



Signatures of de-domestication in autochthonous pig breeds and of domestication in wild boar populations from *MC1R* and *NR6A1* allele distribution

A. Ribani*, V. J. Utzeri*, C. Geraci*, S. Tinarelli*[†], M. Djan[‡], N. Veličković[‡], R. Doneva[§], S. Dall'Olio*, L. Nanni Costa*, G. Schiavo*, S. Bovo*, G. Usai[¶], M. Gallo[†], Č. Radović**[†], R. Savić^{††}, D. Karolyi^{‡‡}, K. Salajpal^{‡‡}, K. Gvozdanić^{§§}, I. Djurkin-Kušec^{§§}, M. Škrlep^{¶¶}, M. Čandek-Potokar^{¶¶}, C. Ovilo*** and L. Fontanesi*

*Division of Animal Sciences, Department of Agricultural and Food Sciences, University of Bologna, Viale Fanin 46, 40127 Bologna, Italy.

[†]Associazione Nazionale Allevatori Suini, via Nizza 53, 00198 Roma, Italy. [‡]Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovica 2, 21000, Novi Sad, Serbia. [§]Association for Breeding and Preserving of the East Balkan Swine, 3 Simeon Veliki Blvd., Shumen 9700, Bulgaria. [¶]Servizio Ricerca per la Zootecnia, Agris Sardegna, Loc. Bonassai SS 291 km 18,600, 07100 Sassari, Italy. **Department of Pig Breeding and Genetics, Institute for Animal Husbandry, 11080 Belgrade-Zemun, Serbia. ^{††}Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia. ^{‡‡}Department of Animal Science, Faculty of Agriculture, University of Zagreb, Svetosimunska 25, 10000, Zagreb, Croatia. ^{§§}Faculty of Agrobiotechnical Sciences, University of Osijek, Vladimira Preloga 1, 31000, Osijek, Croatia. ^{¶¶}Kmetijski inštitut Slovenije, Hacquetova ulica 17, 1000 Ljubljana, Slovenia. ***Departamento Mejora Genética Animal, INIA, Crta. de la Coruña, km. 7,5, 28040 Madrid, Spain.

Summary

Autochthonous pig breeds are usually reared in extensive or semi-extensive production systems that might facilitate contact with wild boars and, thus, reciprocal genetic exchanges. In this study, we analysed variants in the *melanocortin 1 receptor (MC1R)* gene (which cause different coat colour phenotypes) and in the *nuclear receptor subfamily 6 group A member 1 (NR6A1)* gene (associated with increased vertebral number) in 712 pigs of 12 local pig breeds raised in Italy (Apulo-Calabrese, Casertana, Cinta Senese, Mora Romagnola, Nero Siciliano and Sarda) and south-eastern European countries (Krškopolje from Slovenia, Black Slavonian and Turopolje from Croatia, Mangalitsa and Moravka from Serbia and East Balkan Swine from Bulgaria) and compared the data with the genetic variability at these loci investigated in 229 wild boars from populations spread in the same macro-geographic areas. None of the autochthonous pig breeds or wild boar populations were fixed for one allele at both loci. Domestic and wild-type alleles at these two genes were present in both domestic and wild populations. Findings of the distribution of *MC1R* alleles might be useful for tracing back the complex genetic history of autochthonous breeds. Altogether, these results indirectly demonstrate that bidirectional introgression of wild and domestic alleles is derived and affected by the human and naturally driven evolutionary forces that are shaping the *Sus scrofa* genome: autochthonous breeds are experiencing a sort of 'de-domestication' process, and wild resources are challenged by a 'domestication' drift. Both need to be further investigated and managed.

Keywords coat colour, conservation, introgression, genetic resources, SNP, *Sus scrofa*, vertebrae

The domestication process in all livestock species has been determined by a complex series of spatial-temporal events

Address for correspondence

L. Fontanesi, Division of Animal Sciences, Department of Agricultural and Food Sciences, University of Bologna, Viale Fanin 46, 40127 Bologna, Italy.

E-mail: luca.fontanesi@unibo.it

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causing continuous genetic changes derived from population admixture and isolation that have shaped the animal genome from the corresponding ancestral wild genetic pools (Larson & Burger 2013). The reconstruction of the domestication history of the pig (*Sus scrofa*), from the earliest events (that might date back some 9000–10 000 years ago) until the constitution of the modern breeds, has largely relied on local wild boar populations that were the sources of the domestic pools (e.g. Larson *et al.* 2007, 2010; Ramos-

Onsins *et al.* 2014; Iacolina *et al.* 2016; Yang *et al.* 2017). It also seems clear that the domestication of the pig was not based on few fixed events but, rather, occurred over many millennia and included repeated admixture of domestic populations with local wild boars that, in most cases, shared the same environments (Larson & Burger 2013). The results of this continuous process determined several morphological, behavioral and physiological modifications of the pigs that satisfied the primary farmers' needs and led to the fixation of a few traits (e.g. coat colour and shape of the animals), regarded as among the first domestication-derived phenotypes (Clutton-Brock 1999).

Coat colour in pigs is largely affected by variability at the *Extension* locus. This locus is characterized by different alleles at the *melanocortin 1 receptor* (*MC1R*) gene (Kijas *et al.* 1998, 2001). Among the described *MC1R* alleles, the wild-type allele (E^+ , indicated also as allele *0101*; Fang *et al.* 2009) is the typical form in European wild boars (determining the classical grey/brown coat colour), whereas several other alleles are considered domestic variants. These include (i) alleles E^{D1} (indicated as alleles *0201*, *0202* and *0203*; of Asian origin) and E^{D2} (allele *0301*; of European origin), which cause the dominant black coat colour; (ii) allele E^P (identified also as alleles *0501*, *0502* and *0503*), which is common in spotted and completely white pigs; and (iii) allele e , which is the recessive allele producing the reddish coat colour of the Duroc breed. Most of the commercial pig breeds are fixed for one of the domestic alleles (Kijas *et al.* 1998, 2001; Fontanesi *et al.* 2010).

Another mutation affecting a domestication-selected trait has been reported in the *nuclear receptor subfamily 6 group A member 1* (*NR6A1*) gene. The variant allele (derived from a missense mutation: p.Pro192Leu) is associated with an increased number of thoracic and dorsal vertebrae (21–23 vs. 19 vertebrae) as compared to the wild-type allele (Mikawa *et al.* 2007). The positive effect of the variant allele on the number of vertebrae is, in turn, associated with increased length of the animals, more meat, greater number of teats (1–3 more teats) and thus increased reproduction potential of sows (Borchers *et al.* 2004; Mikawa *et al.* 2007). These effects indirectly determined its fixation in commercial pig breeds and lines through a directional selection pressure on these traits (e.g. Rubin *et al.* 2012; Fontanesi *et al.* 2014).

Domestic alleles at both the *MC1R* and *NR6A1* genes have been also described in wild boar populations as the result of introgression from domestic populations, probably derived by accidental crossbreeding with free-ranging domestic pigs or by deliberate crosses in captive farming systems and subsequent release of crossbred animals to improve performances of wild boar populations (e.g. Koutsogiannouli *et al.* 2010; Frantz *et al.* 2012, 2013; Fontanesi *et al.* 2014).

Local pig breeds are usually constituted by small populations that have recently (or at some time during their

developmental history) experienced bottlenecks, genetic drifts and, in some cases, introgression from other populations that contributed to increased variability and reduced inbreeding (Porter 1993). Local pig breeds are usually reared in extensive or semi-extensive production systems and are less productive than are commercial breeds (considering both performance and reproductive traits). As the populations of many local breeds are usually too small to run effective selection programmes, only conservation programmes can be considered. Polymorphisms in coat colour genes have been proposed as useful markers for the authentication of mono-breed pork products and might also be considered targets for their characterization (Kijas *et al.* 1998; D'Alessandro *et al.* 2007; Fontanesi 2009; Fontanesi *et al.* 2016).

In this study, we analysed *MC1R* and *NR6A1* gene variants in 12 local pig breeds raised in Italy and south-eastern (SE) Europe (Slovenia, Croatia, Serbia and Bulgaria) and compared the data with the genetic variability at these loci investigated in wild boar populations spread in the same three separate macro-geographic areas: one represented by Sardinia (isolated because of a geographical barrier and recently strengthened by the ban of exchange of pigs and wild boars since 1978, caused by the presence of African Swine Fever; Jurado *et al.* 2018); another constituted by the Italian peninsula, which traditionally has a continuous genetic flow with Sicily; and the third represented by the Balkan countries.

A total of 712 pigs belonging to six Italian [Apulo-Calabrese, $n = 73$; Casertana, $n = 114$; Cinta Senese (Siena Belted), $n = 80$; Mora Romagnola, $n = 74$; Nero Siciliano (Sicilian Black), $n = 70$; Sarda, $n = 58$], one Slovenian (Krškopolje, $n = 31$), two Croatian (Black Slavonian, $n = 27$; Turopolje, $n = 47$), two Serbian (Mangalitsa, $n = 47$; Moravka, $n = 47$) and one Bulgarian (East Balkan Swine, $n = 44$) autochthonous breeds were investigated (detailed information on the geographical distribution, standard coat colour, thoracic vertebrae and teat range numbers of these breeds are reported in Table S1). In addition to autochthonous pig populations, a total of 229 wild boars were sampled. Collection was opportunistic, derived by hunting or via inspection of carcasses/hunted animals by forest policemen. Of these wild boars, 139 were sampled in two isolated Italian areas (113 in the Appennini mountains in northern Italy and 26 on the island of Sardinia). The remaining 90 wild boars were from western and central Balkan countries (16 from Bosnia and Herzegovina, 10 from Croatia, 16 from Montenegro, 16 from the north of Macedonia, 17 from Serbia and 15 from Slovenia) that altogether were thereafter referred to as the SE European population. Information about the investigated wild boars is reported in Table S2. Blood, hair roots or meat were sampled from these animals. DNA was extracted using the Wizard (R) Genomic DNA Purification kit (Promega Corp.) or by standard phenol–chloroform–isoamyl alcohol extraction (Sambrook *et al.* 1989).

Five autosomal polymorphisms were genotyped: three single nucleotide polymorphisms (SNPs) and one insertion/deletion in the *MC1R* gene that, on the whole, can distinguish all major alleles at the *Extension* locus (i.e. E^+ , E^{D1} , E^{D2} , E^P and e), described by Kijas *et al.* (1998, 2001), and the missense mutation in the *NR6A1* gene (rs326780270:C>T or p.Pro192Leu), which is considered the causative mutation of the QTL for the number of vertebrae reported on porcine chromosome 1 (Mikawa *et al.* 2007). Genotyping protocols were based on PCR-RFLP, fragment analysis of PCR amplicons and on an OpenArray™ genotyping platform (details are reported in Table S3).

Genotyping results obtained in the autochthonous pig breeds and in the wild boar populations are reported in Tables S4 & S5 respectively. Allelic frequencies at the *MC1R* and *NR6A1* genes are summarized in Table 1. Shown in Fig. 1 is a principal component analysis plot based on the first two principal components (PC1 and PC2) obtained by analysing allelic frequency data using the R 'prcomp' function and a cluster representation of the analysed breeds and populations generated with the 'dist' and 'hclust' functions of R using allelic frequency information to calculate the Euclidean distance among groups (R Core Team 2018).

None of the pig breeds and none of the wild boar populations were fixed for one allele at both loci. The East Balkan Swine breed showed all *MC1R* and *NR6A1* alleles. A few breeds showed only one allele at one of the investigated genes. In particular, Turopolje was fixed for the *MC1R* E^P allele and Mangalitsa was fixed for the wild type E^+ allele, as already reported by Fang *et al.* (2009), which confirms the inference determined by its phenotypic description (Porter 1993). The fixation of the wild-type allele in Mangalitsa

could facilitate the expression of the *agouti signalling protein* (*ASIP*) gene, determining the classic black-and-tan phenotype (according to the epistatic interaction between the *MC1R* and *ASIP* genes) of this breed (Drögemüller *et al.* 2006). The wild-type allele was also observed in all Italian breeds (Sarda was the breed with the highest frequency: 30%) and in two eastern European breeds (Moravka, 3%, and East Balkan Swine, 28%). Among the Italian breeds, allele E^{D2} at the *MC1R* gene was the most frequent one (ranging from 57% in Nero Siciliano to 86% in Apulo-Calabrese) except in the Mora Romagnola breed, which showed a quite unique genetic structure with only two alleles: the highest frequency of the recessive e allele (82%) and the presence of the E^+ allele (18%). All other Italian breeds showed at least four of the five analysed *MC1R* alleles.

Allele E^{D1} was highly frequent in the Black Slavonian (88%) and quite frequent in Moravka (37%), testifying to their genetic origin or contamination with Asian-derived populations or breeds including Large Black and Berkshire pigs (which are well known to be derived by crossbreeding with Chinese pigs; Porter 1993; Megens *et al.* 2008; Gvozdanović *et al.* 2018).

Krškopolje showed a high frequency (82%) of the other dominant black allele (E^{D2}). At the *NR6A1* gene, Cinta Senese, Mora Romagnola, Krškopolje and Moravka pigs showed only the domestic allele (i.e. T). This allele was the most frequent in almost all other breeds except in the East Balkan Swine, in which allele C had a frequency of 81%, suggesting a high level of wild boar gene introgression. Based on the *MC1R* and *NR6A1* data, this breed was the closest one to the wild boar populations (Fig. 1).

Only the Sardinian wild boar population was fixed for the *MC1R* E^+ allele even though the frequency of the domestic

Table 1 Frequencies of *melanocortin 1 receptor* (*MC1R*) and *nuclear receptor subfamily 6 group A member 1* (*NR6A1*) alleles in domestic pig breeds and wild boar populations.

Breeds/populations	No. of animals	<i>MC1R</i> alleles					<i>NR6A1</i> alleles	
		E^+	E^{D1}	E^{D2}	E^P	e	C	T
Domestic pigs								
Apulo-Calabrese	73	0.05	0.00	0.86	0.03	0.06	0.23	0.77
Casertana	114	0.17	0.00	0.71	0.10	0.02	0.06	0.94
Cinta Senese	80	0.01	0.01	0.83	0.13	0.02	0.00	1.00
Mora Romagnola	74	0.18	0.00	0.00	0.00	0.82	0.00	1.00
Nero Siciliano	70	0.17	0.07	0.57	0.19	0.00	0.28	0.72
Sarda	58	0.30	0.06	0.58	0.00	0.06	0.01	0.99
Krškopolje	31	0.00	0.00	0.82	0.16	0.02	0.00	1.00
Black Slavonian	27	0.00	0.88	0.04	0.06	0.02	0.07	0.93
Turopolje	47	0.00	0.00	0.00	1.00	0.00	0.43	0.57
Mangalitsa	47	1.00	0.00	0.00	0.00	0.00	0.02	0.98
Moravka	47	0.03	0.37	0.43	0.17	0.00	0.00	1.00
East Balkan Swine	44	0.28	0.17	0.49	0.05	0.01	0.81	0.19
Wild boars								
North of Italy	113	0.92	0.00	0.01	0.05	0.02	0.98	0.02
Sardinia	26	1.00	0.00	0.00	0.00	0.00	0.88	0.12
South-East Europe	90	0.87	0.00	0.11	0.02	0.00	0.93	0.07

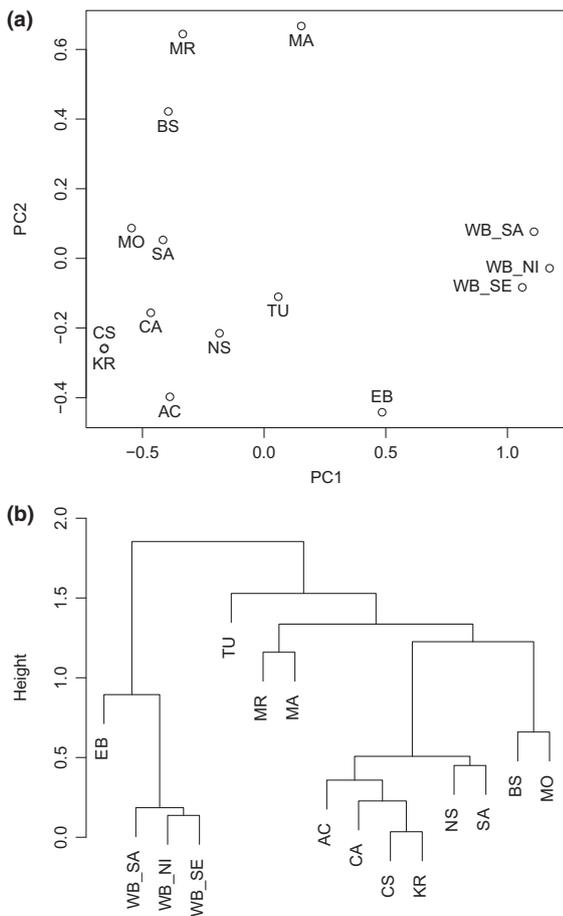


Figure 1 Graphical representations of the analysed breeds and wild boar populations based on *MC1R* and *NR6A1* data. (a) Principal component analysis plot. (b) Cluster dendrogram of the pig breeds and wild populations. AC, Apulo-Calabrese; BS, Black Slavonian; CA, Casertanta; CS, Cinta Senese; EB, East Balkan Swine; KR, Krškopolje; MA, Mangalitsa; MO, Moravka; MR, Mora Romagnola; NS, Nero Siciliano; SA, Sarda; TU, Turopolje; WB_NI, wild boars from northern Italy; WB_SA, wild boars from Sardinia; WB_SE, wild boars from south-eastern Europe.

allele at the *NR6A1* gene was 12%. This allele was also identified in the other two wild boar populations, although at lower frequencies (2% in the North Italian wild boars and 7% in the SE European group). Three (E^{D2} , E^P and e) and two (E^{D2} and E^P) *MC1R* domestic alleles were identified in the North Italian and SE European wild boar populations respectively. The most frequent domestic alleles were E^{D2} (11%) and E^P (5%) in the two populations respectively.

These results clearly indicate that autochthonous breeds have a complex history. These breeds could have recently experienced crossbreeding with other commercial breeds, which might have introduced or re-introduced heterogeneity at the *MC1R* gene. Crossbreeding is mentioned in several historical records (available for some of the investigated breeds; e.g. Mora Romagnola crossed with Duroc) and testified by orally transmitted information (e.g. Nero

Siciliano crossed with Pietrain). The presence of the *MC1R* E^+ allele could be derived by planned (in the case of Mora Romagnola) or accidental crossbreeding with wild boars, which also might be the source of introgression of the wild-type allele (i.e. C) of the *NR6A1* gene observed in local pig breeds. These genetic fluxes could be important for the re-introduction of genetic variability in pig populations that usually have quite a high level of inbreeding. On the other hand, this heterogeneity should be managed to create phenotypically uniform populations that might better match the standard traits defined by their herd books, useful for acquiring a specific identity for these pig genetic resources and making possible the application of DNA-based systems for the authentication of their products (Fontanesi 2009; Fontanesi *et al.* 2016).

Moreover, these results confirm that wild boar genetic integrity has been 'polluted' by domestic alleles (as already described; e.g. Goedbloed *et al.* 2013) that might be putatively derived from commercial pig and/or local breeds (e.g. *MC1R* E^P allele, fixed in commercial white breeds but also in Turopolje; Table 1 and Fontanesi *et al.* 2010) or from autochthonous black pig breeds (i.e. E^{D2}) that are in close contact with wild animals when raised in free-range systems (see Table 1). The introgression direction of the *MC1R* and *NR6A1* alleles from domestic pig breeds to wild boar populations was tested with the four-taxon ABBA/BABA test, computing the Patterson's D -statistic (Dasmahapatra *et al.* 2012; Martin *et al.* 2014). Four populations can be considered in the model and five plausible scenarios were tested, defining different wild boar and domestic populations in the ABBA positions (see Table S6 and Fig. S1). In these scenarios, the outgroup population (P4) was a hypothetical ancestral wild boar population with ancestral allele frequency equal to 1 (as requested by the test), P3 was a domestic population (defined by one single breed or grouping different breeds, using the geographic criteria) and P2 and P1 were different wild boar populations. Even if the test could not be formally used to evaluate the opposite introgression (as it would have assumed that a derived pig is the ancestor of a wild boar, contrasting the coalescent model), we also modelled one BABA scenario assuming an ancestral domestic population, with domestic allele frequency equal to 1. In all tested scenarios for both loci, absolute D values ranged from 0.184 to 1.000 (Table S6). Results indicated an ABBA excess, meaning that there were events of domesticated alleles introgression, whereas the tested BABA scenario, even if not formally appropriate, might indicate an inverse introgression flow.

The relatively high frequency of the domestic *NR6A1* allele in wild boar populations might be derived by a reproductive advantage and, in turn, a slightly higher fitness of the carriers of the domestic allele, which might tend to increase its frequency in natural environments. An increased number of vertebrae associated with the domestic allele would contribute to increased body size and length of

the animals, with subsequent effects on reproduction traits obtained directly with an increment in litter size (derived by a higher uterus capacity) or indirectly with an increased number of teats, as also reported in QTL studies with domestic pigs (Duijvesteijn *et al.* 2014). This matter should be better investigated to acquire phenotypic evidences associated with genotyping data (Fulgione *et al.* 2016). Combining information from the two investigated genes, signals of introgression of domestic alleles were observed in 16% (northern Italy) and 34% (SE Europe) of the investigated wild boars (in Sardinia, 12% carried the *NR6A1* C allele). These regions constitute three contiguous but separate European areas considered among those with the highest number of autochthonous pig breeds (Porter 1993). From a molecular ecology perspective, highly introgressed populations could provide an opportunity to evaluate the effect of natural selection on domestic alleles that have reached wild populations through interdemographic gene flow (Fulgione *et al.* 2016).

Altogether, these results indirectly demonstrate that bidirectional introgression of wild and domestic alleles is part of the human- and naturally driven evolutionary forces that are continuously shaping the *S. scrofa* genome. On one hand, this species is still experiencing a 'de-domestication' process (i.e. the genome of autochthonous breeds, which can be considered at the 'domestication border') and, on the other hand, a 'domestication' drift (i.e. the wild boar populations). In the case of autochthonous breeds, the domestication might still be considered a work in progress against de-domestication forces, led by the way in which these genetic resources are managed. Both aspects need to be further investigated and evaluated in genetic conservation programs of wildlife and domestic populations.

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Conflict of interest

The authors declare that they do not have competing interests.

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Supporting information

- Additional supporting information may be found online in the Supporting Information section at the end of the article.
- Figure S1** ABBA–BABA tree with indicated the direction of the tested introgression flow based on the four populations (P1–P4) defined in the scenarios reported in Table S6.
- Table S1** Information on the sampled animals of the domestic pig breeds.
- Table S2** Information on the investigated wild boars.
- Table S3** PCR–RFLP and fragment analysis protocols used for the genotyping of markers at the *MC1R* and *NR6A1* genes.
- Table S4** *MC1R* and *NR6A1* genotyping data in the autochthonous pig breeds.
- Table S5** *MC1R* and *NR6A1* genotyping data in the wild boar populations.
- Table S6** *D* statistics of the ABBA–BABA test (Dasmahapatra *et al.* 2012; Martin *et al.* 2014) for *MC1R* and *NR6A1* loci.