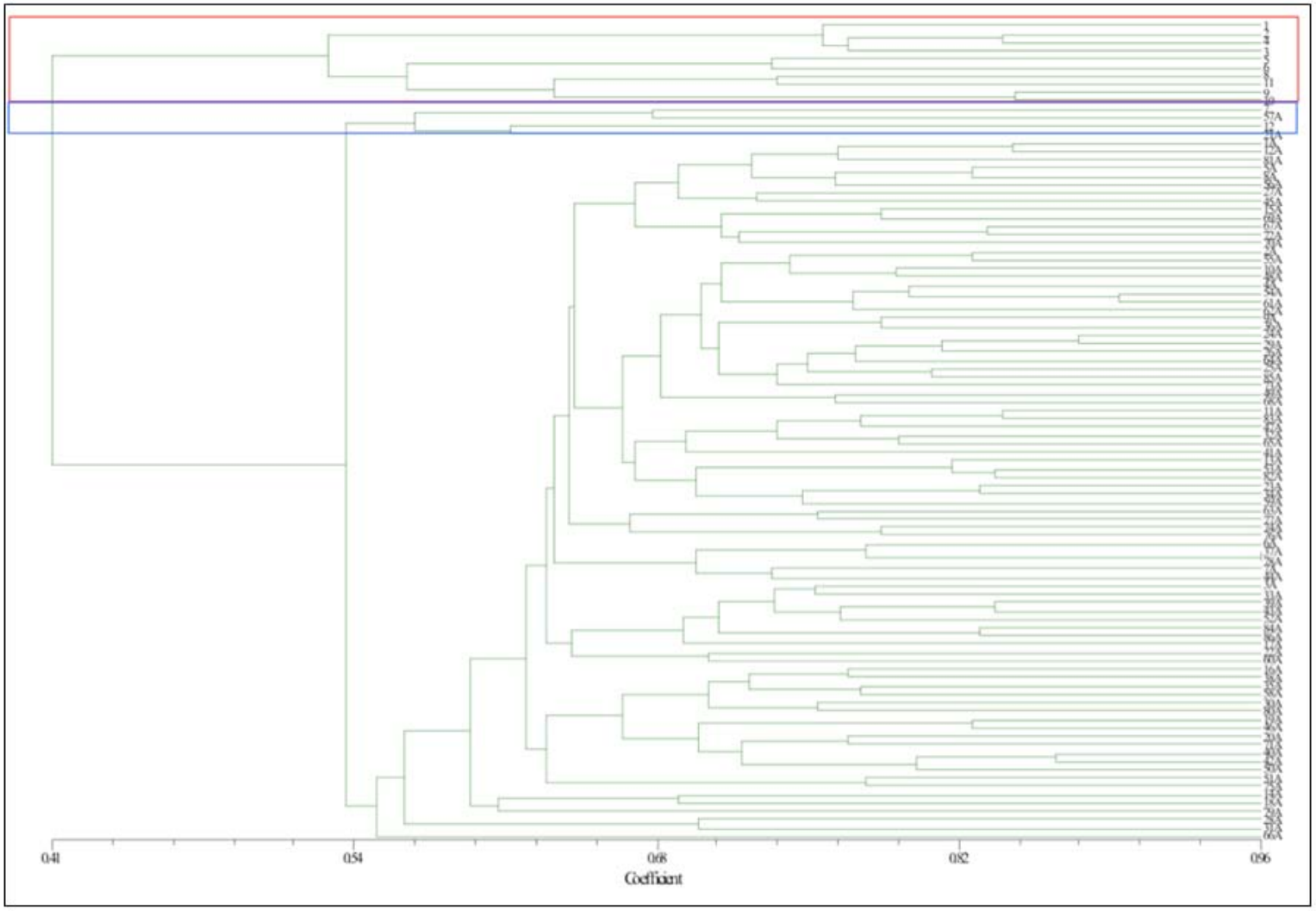

Fig. 2 Dendogram representing genotipe-based genetic distance among CS and white breeds. The unweighted pair-group method with arithmetic mean (UPGMA) topology tree showing the genetic relationship among the pig breeds and relative crosses analyzed. The red square includes all the individuals from the white breeds.The blue square includes the L x CS hybrid (12) one LW (7), which are closest to the CS population. Most of the CS individuals (from 1A to 86A) gather together in the remaining part of the tree.

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In the CS breed deviations from Hardy-Weinberg equilibrium are significant ($P < 0.05$) and are all observed at loci S0155, SW240, SW1067, S0218, SW632, SW1828 and SW936. Furthermore, the deviations observed are associated predominantly to a quite high positive F_{IS} value (Table 4).

Table 4. F_{IS} values per sub-populations, calculated per each locus.

Locus	L	LW	LW x L	L x CS	CS
S0026	-1.000	-0.500	0.000	NA*	-0.113
S0155	0.200	-0.200	-0.333	NA	0.367
S0226	-0.200	-0.200	-0.333	NA	0.118
SW240	NA	NA	NA	NA	0.251
S0068	NA	-1.000	0.000	NA	-0.086
SW857	-0.200	-0.368	-0.500	NA	0.048
S0218	-0.200	NA	NA	NA	0.952
S0228	NA	NA	-0.333	NA	-0.035
SW122	-1.000	0.143	-0.143	NA	0.07
S0005	0.333	1.000	-0.333	NA	0.117
SW632	NA	0.053	-0.333	NA	0.027
IGF1	-0.600	-1.000	NA	NA	-0.039
SW2406	NA	0.333	-0.333	NA	0.069
SW72	-0.200	-0.200	-1.000	NA	0.002
S0090	-0.200	0.368	NA	NA	-0.129
SW1828	-0.091	0.200	0.200	NA	-0.012
SW1067	-0.091	-0.412	NA	NA	0.026
SW936	-0.200	-0.333	0.600	NA	-0.309
All:	-0.222	-0.045	-0.172	NA	0.083

L: Landrace; LW: Large White; CS: Cinta Senese; LW x L: Large White x Landrace; L x CS: Landrace x Cinta Senese. *NA, not applicable.

DISCUSSION

Genetic structure of the CS breed

In the present paper the CS breed diversity was analyzed by use of allelic polymorphisms observed at 18 SSRs loci.

Results showed that CS breed is genotypically distinguishable from L, LW, LW x L and L x CS by use of SSRs genotyping. Due to the complex, multi factorial and multi-genes determined character of the colour coat in pig, the use of a general, neutral SSRs genotyping approach, seems appropriate in determining the pig breed identity.

The fixation indices showed a generally high level of genetic differentiation between breeds, with quite significant differences across loci. The isolation of CS from white breeds studied demonstrates that there were low levels of gene exchange during breed formation in this Tuscan indigenous breed. Likely, the practice of mating CS with white breeds, which was a widespread tendency during the ’60 and ’70 to preserve the CS breed and overcome the risk of extinction, did not modify significantly the peculiarity of CS genotype diversity. The mean of Ho in our study is also similar to that of previously published data (Li et al. 2004). The mean F_{ST} value (0.2858) for CS and the white breeds used evaluated at the 18 SSRs in the present work is comparable with that of other European breeds (Laval et al. 2000). Thus, we can assess that CS breed differentiation with respect to white breeds analyzed is significant and confirmed by the high value of F_{ST} and the clustering based on genetic distances between individuals, which grouped essentially all individuals in two main groups corresponding to CS breed and white breeds. In the CS breed positive F_{IS} values were observed. This could be derived from a certain degree of inbreeding and from a significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$). However, F_{IS} could also measure the excess or the reduction of Ho and He in the population (*e.g.* F_{IS} = He-Ho/He). None of the sub-populations analyzed were in Hardy-Weinberg equilibrium and

the He is quite high for all breeds. A high value of heterozygosity could be due to the existence of multiple lineages within the same breed (*Walhund* effect) or to the absence of a defined selective pressure driven by coordinated breeding programmes.

The analysis of genetic variability in farm animal breeds using DNA neutral markers, such as SSRs could provide additional information that may be considered together with the data obtained with alternative approaches, such as the analysis of single nucleotide polymorphism of coding sequences of candidate genes for production traits. The possibility of establishing a defined genotype for the CS breed allows to set an accurate management of breeding programs for genetic improvement and breed genetic identity preservation over time.

Rare alleles identification in the CS breed, renders it possible the molecular traceability of CS meat over other major white breeds. Products consisting of 100% CS meat can be certified by use of SSRs polymorphism analysis or by quantitative PCR diagnostics.

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