Livestock Science 184 (2016) 71–77

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Authentication of "mono-breed" pork products: Identification of a coat colour gene marker in Cinta Senese pigs useful to this purpose

L. Fontanesi [a,*], E. Scotti [a], M. Gallo [b], L. Nanni Costa [a], S. Dall'Olio [a]

[a] Department of Agricultural and Food Sciences, Division of Animal Sciences, University of Bologna, Viale Fanin 46, 40127 Bologna, Italy
[b] Associazione Nazionale Allevatori Suini (ANAS), Via L. Spallanzani 4, 00161 Roma, Italy

ARTICLE INFO

Article history:
Received 28 June 2015
Received in revised form
9 December 2015
Accepted 18 December 2015

Keywords:
SNP
Coat colour gene
KIT
Traceability
Authenticity
Pig breed

ABSTRACT

The possibility to authenticate food products is crucial to defend local livestock production chains from frauds. Cinta Senese is an autochthonous pig breed reared under extensive or semi-extensive management systems, mainly in the Tuscany (Italy). A Protected Designation of Origin (PDO) brand for Cinta Senese meat was recently obtained. The breed is characterised by a typical black with a white-belted coat colour pattern. We analysed a coat colour gene (*KIT*) to identify a DNA marker that could be useful for Cinta Senese meat product authentication. An informative single nucleotide polymorphism (SNP) was identified among different *KIT* gene haplotypes that were obtained from several pigs of different breeds. This SNP (g.43597545C > T; position on porcine chromosome 8 in the Sscrofa10.2 genome assembly) was genotyped by PCR-restriction fragment length polymorphism (RFLP) in 631 animals of 11 different pig breeds and one wild boar population. Allele T was almost fixed in Cinta Senese (95.9%) and absent in many breeds and was considered the tag SNP of the belted allele. Probability to correctly assign an unknown meat sample to Cinta Senese was 0.97–1.00. This DNA marker can be useful to distinguish meat of Cinta Senese pigs from meat of non-belted pigs. Thus, it could be an important tool not only to defend Cinta Senese pork chain from frauds but also to design breeding plans to eliminate non belted alleles from this pig population.

1. Introduction

The identification of the origin and the authentication of food products are important issues to defend livestock production chains from frauds that produce consumer distrust and undermine commercial valorisation of many local and niche products (Montowska and Pospiech, 2012). Among these products, an increasing interest during the last few years has been directed to the development of "mono-breed" labelled lines of meat and dairy products (Fontanesi, 2009). The marketing link between a breed and its products is positively considered by the consumers in terms of perceived quality and contributes to improve profitability as the products are sold at a higher price compared to undifferentiated ones. This link is mainly used to improve the economic incomes derived by local and endangered breeds that are usually less productive. The market added value is important for a sustainable exploitation of rural economies and is the fundamental driver for the conservation of endangered animal genetic resources (Fontanesi, 2009; Hoffmann, 2011).

Cinta Senese is an autochthonous pig breed that is reared under extensive or semi-extensive management systems, mainly in the Tuscany region (Italy). The breed is characterised by a typical black with a white belted coat colour pattern. Its origin dates back to the XIII–XIV century when belted pigs were raised in the hills around Siena as demonstrated by a famous painting of Ambrogio Lorenzetti in the Palazzo Comunale of Siena (a.d. 1340). The importance of this breed was recognised with the early constitution of the breed national herdbook that worked from the 1936 to 1966 and then by a regional herdbook that was active since 1976. Just after the second world war the breed was also used to produce grey or "tramacchiati" crossbred pigs by crossing with white pigs that were fed with whey produced by the cheese factories of Pianura Padana in the North of Italy. This use was stopped by the transportation ban of the pigs due to an outbreak of diseases in 1968. Since then the number of animals of this breed dropped down, almost leading to the extinction of the breed. At the end of the eighties a few projects started the recovery of this breed and in 1997 the national pig breeders herdbook preliminarily re-activated a section dedicated to Cinta Senese to promote conservation programs that made it possible to constitute a definitive herdbook section for this breed in 2001 (ANAS, 2015; Franci et al., 2007). These alternate periods influenced the number of Cinta Senese

* Corresponding author.
E-mail address: luca.fontanesi@unibo.it (L. Fontanesi).

http://dx.doi.org/10.1016/j.livsci.2015.12.007
1871-1413/

heads: the number increased reaching about 160,000 in the fifties, then it decreased reaching the lowest number of 81 sows and 3 boars recorded in 1986 and after conservation programs the number of pigs raised to about 5000 heads (Franci et al., 2007; Raimondi, 1954). At present about 900 sows and 150 boars are registered in the National Herdbook (ANAS, 2015). The current stabilized number is supported by the constitution of a Protected Designation of Origin (PDO) brand for Cinta Senese meat in 2011 and the development of the Cinta Senese Consortium (Consorzio di Tutela della Cinta Senese). This consortium and the PDO contributed to the visibility of Cinta Senese products and to the added value of the meat of this breed that should be defended from potential frauds.

Coat colour is one of the most important traits that differentiate livestock breeds (Fontanesi, 2009). DNA markers associated with coat colours in different livestock species have been already used to authenticate mono-breed dairy and meat products (D'Alessandro et al., 2007; Russo et al., 2007; Maudet and Taberlet, 2002; Fontanesi et al., 2010a, 2010b, 2011).

As already mentioned, Cinta Senese pigs are characterised by a typical belted coat colour that can be the basis for the development of DNA markers useful for the authentication of Cinta Senese PDO products. The belted allele, in the past thought to be caused by a specific coat colour locus, is one allele of the *Dominant white* (*I*) locus series that lists several alleles derived by complex mutations in the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) gene (whose product is involved in the migration of the melanoblast), namely copy number variations (CNV) and a splice site mutation (Fontanesi et al., 2010a; Johansson Moller et al., 1996; Marklund et al., 1998; Pielberg et al., 2002). The combination of these mutations produces the classical white coat colour phenotype (CNV and the splice site mutation) or the patch phenotype (CNV). The molecular basis of the roan or I^d allele is not completely known even though it is due to variants affecting the *KIT* gene (Fontanesi et al., 2010a). The belted allele was suggested to be derived by an uncharacterised regulatory mutation in the *KIT* gene, as it was not associated to any duplication of the *KIT* gene described for other *I* alleles (Giuffra et al., 1999). Rubin et al. (2012) reported that the belted allele could be due to duplication events in the promoter region. We recently characterised different *KIT* gene haplotypes by Sanger sequencing in several cosmopolitan and local pig breeds including a few Cinta Senese pigs and identified potential breed informative haplotypes (Fontanesi et al., 2010a). In this study we further analysed the *KIT* gene and identified a DNA marker that, by comparing 11 different pig breeds and one wild boar population, was useful to design a simple genotyping test for the authentication of Cinta Senese meat.

2. Materials and methods

2.1. Animals and DNA extraction

DNA was extracted from blood samples, liver and muscle specimens and hair roots collected from a total of 602 pigs of 11 different breeds (110 Cinta Senese; 105 Italian Large White; 52 Italian Landrace; 86 Italian Duroc; 32 Pietrain; 16 Hampshire; 50 Mora Romagnola; 47 Casertana; 50 Apulo Calabrese; 42 Nero Siciliano; and 12 Meishan) and one wild boar population (29 animals) for a total of 631 animals. Samples were mainly obtained from previous projects (Fontanesi et al., 2010a, 2014). Novel blood samples were collected during slaughtering in commercial abattoirs. DNA extraction was carried out using a standard phenol-chloroform protocol (Sambrook et al., 1989) or using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA), following the manufacturer instructions.

2.2. PCR analysis

PCR was carried out using two primer pairs. The first primer pair (forward: 5′-CCTCGCAGCAGGAGCAGT-3′; reverse: 5′-CTCAGGGCTGAGCATTCG-3′) was used to amplify a fragment of 388 bp encompassing a portion of intron 17, exon 18, intron 18 and a portion of exon 19 of the porcine *KIT* gene that was used to re-sequence this gene region in 12 Cinta Senese pigs to confirm previous sequencing data obtained by Fontanesi et al. (2010a). The second primer pair (forward: 5′-TGAACATTGCTGACTCCCCT-3′; reverse: 5′-TGCATTTTACCTAAAGAGAAGAGC-3′) was used to amplify a fragment in all 631 animals. The amplicon of 157 bp was used for the PCR-RFLP analysis described below. The amplification reactions were cycled in a 2720 Life Technologies thermal cycler (Life Technologies, Foster City, CA, USA) with the following steps: 5 min at 95 °C; 35 amplification cycles of 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C; 10 min at 72 °C. The final reaction volume was of 20 μL and included: 50–100 ng of template DNA, 1 U of Taq DNA polymerase (AmpliBioTherm Taq DNA polymerase, Fisher Molecular Biology, Trevose, PA, USA; or EuroTaq DNA polymerase, EuroClone Ltd., Paington, Devon, UK); 1X PCR buffer; 2.5 mM dNTPs; 10 pmol of each primer; 2.0 mM of $MgCl_2$.

2.3. Sequencing and haplotype analysis

Amplified fragments obtained using the PCR primers of the first pair reported above were preliminarily treated with 1 μl of ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) for 15 min at 37 °C. Treated amplicons were sequenced using the same PCR primers and the Big Dye v3.1 cycle sequencing kit (Life Technologies, Foster City, CA, USA). Sequencing reactions were purified using EDTA 0.125 M, Ethanol 100% and Ethanol 70%, following a standard protocol. Then, the purified products were loaded on an ABI3100 Avant sequencer (Life Technologies). Obtained sequences were visually inspected and aligned with the help of the CodonCode Aligner software (http://www.codoncode.com/aligner) using the reference sequence of the corresponding pig *KIT* gene region (Fontanesi et al. 2010a). The 28 *KIT* gene haplotypes previously reported by Fontanesi et al. (2010a) from several pig breeds were aligned and compared with sequences obtained in the current study. These datasets (the previously reported *KIT* haplotypes and the additional sequences obtained here) were used to identify the most informative single nucleotide polymorphism (SNP) of the most frequent Cinta Senese haplotype.

2.4. Genotyping and data analyses

The genotyping protocol of the selected SNP (g.43597545C > T; position of the nucleotide coordinate on porcine chromosome 8 in the Sscrofa10.2 genome assembly of the *Sus scrofa* genome) of the Cinta Senese haplotype was based on PCR-RFLP. The amplified fragments of 157 bp obtained with the second primer pair reported above was digested with the restriction enzyme *Dde*I. Briefly, restriction analysis was carried out overnight at 37 °C in a total of 13 μL of reaction volume including 5 μL of PCR product, 3 U of *Dde*I (Fermentas, Vilnius, Lithuania) and 1X reaction buffer. Produced DNA fragments were electrophoresed in 2.5% agarose gels running in TBE 1X and visualised with 1X GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

Hardy-Weinberg equilibrium of the genotyped SNP in the analysed populations was tested using the HWE software program (Linkage Utility Programs, Rockefeller University, New York, NY, USA). Pairwise allele and genotype frequency differences between Cinta Senese breed and all other populations were evaluated using