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SHORT COMMUNICATION

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

Giuseppina Schiavo[1,*], Francesca Bertolini[1,2;*], Valerio Joe Utzeri[1], Anisa Ribani[1], Claudia Geraci[1], Laura Santoro[3], Cristina Óvilo[4], Ana I. Fernández[4], Maurizio Gallo[5] and Luca Fontanesi[1]

[1] Department of Agricultural and Food Sciences, Division of Animal Sciences, University of Bologna, Viale Fanin 46, 40127 Bologna, Italy

[2] Department of Animal Science, Iowa State University, 2255 Kildee Hall, 50011 Ames, Iowa, USA

[3] ConSDABI - National Focal Point Italiano FAO, Contrada Piano Cappelle, 82100 Benevento, Italy

[4] Departamento de Mejora Genética Animal, Instituto Nacional de Tecnología Agraria y Alimentaria (INIA), Ctra. de la Coruña km. 7.5, 28040 Madrid, Spain

[5] Associazione Nazionale Allevatori Suini, Via L. Spallanzani 4, 00161 Roma, Italy

* These authors contributed equally to this work

Corresponding author: luca.fontanesi@unibo.it

Running title: Hairless in Casertana pigs

Summary

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

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

Text

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

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

Hairless or hairlessness in pigs can be better described as hypotrichosis or congenital deficiency of hairs, as animals classified as “hairless” usually show a small number rather than a complete absence of hairs. Roberts & Carroll (1931) were the first authors that reported a possible inheritance model for this hypotrichotic condition in Mexican pigs, suggesting the presence of a monogenic factor with a recessive mutated *h* allele that could give the hairless phenotype when homozygous. Homozygous pigs for the wild type allele *H* might be normal-haired whereas heterozygous *Hh* pigs might show an intermediate phenotype. This early study was not followed by any other genetic investigations on the hairless condition in pigs. More recently, variability in the porcine *hairless* gene (known as *HR*, *lysine demethylase and nuclear receptor corepressor*), located on porcine chromosome (SSC) 14, was evaluated in a candidate gene approach to study the hairless phenotype in Iberian pigs but no association with this trait was reported (Fernández *et al.* 2003, 2006). Mutations in the *HR* gene have been shown to impair hair growth in different mammals (i.e. Stoye *et al.* 1988; Ahmad *et al.* 1998; Finocchiaro *et al.* 2003). A high number of other genes in humans and rodents have been implicated in abnormal hair development and hypotrichosis (Shimomura & Christiano 2010; Ramot & Zlotogorski 2015), making impractical a candidate gene approach to successfully identify polymorphisms associated with the hairless phenotype in pig populations.

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

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

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

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

The comparison among GEMMA and F_{ST} genome-wide analyses identified two overlapping regions encompassing two SNPs on SSC7 and two SNPs on SSC15 that constituted the 1.14 Mb region previously mentioned (Table 1). A total of eight and nine genes were annotated in the SSC7 and SSC15 regions, respectively (in a window ±500 kb from the first and the last SNPs; Table 1). The most plausible candidate gene in the SSC7 region was the *forkhead box N3* (*FOXN3*) gene (position: 111036492-111454106 bp), that is 66.56 kb far from INRA0028322 (one of the two most significant SNPs in the GWAS; Table 1). This gene has a role in the regulation of hepatic glucose utilization (Karanth *et al.* 2016), craniofacial development (Samaan *et al.* 2010) and growth and migration of colon cancer cells (Dai *et al.* 2017). The *FOXN3* gene was also found differentially expressed in a case-control study for androgenetic alopecia in humans (Mirmirani & Karnik 2010). Forkhead box proteins constitute a family of transcription factors involved in embryo and fetal development and function of adult organisms (Hannenhalli & Kaestner 2009). This group of proteins list about 50 members in mammals, divided in 19 subfamilies indicated with the letters from A to S (Jackson *et al.* 2010; Benayoun *et al.* 2011). Among the N subfamily, forkhead box N1 (FOXN1) regulates keratin gene expression and the gene is responsible for the *nude* locus in rodents (Flanagan 1966; Meier *et al.* 1999). Mutations in this gene determine hairlessness, alopecia and other pleiotropic effects in mice and rats (Nehls *et al.* 1994) and congenital alopecia, nail dystrophy, and primary T-cell immunodeficiency in humans (Frank *et al.* 1999). Therefore, considering the phylogenetic relationships and the partially conserved domains between the *FOXN1* and *FOXN3* genes (Benayoun *et al.* 2011), it seems plausible that FOXN3 might have conserved similar regulatory functions of FOXN1 that could explain the effect of this SSC7 chromosome region on the hairless phenotype in Casertana pigs. This indication might contribute to understand the involvement of forkhead box proteins in hair development and, if confirmed by functional studies, adds another candidate gene to the list of those potentially involved in alopecia and baldness.

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

The combination of the GWAS and F_{ST} results with the annotated gene functions was useful to draft a possible biological explanation of the hairless phenotype in Casertana pigs and to identify significant regions, excluding other regions that reached or were close to the defined thresholds in one or the other genome wide investigation methods derived by several confounding factors that could not be better managed in our study (i.e. genetic drift, population structure, ascertain bias of the SNP chip tool). However, the results obtained in this breed, even if based on a small group of pigs with normal-haired phenotype (that is a quite rare in this breed) in contrast with the hairless group, seems to support the presence of more than one locus affecting this trait. A few of the associated genomic regions contain candidate genes that, based on their function or inferred function may be involved in the hypotricotic condition of the Casertana pigs, with the hypothesis that this trait might be more complex than previously suggested.

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

Competing interests

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

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

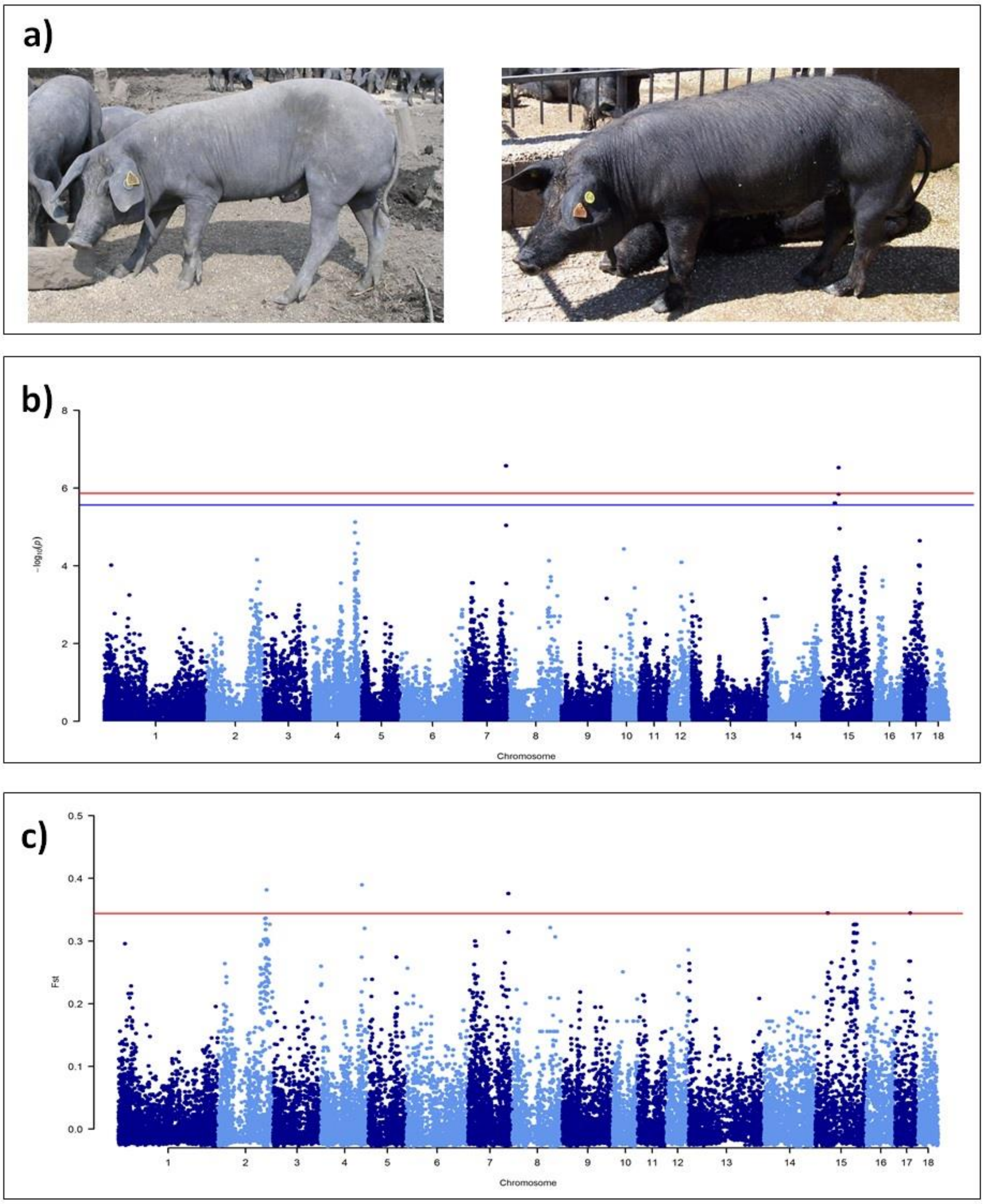


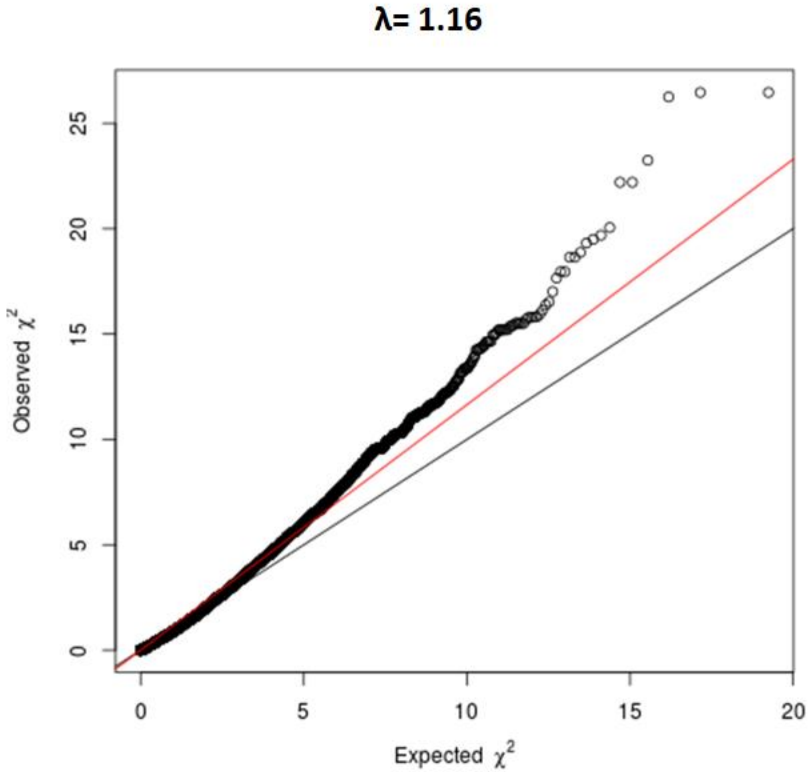
Table 1. List of significant (P<0.05) and suggestively significant (0.05<P<0.10; Bonferroni corrected) single nucleotide polymorphisms (SNPs) obtained in the genome wide association study (GWAS) in the Casertana pigs (GEMMA) and the top 0.998 detected in the F_{ST} analysis. For the overlapping regions among the two approaches, annotated genes nearby the SNPs (±500 kb from the first to the last SNP of the region) were reported (Sscrofa11.1 genome version). The candidate genes that could be involved in the hair phenotype are indicated with the "*" symbol. P, F_{ST} and 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	*LOC106504536, PSMC1, EFCAB11, NRDE2, CALM1, TDP1, KCNK13, FOXN3**
7	ALGA0044817	111690832	2.68E-07	0.376	
15	MARC0009352	33679138	2.45E-06	0.345	*C110257074, CLN8, KBTBD11, DLGAP2, LOC106509653, ARHGEF10*, LOC106506202, CSMD1, MYOM2*
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	-

Supplementary Material

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

Figure S1. Q-Q plot.


λ= 1.16
Observed χ2
Expected χ2
0
5
10
15
20
25
0
5
10
15
20