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Taking advantage from phenotype variability in a local animal genetic resource: identification of genomic regions associated with the hairless phenotype in Casertana pigs

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2 *variability in a local animal genetic resource: identification of genomic regions associated with the*
3 *hairless phenotype in Casertana pigs” by Giuseppina Schiavo, Francesca Bertolini, Valerio Joe*
4 *Utzeri, Anisa Ribani, Claudia Geraci, Laura Santoro, Cristina Óvilo, Ana I. Fernández, Maurizio*
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8

9 **SHORT COMMUNICATION**

10

11 **Taking advantage from phenotype variability in a local animal genetic resource: identification**
12 **of genomic regions associated with the hairless phenotype in Casertana pigs**

13

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31

32 **Running title:** Hairless in Casertana pigs

33 **Summary**

34 Casertana is an endangered autochthonous pig breed (raised in the Central-South of Italy) that
35 is considered the descendant of the influential Neapolitan pig population that was used to improve
36 British breeds in the 19th century. Casertana pigs are characterized by a typical, almost complete,
37 hairless phenotype. Despite this phenotype is the characteristic trait of this breed, few Casertana pigs
38 are normal-haired. In this work, using Illumina PorcineSNP60 BeadChip data, we carried out a
39 genome wide association study (GWAS) and an F_{ST} analysis in this breed by comparing animals
40 showing the classical hairless phenotype (n. 81) versus pigs classified as haired (n. 15). Combining
41 results obtained with the two approaches, we identified two significant regions, one on porcine
42 chromosome (SSC) 7 and one on SSC15. The SSC7 region contains the *forkhead box N3 (FOXN3)*
43 gene, the most plausible candidate gene of this region, considering that mutations in another gene of
44 the same family (forkhead box N1; *FOXN1*) are responsible for the *nude* locus in rodents and alopecia
45 in humans. Another potential candidate gene, *Rho guanine nucleotide exchange factor 10*
46 (*ARHGEF10*) is located on the SSC15 region. *FOXN3* and *ARHGEF10* have been detected as
47 differentially expressed in androgenetic and senescent alopecia, respectively. This study in an
48 autochthonous pig breed contributed to shed some lights on novel genes potentially involved in hair
49 development and growth, demonstrating that local animal breeds can be valuable genetic resources
50 to disclose genetic factors affecting unique traits, taking advantage from phenotype variability
51 segregating in small populations.

52

53 **Key words:** alopecia, animal genetic resource, animal model, baldness, hairless, F_{ST} , GWAS, SNP.

54 **Text**

55 Local animal genetic resources might be characterized by specific and inheritable phenotypes
56 with relevant importance for current or potential future use in breeding programs or for many other
57 purposes, including the definition of new biological models or to understand mechanisms of
58 biological adaptations to different environments (Leroy *et al.* 2016).

59 Casertana is an endangered autochthonous pig breed mainly raised in the Central-South of Italy,
60 accounting for about 100 boars and sows currently registered to its herd book (ANAS 2016).
61 Casertana pigs are usually raised in extensive or semi-extensive systems to produce niche pork
62 products. This local breed is considered the descendant of the influential Neapolitan breed of the late
63 18th and 19th centuries that was used to improve British pig populations from which several modern
64 commercial breeds were derived (Porter 1993). Casertana pigs are characterized by a black or grey
65 coat colour, wrinkled skin, forward ears, two goatlike wattles (not always present) and a typical,
66 almost complete, hairless phenotype. This later characteristic is also reported in one of its local names,
67 i.e. *Pelatella* (that means plucked or bald). Despite the hairless phenotype is the characteristic
68 phenotype of this breed, Casertana population shows some variability for this trait, including animals
69 having from almost complete absence of hairs (hairless; the most common pigs) to few animals
70 having abundant hairs (normal-haired pigs; Figure 1a). The hairless phenotype is also present in other
71 pig breeds like the Creole hairless Mexican breed (also known as Pelón Mexicano) and the black
72 hairless Iberian strains, including the Guadyerbas strain maintained as isolated population (Toro *et*
73 *al.* 2000; Lemus-Flores *et al.* 2001). Casertana and all these other hairless pigs seem historically
74 connected through exchange of pig genetic material determined by commercial activities in the 18th
75 and 19th centuries (Porter 1993), suggesting a potential common origin of the hairless phenotype.

76 Hairless or hairlessness in pigs can be better described as hypotrichosis or congenital deficiency
77 of hairs, as animals classified as “hairless” usually show a small number rather than a complete
78 absence of hairs. Roberts & Carroll (1931) were the first authors that reported a possible inheritance
79 model for this hypotrichotic condition in Mexican pigs, suggesting the presence of a monogenic factor

80 with a recessive mutated *h* allele that could give the hairless phenotype when homozygous.
81 Homozygous pigs for the wild type allele *H* might be normal-haired whereas heterozygous *Hh* pigs
82 might show an intermediate phenotype. This early study was not followed by any other genetic
83 investigations on the hairless condition in pigs. More recently, variability in the porcine *hairless* gene
84 (known as *HR*, *lysine demethylase and nuclear receptor corepressor*), located on porcine
85 chromosome (SSC) 14, was evaluated in a candidate gene approach to study the hairless phenotype
86 in Iberian pigs but no association with this trait was reported (Fernández *et al.* 2003, 2006). Mutations
87 in the *HR* gene have been shown to impair hair growth in different mammals (i.e. Stoye *et al.* 1988;
88 Ahmad *et al.* 1998; Finocchiaro *et al.* 2003). A high number of other genes in humans and rodents
89 have been implicated in abnormal hair development and hypotrichosis (Shimomura & Christiano
90 2010; Ramot & Zlotogorski 2015), making impractical a candidate gene approach to successfully
91 identify polymorphisms associated with the hairless phenotype in pig populations.

92 In this work, with the aim to restrict the number of potential causative genes involved in the
93 hypotrichotic phenotype in pigs, we carried out a genome wide association study (GWAS) and a
94 genome wide *F_{ST}* analysis in the Casertana breed by comparing animals showing the classical hairless
95 phenotype (n. 81) versus pigs classified as haired (n. 15; a quite rare phenotype in this breed), without
96 any distinction between possible different hair levels that could not be precisely recorded in outdoor
97 animals. Casertana breed offers a unique possibility to investigate this phenotype that is segregating
98 within the same population. This is one of the first population based genome wide study in a local pig
99 breed that is not only useful to characterize a breed specific trait but also to obtain basic biology
100 information that could be important to better define an interesting animal model for alopecia or related
101 phenotypes in humans (Shimomura 2012).

102 Blood or hair roots were collected from all these Casertana pigs raised in six different farms
103 (having from 5 to 49 pigs each, with unknown relationships) and extracted DNA was used for
104 genotyping with the Illumina PorcineSNP60 BeadChip v.2 (Illumina, Inc., San Diego, CA, USA)
105 interrogating 61,565 single nucleotide polymorphisms (SNPs). Genotyping data were processed with

106 PLINK 1.9 software (Chang *et al.* 2015) using the following criteria to filter SNPs: call rate >0.9,
107 minor allele frequency >0.01 and Hardy-Weinberg equilibrium $P > 0.001$. A total of 36,533 autosomal
108 SNPs assigned to a unique position in the Sscrofa11.1 genome version was then used in the GWAS
109 that was carried out by applying the univariate mixed model of GEMMA (Zhou & Stephens 2012).
110 The centered relatedness matrix calculated from SNP genotypes was included in the model to correct
111 for population stratification. Figure S1 reports the genomic inflation factor (λ) and quantile–quantile
112 (Q–Q) plot, obtained with GenABEL (Aulchenko *et al.* 2007). Figure 1b reports the Manhattan plot
113 obtained in this GWAS. At the $P < 0.05$ Bonferroni corrected level (P nominal value $< 1.37E-06$),
114 three SNPs were significant whereas at the $P < 0.1$ Bonferroni corrected threshold (P nominal value =
115 $2.74E-06$) other three SNPs were suggestively significant (Table 1). Two of these SNPs were located
116 on SSC7 (170.17 kb apart) and four on SSC15, in two distinct regions of approximately 1.14 Mb and
117 338.61 kb.

118 F_{ST} analysis was performed on the same dataset using PLINK 1.9 software. Missing SNPs were
119 imputed using the Beagle 3.3.2 software (Browning and Browning, 2009). Figure 1c reported the
120 Manhattan plot of the F_{ST} analysis. The top 0.9998 SNPs of the percentile distribution ($F_{ST}=0.345$)
121 were considered as the most divergent across the comparison and therefore retained for subsequent
122 evaluation (Table 1). A total of 8 SNPs was above the selected threshold: one on SSC4, one on SSC2,
123 two on SSC7 (170.17 kb apart), two on SSC15 (1.14 Mb apart) and two on SSC17 (32.00 kb apart).

124 The comparison among GEMMA and F_{ST} genome-wide analyses identified two overlapping
125 regions encompassing two SNPs on SSC7 and two SNPs on SSC15 that constituted the 1.14 Mb
126 region previously mentioned (Table 1). A total of eight and nine genes were annotated in the SSC7
127 and SSC15 regions, respectively (in a window ± 500 kb from the first and the last SNPs; Table 1).
128 The most plausible candidate gene in the SSC7 region was the *forkhead box N3* (*FOXP3*) gene
129 (position: 111036492-111454106 bp), that is 66.56 kb far from INRA0028322 (one of the two most
130 significant SNPs in the GWAS; Table 1). This gene has a role in the regulation of hepatic glucose
131 utilization (Karanth *et al.* 2016), craniofacial development (Samaan *et al.* 2010) and growth and

132 migration of colon cancer cells (Dai *et al.* 2017). The *FOXN3* gene was also found differentially
133 expressed in a case-control study for androgenetic alopecia in humans (Mirmirani & Karnik 2010).
134 Forkhead box proteins constitute a family of transcription factors involved in embryo and fetal
135 development and function of adult organisms (Hannenhalli & Kaestner 2009). This group of proteins
136 list about 50 members in mammals, divided in 19 subfamilies indicated with the letters from A to S
137 (Jackson *et al.* 2010; Benayoun *et al.* 2011). Among the N subfamily, forkhead box N1 (FOXN1)
138 regulates keratin gene expression and the gene is responsible for the *nude* locus in rodents (Flanagan
139 1966; Meier *et al.* 1999). Mutations in this gene determine hairlessness, alopecia and other pleiotropic
140 effects in mice and rats (Nehls *et al.* 1994) and congenital alopecia, nail dystrophy, and primary T-
141 cell immunodeficiency in humans (Frank *et al.* 1999). Therefore, considering the phylogenetic
142 relationships and the partially conserved domains between the *FOXN1* and *FOXN3* genes (Benayoun
143 *et al.* 2011), it seems plausible that FOXN3 might have conserved similar regulatory functions of
144 FOXN1 that could explain the effect of this SSC7 chromosome region on the hairless phenotype in
145 Casertana pigs. This indication might contribute to understand the involvement of forkhead box
146 proteins in hair development and, if confirmed by functional studies, adds another candidate gene to
147 the list of those potentially involved in alopecia and baldness.

148 No strong candidate gene could be identified in the SSC15 region. A possible candidate could
149 be *Rho Guanine Nucleotide Exchange Factor 10 (ARHGEF10)* gene. ARHGEF10 is involved in
150 neural morphogenesis and connectivity and in the regulation of small RhoGTPases (Verhoeven *et al.*
151 2003). The *ARHGEF10* has been reported to be differentially expressed in a case-control study of
152 senescent alopecia in human (Mirmirani & Karnik 2010), supporting, to some extent, its possible role
153 in the hairless phenotype in the Casertana breed. According to the available functional information,
154 no other gene in the two identified regions might be involved in hair or follicle development or
155 phenotypes similar to the hairless condition we investigated.

156 The combination of the GWAS and F_{ST} results with the annotated gene functions was useful to
157 draft a possible biological explanation of the hairless phenotype in Casertana pigs and to identify

158 significant regions, excluding other regions that reached or were close to the defined thresholds in
159 one or the other genome wide investigation methods derived by several confounding factors that
160 could not be better managed in our study (i.e. genetic drift, population structure, ascertain bias of the
161 SNP chip tool). However, the results obtained in this breed, even if based on a small group of pigs
162 with normal-haired phenotype (that is a quite rare in this breed) in contrast with the hairless group,
163 seems to support the presence of more than one locus affecting this trait. A few of the associated
164 genomic regions contain candidate genes that, based on their function or inferred function may be
165 involved in the hypotricotic condition of the Casertana pigs, with the hypothesis that this trait might
166 be more complex than previously suggested.

167 This work demonstrated that endangered animal genetic resources could be investigated to
168 disclose genetic factors affecting unique traits taking advantage from phenotype variability
169 segregating within a small population. Other investigations are needed to refine these results obtained
170 in Casertana and to evaluate if the hairless condition in other pig breeds is derived by the same genetic
171 factors identified in this study.

172

173 **Competing interests**

174 The authors declare that they do not have competing interests. Data reported in this work can be
175 shared after signature of an agreement on their use with University of Bologna.

176

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183

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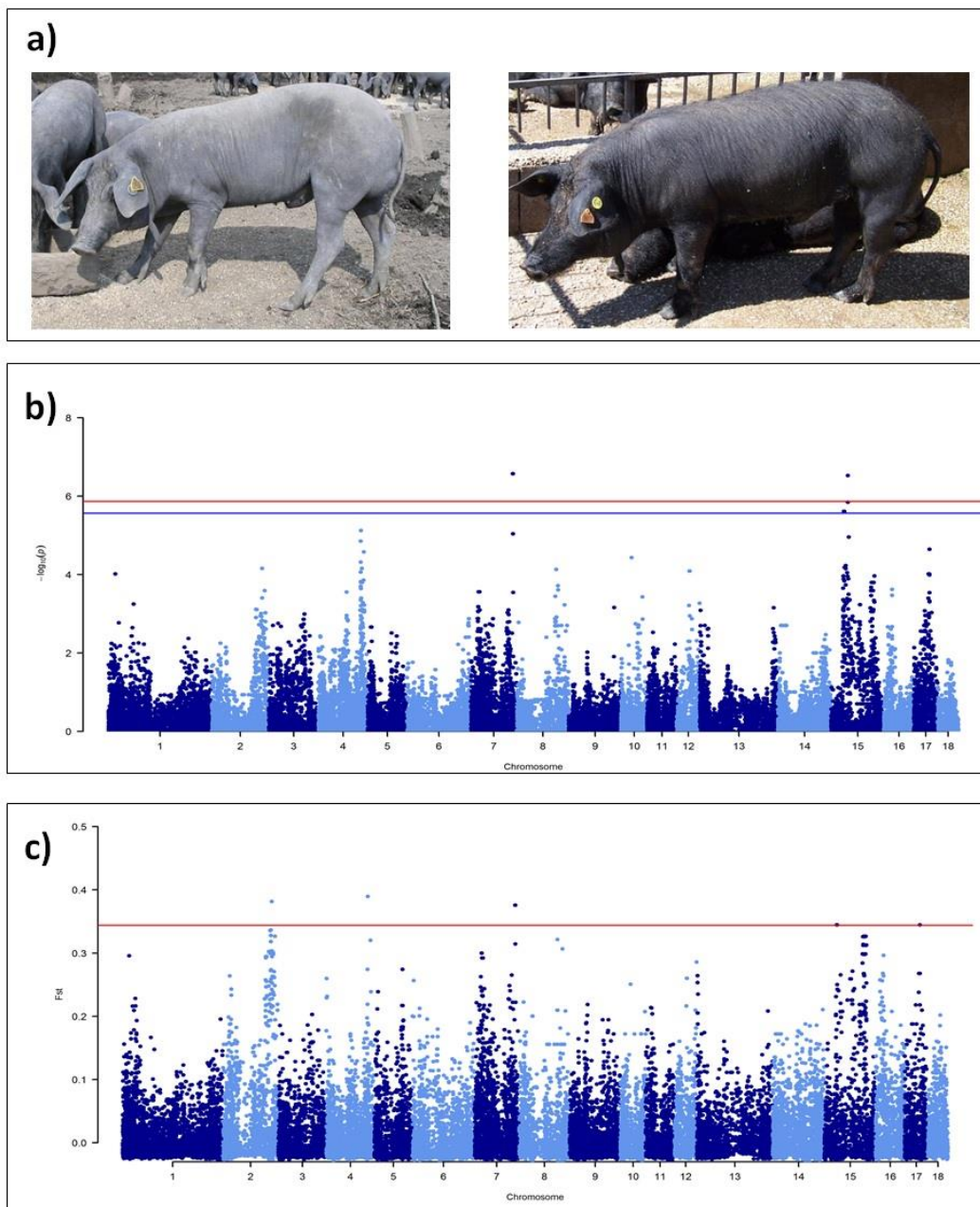
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250 **Figure 1.** Casertana pigs and results of the genome wide association study (GWAS). a) Casertana
251 pigs with the hairless (left) and haired (right) phenotypes. b) Manhattan plot of the GWAS results
252 showing Bonferroni significant (red line: $P < 0.05$) and suggestively significant (blue line: $P < 0.10$)
253 single nucleotide polymorphisms (SNPs; thresholds are Bonferroni corrected P values). c) F_{ST} plot.
254 Single nucleotide polymorphisms above the red line ($F_{ST} = 0.345$) are the top 0.9998 SNPs.
255



256

257

258 **Table 1.** List of significant ($P < 0.05$) and suggestively significant ($0.05 < P < 0.10$; Bonferroni corrected) single nucleotide polymorphisms (SNPs)
259 obtained in the genome wide association study (GWAS) in the Casertana pigs (GEMMA) and the top 0.998 detected in the F_{ST} analysis. For the
260 overlapping regions among the two approaches, annotated genes nearby the SNPs (± 500 kb from the first to the last SNP of the region) were reported
261 (Sscrofa11.1 genome version). The candidate genes that could be involved in the hair phenotype are indicated with the “*” symbol. P , F_{ST} and
262 annotated genes are reported only for the SNPs and regions for which both P and F_{ST} values trespassed the indicated thresholds.

SSC	SNP	position	GWAS, P nominal value	F_{ST} value	Annotated genes
2	ALGA0016212	134598604	-	0.381	-
4	INRA0016870	113277535	-	0.390	-
7	INRA0028322	111520662	2.68E-07	0.376	<i>LOC106504536, PSMC1, EFCAB11, NRDE2, CALM1, TDPI, KCNK13, FOXN3*</i>
7	ALGA0044817	111690832	2.68E-07	0.376	
15	MARC0009352	33679138	2.45E-06	0.345	<i>C110257074, CLN8, KBTBD11, DLGAP2, LOC106509653, ARHGEF10*, LOC106506202, CSMD1, MYOM2</i>
15	ALGA0084906	34793592	2.45E-06	0.345	
15	H3GA0044265	44006149	3.00E-07	-	-
15	INRA0049225	44344760	1.43E-06	-	-
17	DRGA0016747	41675886	-	0.345	-
17	H3GA0049027	41643251	-	0.345	-

263
264

265 **Supplementary Material**

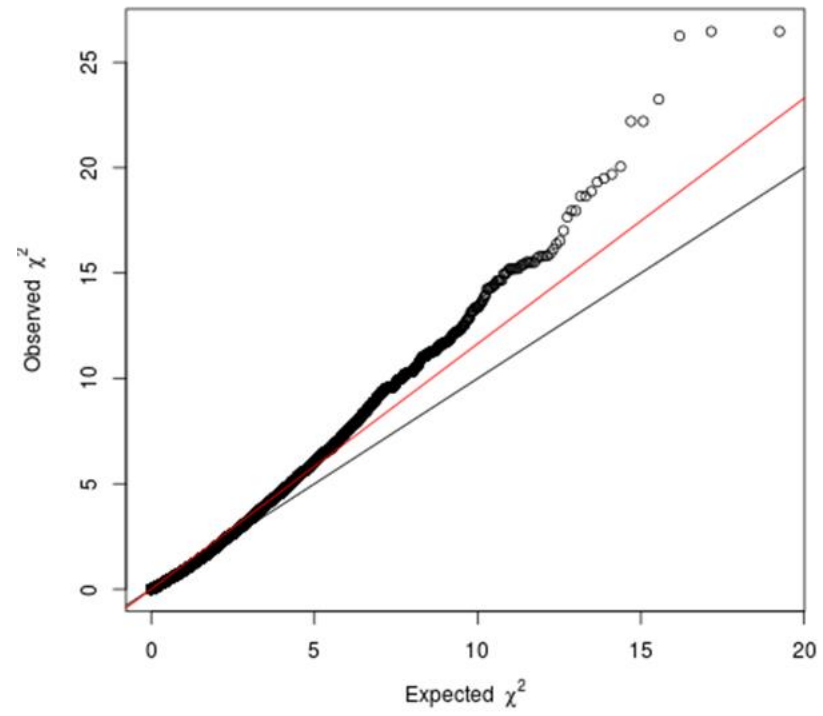
266

267 **Taking advantage from phenotype variability in a local animal genetic resource: identification of genomic regions associated with the**
268 **hairless phenotype in Casertana pigs**

269

270 **Figure S1. Q-Q plot.**

$\lambda = 1.16$



271

272

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