

1           **Sequence monomorphism at MC1R *wild-type* allele and evidences of introgression of**  
2                   **domestic alleles at two nuclear loci across European wild boar populations**

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19          introgression

22 **Summary**

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24 Domestication promotes the emergence of novel phenotypic and behavioural traits in  
25 domesticated animals compared to their wild ancestors. We analyzed variation at the  
26 *MC1R* and *NR6A1* genes in European wild boar populations, two loci which have been  
27 under strong artificial selection during domestication, influencing respectively coat colour  
28 and number of vertebrae.

29 A total of 145 wild boars were sampled throughout Europe, to evaluate frequency and  
30 spatial distribution of domestic alleles, in order to investigate patterns of hybridization  
31 between wild and domestic forms. As expected, most of the wild boars (94%) were  
32 homozygous for the European *wild-type* ( $E^+$ ) *MC1R* allele. We did not observe any  
33 synonymous substitution in the European  $E^+$  allele, confirming its monomorphism even in  
34 areas known to be hotspots of wild boar genetic diversity. The remaining wild boars  
35 showed genetic introgression of three different European domestic alleles.

36 No Asian *MC1R* allele was found in our sample, suggesting that extensive introgression  
37 from Asian wild boars or domestic pigs is unlikely. Furthermore, domestic *NR6A1* alleles  
38 were observed in 6% of wild boars. Considering the two loci analyzed, 11% of boars,  
39 sampled all over Europe, showed signs of recent or past introgression in their genome.

40 These data agree with previous investigations on other molecular markers, confirming the  
41 relatively low genetic diversity of European wild boars, which was locally increased by the  
42 introgression of allelic variants from the domestic counterpart.

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45 **Main text**

46 The variation in pigmentation in mammals depends on the spatial distribution of  
47 pigmentation across the body and along hairs, and the balance between the black  
48 eumelanin and the yellowish pheomelanin. This balance is controlled primarily by two  
49 loci, *Extension* and *Agouti*, the former one encoding the melanocortin receptor I gene  
50 (*MC1R*), a G protein-coupled receptor expressed in melanocytes (Fang et al. 2009).

51 A typical *wild-type* coat colour in mammals is composed of a mixture of eumelanin and  
52 pheomelanin. Mutations of the *MC1R* gene leading to a constitutively active receptor are  
53 dominant and induce the production of eumelanin only, causing dark/black coat colour.  
54 Nonsense and frameshift mutations, that prevent the formation of functional receptors, are  
55 recessive, and cause the production of pheomelanin only, leading to yellow/reddish coat  
56 colour (Suzuki 2013). Classical genetics had established an allelic series including four  
57 alleles at the *Extension* locus in pigs: Dominant black ( $E^D$ ), *wild-type* ( $E^+$ ), black spotting  
58 ( $E^P$ ) and recessive red ( $e$ ) (Legault 1998). Subsequent sequence analyses on the porcine  
59 *MC1R* gene revealed several allelic variants of these four phenotypically-defined alleles  
60 (Andersson 2003).

61 Asian wild boar populations show high diversity, having at least 14 different *wild-type*  
62 *MC1R* allelic variants (in which only synonymous substitutions occur) (Li et al. 2010); on  
63 the opposite, no polymorphism has been detected so far in European wild boars for the  
64 *wild-type MC1R* allele (Fang et al. 2009). Nevertheless, the latter result can be influenced  
65 by the small number and narrow geographic range of the European samples that have been  
66 sequenced so far (Fang et al. 2009; Giuffra et al. 2000; Kijas et al. 1998, 2001).

67 Since the *wild-type* allele is private to wild populations (with the exception of the  
68 Mangalica domestic breed), DNA polymorphisms at the *MC1R* locus have been  
69 successfully used for the traceability of meat products (Fontanesi et al. 2014) and to  
70 detect introgression of domestic pig genes into wild boar populations (e.g. Frantz et al.

71 2013, Koutsogiannouli et al. 2010). Domestic *MC1R* alleles have been found in 5% of 119  
72 free-ranging and 16% of 12 reared wild boars in Greece (Koutsogiannouli et al. 2010), and  
73 in one out of 153 wild boars from Western Europe (Frantz et al. 2013).

74 Another trait that differentiates wild boars and European commercial pigs is the number of  
75 vertebrae: 19 in the wild boar and 21-23 in ameliorated breeds. A proline to leucine  
76 substitution at codon 192 (p.Pro192Leu) in the nuclear receptor subfamily 6, group A,  
77 member 1 (*NR6A1*) gene was shown to be the most likely causative mutation underlying  
78 the QTL effect on the number of vertebrae in the pig (Mikawa et al. 2007). The mutant  
79 allele is fixed in most European commercial pig breeds, while wild boar populations carry  
80 the *wild-type* allele only.

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82 We analyzed the variation at the *MC1R* and *NR6A1* genes in wild boars sampled  
83 throughout Europe and in a sample of local Italian pigs, with the following aims: i)  
84 evaluate the *MC1R* genetic diversity of European wild boar populations at a wider scale  
85 compared to previous studies, in order to verify the supposed monomorphism of the  
86 European *wild-type MC1R* allelic variant; ii) investigate the presence, frequency and  
87 spatial distribution of *MC1R* and *NR6A1* domestic alleles in European wild boar  
88 populations.

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90 We obtained DNA samples, or tissue samples provided by local hunters, for a total of 145  
91 European wild boars from 12 different localities (Belarus,  $N = 10$ ; Bulgaria,  $N = 5$ ;  
92 Croatia,  $N = 6$ ; France,  $N = 15$ ; Greece,  $N = 10$ ; mainland Italy,  $N = 33$ ; Luxembourg,  $N =$   
93  $8$ ; Poland,  $N = 7$ ; Portugal,  $N = 11$ , Romania,  $N = 8$ ; Sardinia,  $N = 21$ ; Spain,  $N = 11$ ).  
94 Additionally, we obtained DNA samples from 20 Sardinian and four Italian "Cinta Senese"  
95 pigs raised in free or semi-free conditions, possibly affected by introgression of *wild-type*  
96 alleles, given their possibility to crossbreed with wild boars.

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98 We amplified the *MC1R* gene as a single 1.2 kb fragment (the entire 963 bp coding region  
99 plus 6 bp of the 5'-untranslated region, and 208 bp of the 3'-untranslated region) employing  
100 the PCR2 (Fang et al. 2009) as reverse and a new forward primer (PCR4: 5'-  
101 GGGAGCCATGAGCTGAGCAGG-3'). We used the QIAGEN Fast Cycling PCR kit  
102 (Valencia, CA, USA), with 66°C annealing. Both forward and reverse strands were  
103 sequenced. Due to the presence of frameshift mutations, putative heterozygotes were  
104 cloned and sequenced to confirm allele status.

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106 The results of sequence analysis revealed that 94% of wild boars were homozygous for the  
107 already known European variant of the *wild-type* allele (Tab.1), which had an overall  
108 frequency of 96%. We did not find any synonymous substitutions at this allele in our  
109 sample, in confirmation of the supposed monomorphism of the European *wild-type* allele.

110 The first studies on *MC1R* variation in *Sus scrofa* (Kijas et al. 1998) highlighted a  
111 substantial genetic distance between Asian and European pigs, providing one of the first  
112 indications of independent domestication events in the two continents. Asian domestic pigs  
113 originated from a more diverse wild stock, whereas European domestic pigs originated  
114 from European wild boars (Andersson and Plastow 2011), which had faced a pronounced  
115 population bottleneck prior to domestication (Groenen et al. 2012). This can partially  
116 explain the relatively low diversity of both European wild boars and domestic pigs in  
117 comparison to Asian conspecifics (Groenen et al. 2012, Megens et al. 2008), and the  
118 absence of variation at the *MC1R* European *wild-type* allele.

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120 No *wild-type* Asian allele was detected among 145 individuals, indicating that extensive  
121 introgression from Asian wild boars into European populations is unlikely, in agreement  
122 with Vilaça et al. (2014), which detected less than 1% of Asian mtDNA haplotypes among

123 828 European wild boar sequences. Three domestic alleles only, already described and all  
124 of European origin, were detected among wild boars: dominant black and two variant of  
125 black spotting, with overall frequency of 0.7-3.1% (up to 10-20% in some countries like  
126 Bulgaria and Sardinia, where pigs are often reared in semi-free conditions and  
127 crossbreeding with the wild form is possible, Scandura et al. 2008). No Asian domestic  
128 allele was found among the analyzed wild and domestic populations, even if many cases of  
129 introgressions of European pigs/wild boars with Chinese pigs have been documented  
130 (Goedbloed et al. 2013, Groenen et al. 2012, Megens et al. 2008). Interestingly, 4 out 9  
131 boars carrying domestic alleles did not carry any *wild type* allele, indicating that  
132 introgression may be high at a very local scale, and/or that intentional hybridization in  
133 captivity may be an important source of introgression (see Canu et al. 2014).

134 Additionally, the Sardinian domestic stock was affected by substantial genetic  
135 introgression from wild boar, with a frequency of the *MC1R* *wild-type* allele of 7.5%, to  
136 our knowledge never reported for domestic pigs. However, this result is in line with the  
137 previous evidences of crossbreeding between wild and domestic *Sus scrofa* in the island.  
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139 The *NR6A1* gene polymorphism was investigated by PCR-RFLP with the method  
140 described by Fontanesi et al. (2014). The occurrence of either mutant alleles in wild boars  
141 or *wild-type* alleles among domestic pigs was further verified through sequencing. The  
142 *wild-type* allele turned out to be fixed in most wild populations, and, just as the *MC1R*  
143 allele E<sup>+</sup>, had an overall frequency of 96% across European wild populations. Italian,  
144 Spanish, Greek, Bulgarian and French wild boars showed signs of introgression (Tab.1).  
145 Conversely, the *wild type* *NR6A1* allele was detected in 12.5% of domestic pigs (allele  
146 frequency = 6.3%), suggesting that present or past gene flow between wild and domestic  
147 forms was not negligible and bidirectional, at least in Sardinia and continental Italy.

148 With few exceptions (in Greece and Bulgaria, see Tab.1) introgression at the *NR6AI* locus  
149 in wild populations did not match introgression at the *MCIR* locus, though perhaps this is  
150 not surprising, given that selective pressures acting on the two loci are expected to be  
151 different (there is strong selection against non-camouflaged coats, while having an extra  
152 vertebra may not be a major disadvantage).

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154 In the present work, we confirmed the absence of sequence variation at the *MCIR* E<sup>+</sup>  
155 allele in European wild populations, even in Southern peninsulas, known to be hotspots of  
156 wild boar genetic diversity. We also detected relatively high genetic introgression from  
157 domestic pig into European wild boar populations, in agreement with previous studies  
158 (e.g., Goedbloed et al. 2013). Considering both the *MCIR* and *NR6AI* loci, introgression  
159 can be found all over Europe. As many as 11% of wild boars carried domestic genes (only  
160 1.4% at both the considered loci), and the gene flow was bidirectional at least in some  
161 areas. The *MCIR* and *NR6AI* loci have proved to be useful markers to complement the  
162 information provided by other more widely employed genetic markers (e.g., mtDNA and  
163 microsatellites) to disclose patterns of gene flow among wild and domestic European and  
164 Asian forms of *Sus scrofa*.

165

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**Tables**

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Area	<i>MC1R</i>									<i>NR6A1</i>				
	$E^P/E^P$	$E^P/E^{P2}$	$E^P/e$	$E^D/E^P$	$E^D/E^D$	$E^+/E^P$	$E^+/E^{P2}$	$E^+/E^D$	$E^+/E^+$	CC	CT	TT	Tot	
DP	Italy			1	3						1	3	4	
	Sardinia	8		2	7	1		<b>1<sub>A</sub></b>	<b>1<sub>B</sub></b>		<b>2<sub>C,D</sub></b>	18	20	
	Tot	8		2	8	4		<b>1</b>	<b>1</b>		<b>3</b>	21	24	
WB	Belarus						<b>1</b>	<b>1</b>		8	10		10	
	Bulgaria				<b>1<sub>E</sub></b>					4	4	<b>1<sub>E</sub></b>	5	
	Croatia									6	6		6	
	France									15	13	<b>2</b>	15	
	Greece		<b>1<sub>F</sub></b>							9	9	<b>1<sub>F</sub></b>	10	
	Italy									33	29	<b>4</b>	33	
	Luxembourg									8	8		8	
	Poland						<b>1</b>			6	7		7	
	Portugal									11	11		11	
	Romania									8	8		8	
	Sardinia	<b>2</b>						<b>1</b>		18	21		21	
	Spain							<b>1<sub>G</sub></b>		10	10	<b>1<sub>H</sub></b>	11	
	Tot	<b>2</b>	<b>1</b>			<b>1</b>	<b>4</b>	<b>1</b>		136	136	<b>7</b>	<b>2</b>	145

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**Tab. 1**

244

Individual genotypes identified at the *MC1R* locus and at the *NR6A1* g.299084751C>T (p.Pro192Leu)

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polymorphism in domestic pigs (DP) and European wild boars (WB). Introgressed individuals are shown in

246

bold. Each lower case letter identifies a given individual in the population.  $E^+$  = European *wild-type* allele;  $e$

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= recessive red;  $E^D$  = dominant black, European form;  $E^P$  and  $E^{P2}$  = black spotting (respectively,

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corresponding to the 0101, 0401, 0301, 0501 and 0502 alleles in Fang et al. 2009). CC = individual carrying

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two wild-type *NR6A1* alleles; TT = individual carrying two domestic *NR6A1* alleles; CT = heterozygous

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individual.

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