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1 **Authentication of “mono-breed” pork products: identification of a coat colour gene marker in**
2 **Cinta Senese pigs useful to this purpose**

3

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13

14 **Running title:** A DNA marker useful for pork product authentication

15

16 **Highlights**

- 17 • Cinta Senese is a local Italian pig breed with a belted coat colour
- 18 • The value of its meat production chain should be protected from frauds
- 19 • We analyzed the *KIT* gene to identify a suitable DNA marker for this purpose
- 20 • A single nucleotide polymorphism (g.43597545C>T) was almost fixed in Cinta Senese
- 21 • Probability to correctly assign an unknown meat sample to Cinta Senese was ~1.00.

22 **Abstract**

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

39

40 **Keywords:** SNP; coat colour gene; *KIT*; traceability; authenticity; pig breed

41 **1. Introduction**

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

53 Cinta Senese is an autochthonous pig breed that is reared under extensive or semi-extensive
54 management systems, mainly in the Tuscany region (Italy). The breed is characterised by a typical
55 black with a white belted coat colour pattern. Its origin dates back to the XIII-XIV century when
56 belted pigs were raised in the hills around Siena as demonstrated by a famous painting of Ambrogio
57 Lorenzetti in the Palazzo Comunale of Siena (a.D. 1340). The importance of this breed was
58 recognized with the early constitution of the breed national herdbook that worked from the 1936 to
59 1966 and then by a regional herdbook that was active since 1976. Just after the second world war
60 the breed was also used to produce grey or “tramacchiati” crossbred pigs by crossing with white
61 pigs that were fed with whey produced by the cheese factories of Pianura Padana in the North of
62 Italy. This use was stopped by the transportation ban of the pigs due to an outbreak of diseases in
63 1968. Since then the number of animals of this breed dropped down, almost leading to the
64 extinction of the breed. At the end of the eighties a few projects started the recovery of this breed
65 and in 1997 the national pig breeders herdbook preliminarily re-activated a section dedicated to
66 Cinta Senese to promote conservation programs that made it possible to constitute a definitive

67 herdbook section for this breed in 2001 (ANAS, 2015; Franci et al., 2007). These alternate periods
68 influenced the number of Cinta Senese heads: the number increased reaching about 160,000 in the
69 fifties, then it decreased reaching the lowest number of 81 sows and 3 boars recorded in 1986 and
70 after conservation programs the number of pigs raised to about 5000 heads (Franci et al., 2007;
71 Raimondi, 1954). At present about 900 sows and 150 boars are registered in the National Herdbook
72 (ANAS, 2015). The current stabilized number is supported by the constitution of a Protected
73 Designation of Origin (PDO) brand for Cinta Senese meat in 2011 and the development of the Cinta
74 Senese Consortium (Consorzio di Tutela della Cinta Senese). This consortium and the PDO
75 contributed to the visibility of Cinta Senese products and to the added value of the meat of this
76 breed that should be defended from potential frauds.

77 Coat colour is one of the most important traits that differentiate livestock breeds (Fontanesi,
78 2009). DNA markers associated with coat colours in different livestock species have been already
79 used to authenticate mono-breed dairy and meat products (D'Alessandro et al., 2007; Russo et al.,
80 2007; Fontanesi et al., 2010, 2011).

81 As already mentioned, Cinta Senese pigs are characterised by a typical belted coat colour that
82 can be the basis for the development of DNA markers useful for the authentication of Cinta Senese
83 PDO products. The belted allele, in the past thought to be caused by a specific coat colour locus, is
84 one allele of the *Dominant white (I)* locus series that lists several alleles derived by complex
85 mutations in the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) gene
86 (whose product is involved in the migration of the melanoblast), namely copy number variations
87 (CNV) and a splice site mutation (Fontanesi et al., 2010; Johansson Moller et al., 1996; Marklund et
88 al., 1998; Pielberg et al., 2002). The combination of these mutations produces the classical white
89 coat colour phenotype (CNV and the splice site mutation) or the patch phenotype (CNV). The
90 molecular basis of the roan or I^d allele is not completely known even though it is due to variants
91 affecting the *KIT* gene (Fontanesi et al., 2010). The belted allele was suggested to be derived by an
92 uncharacterised regulatory mutation in the *KIT* gene, as it was not associated to any duplication of

93 the *KIT* gene described for other *I* alleles (Giuffra et al., 1999). Rubin et al. (2012) reported that the
94 belted allele could be due to duplication events in the promoter region. We recently characterised
95 different *KIT* gene haplotypes by Sanger sequencing in several cosmopolitan and local pig breeds
96 including a few Cinta Senese pigs and identified potential breed informative haplotypes (Fontanesi
97 et al., 2010). In this study we further analyzed the *KIT* gene and identified a DNA marker that, by
98 comparing 11 different pig breeds and one wild boar population, was useful to design a simple
99 genotyping test for the authentication of Cinta Senese meat.

100

101 **2. Materials and methods**

102 *2.1. Animals and DNA extraction*

103 DNA was extracted from blood samples, liver and muscle specimens and hair roots collected
104 from a total of 602 pigs of 11 different breeds (110 Cinta Senese; 105 Italian Large White; 52
105 Italian Landrace; 86 Italian Duroc; 32 Pietrain; 16 Hampshire; 50 Mora Romagnola; 47 Casertana;
106 50 Apulo Calabrese; 42 Nero Siciliano; and 12 Meishan) and one wild boar population (29 animals)
107 for a total of 631 animals. Samples were mainly obtained from previous projects (Fontanesi et al.,
108 2010, 2014). Novel blood samples were collected during slaughtering in commercial abattoirs.
109 DNA extraction was carried out using a standard phenol-chloroform protocol (Sambrook et al.,
110 1989) or using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI,
111 USA), following the manufacturer instructions.

112

113 *2.2. PCR analysis*

114 PCR was carried out using two primer pairs. The first primer pair (forward: 5'-
115 CCTCGCAGCAGGAGCAGT-3'; reverse: 5'-CTCAGGGCTGAGCATTCG-3') was used to
116 amplify a fragment of 388 bp encompassing a portion of intron 17, exon 18, intron 18 and a portion
117 of exon 19 of the porcine *KIT* gene that was used to re-sequence this gene region in 12 Cinta Senese
118 pigs to confirm previous sequencing data obtained by Fontanesi et al. (2010). The second primer

119 pair (forward: 5'-TGAACATTGCTGACTCCCCT-3'; reverse: 5'-
120 TGCATTTTACCTAAAGAGAAGAGC-3') was used to amplify a fragment in all 631 animals.
121 The amplicon of 157 bp was used for the PCR-RFLP analysis described below. The amplification
122 reactions were cycled in a 2720 Life Technologies thermal cycler (Life Technologies, Foster City,
123 CA, USA) with the following steps: 5 min at 95 °C; 35 amplification cycles of 30 sec at 95 °C, 30
124 sec at 56 °C, 30 sec at 72 °C; 10 min at 72 °C. The final reaction volume was of 20 µL and
125 included: 50-100 ng of template DNA, 1 U of Taq DNA polymerase (AmpliBioTherm Taq DNA
126 polymerase, Fisher Molecular Biology, Trevose, PA, USA; or EuroTaq DNA polymerase,
127 EuroClone Ltd., Paington, Devon, UK); 1X PCR buffer; 2.5 mM dNTPs; 10 pmol of each primer;
128 2.0 mM of MgCl₂.

129

130 *2.3. Sequencing and haplotype analysis*

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

144

145 2.4. Genotyping and data analyses

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

155 Hardy-Weinberg equilibrium of the genotyped SNP in the analysed populations was tested
156 using the HWE software program (Linkage Utility Programs, Rockefeller University, New York,
157 NY, USA). Pairwise allele and genotype frequency differences between Cinta Senese breed and all
158 other populations were evaluated using absolute delta (δ) allele frequency differential. GENEPOP
159 software version 4.0.7 (Rousset, 2008) was used to calculate population pairwise F_{st} genetic
160 distance and G genic differentiation for each population pair (exact G test; Markov chain
161 parameters were: Dememorisation: 10000; Batches: 100; Iterations per batch: 5000). GenAIEx 6.5
162 software (Peakall and Smouse, 2012) was used to calculate population assignment of the pigs to the
163 12 populations using the leave one out option. Probability to incorrectly assign an unknown meat
164 sample to populations different from Cinta Senese or crossbred products of this breed versus all
165 other populations (error rate) was calculated using the frequency of occurrence of Cinta Senese pigs
166 carrying allele C. *Vice versa* probability to correctly assign an unknown meat sample to Cinta
167 Senese (P_{CS}) was calculated using the following formula:

168
$$P_{CS} = 1 - (f_{TT} + f_{CT}) \quad (1)$$

169 where f_{TT} and f_{CT} are the frequency of occurrence of pigs with genotype TT or CT in the other
170 populations.

171

172 **3. Results**

173 *3.1. Identification and analysis of a breed-informative SNP*

174 Haplotype analyses of sequencing data reported by Fontanesi et al. (2010) who sequenced the
175 *KIT* gene in 35 pigs of different breeds (including 6 Cinta Senese pigs) showed that putative Cinta
176 Senese informative haplotypes could be identified (Figure 1). From these preliminary data, only two
177 haplotypes were observed in Cinta Senese pigs (Haplotype 9 and Haplotype 24). However
178 Haplotype 24 was identified in just one animal of this breed (in heterozygous condition with
179 Haplotype 9). In addition, based on this information, it seemed that just a fragment of this gene,
180 including exon 18 and part of exon 19 (Figure 2) could capture most of Haplotype 9 sequence
181 information due to the presence of a tag marker (g.43597545C>T SNP; a synonymous SNP on exon
182 18). Resequencing 24 other Cinta Senese haplotypes (from 12 unrelated Cinta Senese pigs), we
183 confirmed that obtained sequences were the same as already reported for the most frequent Cinta
184 Senese haplotype. Allele T of the tag g.43597545C>T marker was present in Cinta Senese pigs
185 whereas allele C was present in all other haplotypes except in one rare haplotype observed in a Nero
186 Siciliano pig (as reported from the previous data ; Fontanesi et al., 2010; Figure 1). To further
187 validate these results and to identify a marker that could be useful to authenticate Cinta Senese meat
188 products we set up a PCR-RFLP genotyping protocol to analyze a larger number of animals (a total
189 of 631 pigs from different populations were genotyped). The digestion of the amplified product
190 with *DdeI* produced two fragments of 93 + 64 bp when the amplicon contained allele T whereas
191 when allele C was present the fragment of 157 bp remained undigested (Figure 3).

192 Allele and genotype frequencies at the g.43597545C>T SNP were obtained from 11 pig
193 breeds including Cinta Senese and four other local Italian pig breeds (Mora Romagnola, Casertana,
194 Apulo Calabrese and Nero Siciliano), three commercial heavy pig breeds (Italian Large White,
195 Italian Landrace and Italian Duroc), two cosmopolitan breeds (Pietrain and Hampshire, the other
196 belted breed included in this study), one Chinese breed (Meishan) and a European wild boar

197 population sampled in Italy (Table 1). None of the populations in which at least two alleles of the
198 g.43597545C>T SNP were detected were in Hardy-Weinberg disequilibrium. Allele T was the most
199 frequent in Cinta Senese pigs (95.9%). The same allele was the most frequent in the other belted
200 breed (Hampshire) included in our survey (89.9%). In all other populations, allele T was not
201 identified (Italian Large White, Italian Landrace, Pietrain, Mora Romagnola, Apulo-Calabrese,
202 Meishan and European wild boars, in which only allele C was detected) or its frequency was $\leq 6\%$
203 (Italian Duroc, Casertana and Nero Siciliano). Comparing the frequency of allele T in Cinta Senese
204 vs all other populations (Table 1), δ was ≥ 0.90 in all comparisons (δ was equal to 0.899 in Nero
205 Siciliano) except against the Hampshire breed ($\delta = 0.084$). The assignment test indicated that 91.8%
206 of Cinta Senese pigs could be correctly assigned to their breed based on just the genotyped SNP
207 whereas for all other breeds this test assigned only 25% of Hampshire pigs to the correct breed and
208 for all other breeds assignment was 0%, mainly because the genotyped SNP was not very
209 informative or was fixed for allele C.

210 Pairwise F_{st} measure based on the g.43597545C>T SNP indicated that all comparisons of
211 Cinta Senese breed against all other breeds and populations were highly significant ($P < 0.0001$) or in
212 the case of Hampshire the comparison identified closeness with the Cinta Senese breed but was still
213 significant ($P < 0.05$) due to the higher frequency of allele C (0.125) in this cosmopolitan breed
214 (Table 2). Genic differentiation for each population pair (exact G test) including Cinta Senese was
215 highly significant for all other populations except against the Hampshire pigs confirming indirectly
216 the results of the pairwise population matrix of mean genotypic genetic distance (Table 2).

217

218 *3.2. Usefulness of the KIT g.43597545C>T SNP to differentiate Cinta Senese meat*

219 Based on the genotyping data produced for the g.43597545C>T SNP it is interesting to
220 evaluate if this *KIT* gene marker could be useful to differentiate Cinta Senese meat from meat of
221 other pig breeds and from wild boars. In this first analysis, we will exclude the other belted breed

222 (Hampshire) investigated in this study. The Hampshire breed is not present in Italy and it should not
223 be a problem for animals and meat coming from the same country in which Cinta Senese is raised.

224 None of the analysed Cinta Senese pigs had genotype CC. That means that if genotyping
225 results obtained from meat of unknown origin produce genotype CC, origin from Cinta Senese can
226 be excluded with high confidence. If we assume that there is no selection against the CC genotype
227 in Cinta Senese pigs, the frequency of CC animals in this breed would be very low and equal to the
228 square of the frequency of allele C, according to the formula of Hardy Weinberg equilibrium (0.041
229 $\times 0.041 = 0.00168$). This value would be the error rate that we might have when the genotyping test
230 produces CC. However, we should consider that animals of this genotype, based on results obtained
231 in this study and from our previous work (Fontanesi et al., 2010), might not have the classical
232 belted coat colour pattern. This is because allele T can be considered the “belted allele”. Therefore,
233 the real situation should consider a selection against the genotype CC in the Cinta Senese breed, as
234 animals may not have the characteristic belted trait that is used to register Cinta Senese pigs as
235 animals of this breed.

236 If we consider the case in which the genotyping result of a meat of unknown origin is CT, the
237 error rate in assigning this meat to a hybrid pig (obtained by crossing Cinta Senese animals with
238 genotype TT with other animals with genotype CC) different from Cinta Senese is equal to the
239 frequency of CT pigs in the Cinta Senese population (0.089).

240 If we suppose that our comparison would only include pigs of the investigated breeds
241 (excluding Hampshire) we should add information on the frequency of the CT genotype in all other
242 groups of pigs (Table 1). In this case we could also consider the frequency of this genotype to
243 define the error rate in assigning meat of unknown origin with genotype TT to a hybrid pig
244 (obtained by crossing Cinta Senese animals with genotype TT with other animals with genotype
245 CT) different from Cinta Senese is equal to half the frequency of the CT genotypes in the other
246 populations, considering that crossing TT x CT, only half of the F1 animals would have genotype
247 TT. In this way, based on a total of 505 pigs deduced from Table 1 (all genotyped animals

248 excluding Cinta Senese and Hampshire) the number of CT non-Cinta Senese pigs was 15; therefore
249 $(15/505)/2 = 0.015$, is the error rate for this specific question.

250 *Vice versa* probability to correctly assign an unknown meat sample to Cinta Senese (P_{CS})
251 obtained from the data extracted from Table 1 could be $P_{CS} = 1 - 0.0317 = 0.9683$ that derives from
252 the formula (1) of the Materials and methods section in which $f_{TT} = 1/505$ and $f_{CT} = 15/505$, where
253 1 is the only TT individual identified in the Duroc breed, 15 is the number of CT animals identified
254 in Duroc (n. = 6), Casertana (n. = 4) and Nero Siciliano (n. = 5), and 505 is the total number of
255 analysed animals, excluding Cinta Senese and Hampshire.

256 Following these procedures it is possible to easily calculate pairwise statistics for all breeds
257 against Cinta Senese (data not shown). Considering that potential frauds would not be originated by
258 substituting meat coming from other local breeds (that are the only ones that have the T allele,
259 excluding the Duroc and the Hampshire breeds) but only using cheaper meat originated from
260 commercial populations or breeds that are usually of white belted coat colour and in which allele T
261 is not present (or it could be present at a very low frequency, that was not possible to detect in our
262 survey), i) P_{CS} would be 1 and ii) the error rate in assigning to Cinta Senese a meat with genotype
263 TT in case there would be doubts from its possible hybrid origin would be equal to 0.00.

264

265 **4. Discussion**

266 Authentication of meat products obtained from DNA-based approaches is becoming more
267 precise and powerful considering the large amount of genomics data that is currently available and
268 that will be available in the future to extract useful information to answer a large number of new
269 questions and problems arising in this field (Bertolini et al., 2015). One of the most challenging
270 problems is the authentication of mono-breed labelled products usually obtained with local and less
271 productive breeds (Fontanesi, 2009; Montowska and Pospiech, 2012). The problems arise by the
272 fact that it is difficult to identify breed-specific markers as animals of different breeds can
273 interbreed producing fertile hybrids and, for this reason, might share a large number of common

274 variants. Methods for the authentication of breed-specific products are key tools to defend the added
275 economic value of these products that is the strategy that can obtain a sustainable conservation of
276 local animal genetic resources, as part of an integrated development of their production chains
277 (Fontanesi, 2009).

278 Local pig breeds represent an important resource that should be preserved and distinguished
279 to generate economic values for niche markets that are based on their meat products. Cinta Senese
280 products are probably one of the most valuable examples of niche pork production chain (Pugliese
281 and Sirtori, 2012).

282 In this study, mining data obtained in genes affecting breed specific traits (in our case, coat
283 colour), we identified a DNA marker in the *KIT* gene (g.43597545C>T) that can be useful to
284 distinguish meat from belted pigs (the characteristic trait differentiating Cinta Senese pigs from
285 many other pig breeds and commercial populations as well as from wild boars). Allele T was almost
286 fixed in this breed (0.959) and for this reason can be considered its breed-characteristic allele,
287 associated with the belted phenotype. This allele was also the most frequent in Hampshire (0.875),
288 that is the other belted pig breed included in this study. However, at present Hampshire is not raised
289 in Italy (only a few animals of this breed might be present in Italy), therefore there is no risk of
290 substitution of Cinta Senese meat with meat obtained from Hampshire pigs. A potential risk could
291 be derived by imported Hampshire meat but this is a remote possibility for the fact that pure
292 Hampshire populations are maintained in breeding stocks for crossbreeding programs and are not
293 commonly used for large commercial productions of final slaughtered pigs. Anyway, in this case
294 the cost of the fraud would be high and not economically convenient.

295 Allele T was also observed in a few other breeds (Casertana, Nero Siciliano and Italian
296 Duroc) in which it segregates at very low frequency. Only one TT animal, that apparently not had a
297 belted phenotype, was observed in a non-belted breed. It could be possible that another very rare
298 haplotype including allele T would be present in a few breeds or populations, as also suggested by
299 Fontanesi et al. (2010). Moreover, the belted haplotype is also expected to segregate in non-belted

300 breeds as deduced by the results of crossbreeding programs that sometimes produce belted pigs.
301 However, the very low frequency of allele T in other pig populations is not a big problem for the
302 authentication of Cinta Senese products. This is due to the fact that Casertana and Nero Siciliano
303 are local pig populations for which there is no convenience in using their meat to substitute Cinta
304 Senese pork products and usually pure Duroc pigs are not produced on large scale, as this breed is
305 constituted by a small nucleus used to produce sires useful for crossbreeding programs. Therefore
306 the frequency of the CT genotype in these breeds could not be considered to calculate the error rate
307 and P_{CS} derived by this SNP for Cinta Senese products. On the other hand, the low frequency of the
308 alternative C allele in Cinta Senese does not substantially affect error rate and P_{CS} in this pig breed.
309 The presence of this allele in Cinta Senese (but also in Hampshire) can indirectly confirm what is
310 obtained from crossbreeding within the breed that sometimes produces piglets without the typical
311 belted phenotype.

312 Other studies have investigated the possibility to authenticate mono-breed pork products or to
313 distinguish meat of domesticated pig breeds from meat of wild boars using DNA markers. For
314 example, markers in genes affecting coat colour (*MC1R*; *OCA2*; and *KIT*) or other phenotypic traits,
315 like vertebral number (*NR6A1*), that are breed or population specific traits, have been already
316 proposed to this purpose (Carrión et al., 2003; Chung and Chung, 2010; Crovetti et al., 2007;
317 D'Alessandro et al., 2007; Fernández et al., 2004; Fontanesi et al., 2005, 2014; Okumura et al.,
318 2000). Other approaches have used a large number of SNPs derived from the Illumina Porcine
319 SNP60 BeadChip array (that can genotype more than 60,000 SNPs) or identified by next generation
320 sequencing of breed specific DNA pools to identify a subset of 96 SNPs (Wilkinson et al., 2012) or
321 193 SNPs (Ramos et al., 2011) for breed genetic discrimination among several pig breeds using
322 different statistical approaches. Panels of microsatellites were also proposed for the same aim or to
323 identify the level of admixture composition between different breeds (García et al., 2006; Oh et al.,
324 2014). However the use of many markers for authentication of breed specific products has some
325 practical and cost-limiting aspects to be solved for routine applications. A few other attempts based

326 on coat colour gene markers and multilocus microsatellite genotyping were also tested for
327 traceability of Cinta Senese products. In particular, Crovetto et al. (2007) analysed three *MC1R*
328 polymorphisms, one of which can distinguish Duroc pigs from black pigs (Kijas et al., 1998), and
329 the duplication breakpoint test for the *KIT* gene that should give positive results only in white pigs
330 (Giuffra et al., 2002). However, these markers have some limits derived by the fact that Cinta
331 Senese breed is not fixed (or not almost fixed) for only one *MC1R* allele (Crovetto et al., 2007;
332 Fontanesi et al., 2005). In addition, as the test for the duplication breakpoint of the *KIT* gene is
333 designed to have an amplified product in case there is a duplication of the *KIT* gene (usually in
334 white pigs) or absence of amplification in case there is no duplication (all other pigs with different
335 coat colours), it could be possible that the absence of amplification might be derived by PCR failure
336 that would prevent the correct identification of the type of tested pigs. To avoid this problem, a
337 multiplex PCR should be designed including a control amplified fragment that should always be
338 produced in any types of pigs (D'Alessandro et al., 2007; Fontanesi et al., 2010). Anyway, the
339 duplication breakpoint *KIT* gene test cannot give specific indications about the breed of the pigs.
340 Scali et al. (2012) proposed to use genotype information from 18 microsatellites to differentiate
341 Cinta Senese pigs from Landrace, Large White, Large White x Landrace and Landrace x Cinta
342 Senese pigs. However, their approach was not statistically supported as just 3 or 4 pigs for the other
343 breeds or populations were included in the study that did not report the use of any standard sample
344 to refer microsatellite allele size. In addition, more than one PCR and capillary electrophoresis
345 might be needed to obtain the multilocus microsatellite information, even if these details were not
346 reported in their work (Scali et al., 2012).

347 Our method based on the analysis of just one SNP (g.43597545C>T) that is highly
348 informative for belted pigs can directly provide information from the type of pigs from which the
349 meat is originated and is much more precise, cheaper and useful than the methods reported above
350 based on *MC1R* and duplication breakpoint *KIT* gene analysis (Crovetto et al., 2007; Fontanesi et
351 al., 2005) or microsatellite genotyping (Scali et al., 2012).

352

353 **5. Conclusions**

354 We have identified an SNP that is useful to distinguish meat of Cinta Senese pigs from meat
355 of other non-belted pigs. This marker can be easily genotyped by PCR-RFLP using basic
356 instruments commonly available in a molecular genetics laboratory. Therefore, it can be considered
357 as an important tool to defend Cinta Senese pork chain from frauds. In addition, as this marker
358 might capture the belted coat colour phenotype in pigs, it could be used to fix the belted phenotype
359 in Cinta Senese population reducing the out-of-type animals in this breed obtained sometimes by
360 crossing Cinta Senese pigs. Moreover, it will be interesting to evaluate if this marker could be
361 associated with the belted phenotype in other local belted pigs that are present in Europe (i.e.
362 Schwäbisch-Hällisches in Germany and Krškopoljski in Slovenia) and for which mono-breed
363 products have been already proposed or could be marketed as a possible way to improve economic
364 incomes for the farmers.

365

366 **Conflict of interest statement**

367 The authors declare that there are no conflicts of interest.

368

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376

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476

477 **Figure captions**

478

479 **Figure 1.** *KIT* gene haplotypes identified in different pig populations (D = Italian Duroc; CS =
480 Cinta Senese; G = Gray pigs; H = Hampshire; LW = Italian Large White; M = Meishan; NS = Nero
481 Siciliano; P = Pietrain; WB = Wild boar) as defined in Fontanesi et al. (2010) with indicated the
482 allele of the tag marker (g.43597545C>T). Haplotypes identified in Cinta Senese pigs are
483 evidenced.

484

485 **Figure 2.** Resequenced *KIT* gene region including the tag marker g.43597545C>T (within squared
486 brackets) and encompassing part of intron 17, exon 18, intron 18 and part of exon 19. Exon regions
487 are indicated in bold and evidenced. Primer regions are underlined.

488

489 **Figure 3.** PCR-RFLP patterns obtained genotyping the g.43597545C>T SNP (M = molecular DNA
490 ladder; the genotypes are indicated above each gel line).

491

492 **Table 1.** Several statistics and data obtained for the g.43597545C>T single nucleotide
 493 polymorphism of the *KIT* gene in different pig breeds and in wild boars.

494

| Breed/population | No. of pigs | Genotypes (no. of pigs) | | | Allele frequencies | | HWE ¹ | δ^2 |
|---------------------|-------------|-------------------------|----|-----|--------------------|-------|------------------|------------|
| | | CC | CT | TT | C | T | | |
| Cinta Senese | 110 | 0 | 9 | 101 | 0.041 | 0.959 | 0.535 | - |
| Italian Large White | 105 | 105 | 0 | 0 | 1.000 | 0.000 | - | 0.959 |
| Italian Landrace | 52 | 52 | 0 | 0 | 1.000 | 0.000 | - | 0.959 |
| Italian Duroc | 86 | 79 | 6 | 1 | 0.953 | 0.047 | 0.145 | 0.912 |
| Pietrain | 32 | 32 | 0 | 0 | 1.000 | 0.000 | - | 0.959 |
| Hampshire | 16 | 0 | 4 | 12 | 0.125 | 0.875 | 0.449 | 0.084 |
| Mora Romagnola | 50 | 50 | 0 | 0 | 1.000 | 0.000 | - | 0.959 |
| Casertana | 47 | 43 | 4 | 0 | 0.957 | 0.043 | 0.673 | 0.916 |
| Apulo-Calabrese | 50 | 50 | 0 | 0 | 1.000 | 0.000 | - | 0.959 |
| Nero Siciliano | 42 | 37 | 5 | 0 | 0.940 | 0.060 | 0.574 | 0.899 |
| Meishan | 12 | 12 | 0 | 0 | 1.000 | 0.000 | - | 0.959 |
| European wild boars | 29 | 29 | 0 | 0 | 1.000 | 0.000 | - | 0.959 |

495

496 ¹ Hardy Weinberg Equilibrium (P value).

497 ² Absolute delta (δ) allele frequency differential of the T allele between Cinta Senese and all other
 498 breed and populations.

499 **Table 2.** Pairwise population statistics comparing Cinta Senese data versus all other investigated
 500 breeds including F_{st} , genic differentiation (exact G test) and genotypic genetic distance

| Breeds | F_{st} (P value) | P value of the G test | Genotypic distance |
|---------------------|--------------------|-----------------------|--------------------|
| Italian Large White | 0.9580 (<0.0001) | <0.0001 | 3.755 |
| Italian Landrace | 0.9449 (<0.0001) | <0.0001 | 3.755 |
| Italian Duroc | 0.9089 (<0.0001) | <0.0001 | 3.514 |
| Pietrain | 0.9376 (<0.0001) | <0.0001 | 3.755 |
| Hampshire | 0.0532 (0.018) | 0.2315 | 0.291 |
| Mora Romagnola | 0.9443 (<0.0001) | <0.0001 | 3.755 |
| Casertana | 0.9131 (<0.0001) | <0.0001 | 3.513 |
| Apulo-Calabrese | 0.9443 (<0.0001) | <0.0001 | 3.755 |
| Nero Siciliano | 0.9016 (<0.0001) | <0.0001 | 3.417 |
| Meishan | 0.9279 (<0.0001) | <0.0001 | 3.755 |
| European wild boars | 0.9363 (<0.0001) | <0.0001 | 3.755 |

501