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Melanocortin Receptor 1 *(MC1R)* Mutations and Coat Color in Pigs

J. M. H. Kijas,* R. Wales,† A. Törnsten,* P. Chardon,‡ M. Moller* and L. Andersson*

**Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, S-751 24, Uppsala, Sweden,*
†Molecular Biology Group, Pig Improvement Company, Cambridge CB1 2JN, United Kingdom and
‡Laboratoire Mixte Institut National de la Recherche Agronomique-Commissariat à la Énergie Atomique de Radiobiologie Appliquée, Jouy en Josas 78352, France

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ABSTRACT

The melanocortin receptor 1 (MC1R) plays a central role in regulation of eumelanin (black/brown) and phaeomelanin (red/yellow) synthesis within the mammalian melanocyte and is encoded by the classical *Extension* (*E*) coat color locus. Sequence analysis of *MC1R* from seven porcine breeds revealed a total of four allelic variants corresponding to five different *E* alleles. The European wild boar possessed a unique *MC1R* allele that we believe is required for the expression of a wild-type coat color. Two different *MC1R* alleles were associated with the dominant black color in pigs. *MC1R*2* was found in European Large Black and Chinese Meishan pigs and exhibited two missense mutations compared with the wild-type sequence. Comparative data strongly suggest that one of these, L99P, may form a constitutively active receptor. *MC1R*3* was associated with the black color in the Hampshire breed and involved a single missense mutation D121N. This same *MC1R* variant was also associated with E^{p}, which results in black spots on a white or red background. Two different missense mutations were identified in recessive red (*e*/*e*) animals. One of these, A240T, occurs at a highly conserved position, making it a strong candidate for disruption of receptor function.

STUDIES of coat color variation in mammals have identified many genes involved in the development, migration, and regulation of the melanocyte. The relative amount of the two basic types of tyrosine-derived melanin, eumelanin (black/brown) and phaeomelanin (yellow/red), are controlled by the *Extension* (*E*) and *Agouti* (*A*) loci. The wild-type state in many species involves synthesis of both melanin types. However mutations at either locus may commit the melanocyte to exclusive synthesis of a single pigment. In a variety of mammals, dominant alleles at *Extension* act to produce a uniform black coat color (Searle 1968; Jackson 1997), whereas recessive alleles at this locus extend the amount of red/yellow pigment. Conversely, dominant alleles at *Agouti* cause a yellow coat whereas homozygosity for the bottom recessive allele is associated with a uniform black coat (Jackson 1997). Molecular studies have revealed that *Extension* encodes the melanocyte stimulating hormone receptor, also denoted melanocortin receptor 1 (MC1R), which is a G-protein-coupled receptor (Robbins *et al.* 1993). *Agouti* encodes a 131-amino-acid peptide antagonist to MC1R (Lu *et al.* 1994).

MC1R mutations have been shown to alter pigment synthesis in a range of species, including cattle (Klungland *et al.* 1995), horse (Marklund *et al.* 1996a), fox (Våge *et al.* 1997), and chicken (Takeuchi *et al.* 1997). However, the best studied species is the mouse, in which both recessive and dominant alleles have been identified and functionally characterized (Robbins *et al.* 1993). Recessive yellow (*e*) in the mouse encodes a nonfunctional receptor due to a frameshift mutation, thereby eliminating (activity-dependent) eumelanin synthesis. Conversely dominant *Extension* alleles, which cause a range of dark coat color phenotypes, result from gain of function mutations, as, for example, the sombre mouse allele (E^{so}), which encodes a single amino acid substitution leading to a constitutively active receptor (Robbins *et al.* 1993). Studies of human *MC1R* have revealed at least 20 allelic variants, and some of these are in fact associated with red hair and fair skin (Valverde *et al.* 1995; Box *et al.* 1997), as well as melanoma susceptibility (Valverde *et al.* 1996).

A series of three alleles at the *E* locus is well established in pigs (Ollivier and Sellier 1982), where *E* (for uniform black) is dominant to E^{p} (for black spotting) and recessive *e* (for uniform red). Previous analysis of coat color inheritance within a wild boar/Large White intercross pedigree firmly assigned the *Extension* locus to chromosome *6p* (Mariani *et al.* 1996). The objectives of this study were to analyze *MC1R*, a candidate gene for the *Extension* locus in pigs, using this resource and to investigate the possible presence of *MC1R* mutations among a collection of pig breeds representing a variety

Corresponding author: Leif Andersson, Swedish University of Agricultural Sciences, Department of Animal Breeding and Genetics, BMC, Box 597, S-751 24 Uppsala, Sweden.
E-mail: leif.andersson@bmc.uu.se

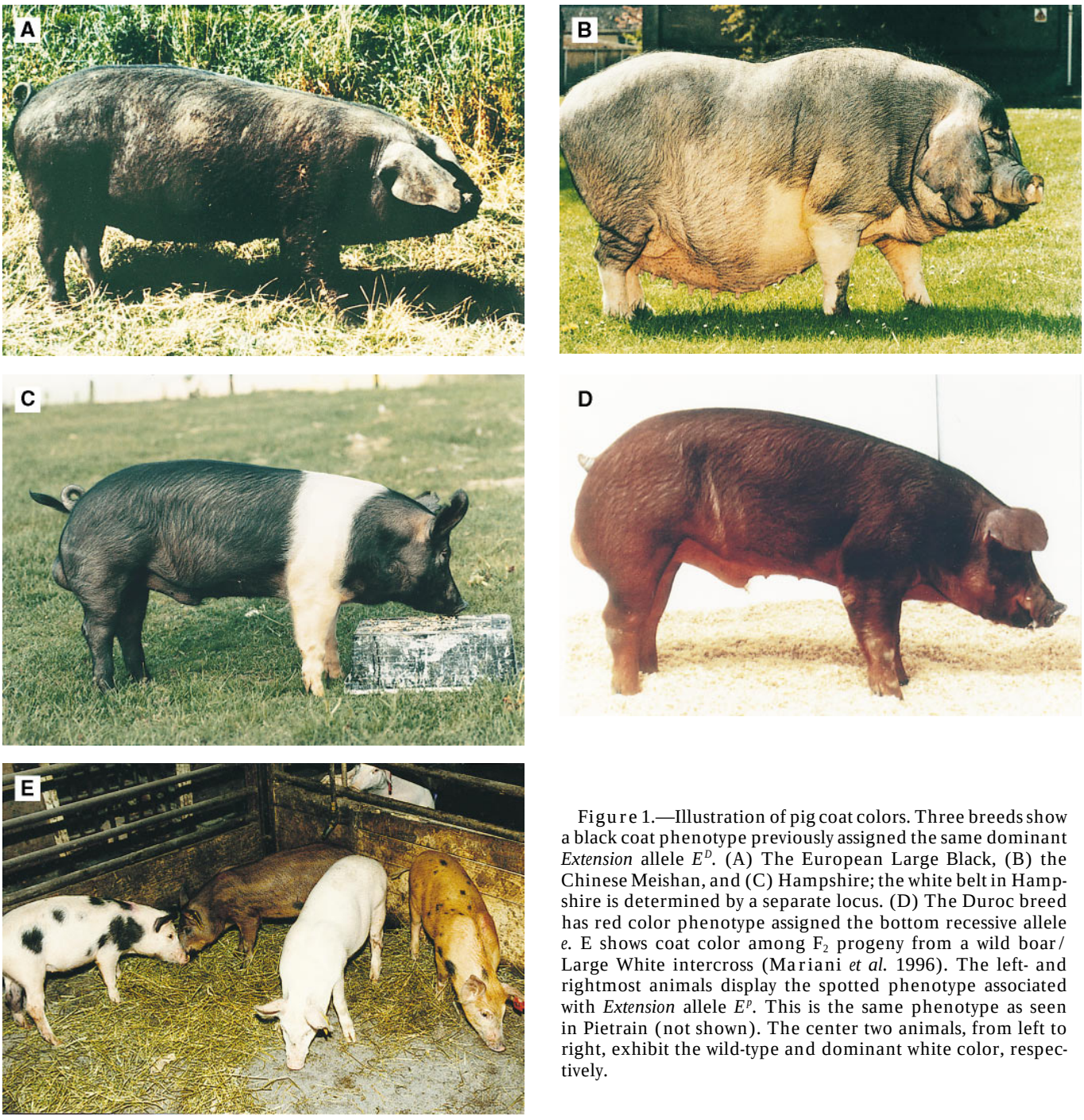

Figure 1.—Illustration of pig coat colors. Three breeds show a black coat phenotype previously assigned the same dominant *Extension* allele E^D. (A) The European Large Black, (B) the Chinese Meishan, and (C) Hampshire; the white belt in Hampshire is determined by a separate locus. (D) The Duroc breed has red color phenotype assigned the bottom recessive allele *e*. E shows coat color among F_2 progeny from a wild boar/Large White intercross (Mariani *et al.* 1996). The left- and rightmost animals display the spotted phenotype associated with *Extension* allele E^p. This is the same phenotype as seen in Pietrain (not shown). The center two animals, from left to right, exhibit the wild-type and dominant white color, respectively.

of coat color phenotypes. We show that the European wild boar possesses a unique *MC1R*/*E* allele and suggest it is required for the expression of a wild-type coat color. This phenotype results from synthesis of both melanin types and appears as variable shades of brown; it includes longitudinal stripes in juvenile animals. The allele was designated E^+ to indicate that it is the wild-type form. *MC1R* sequencing also revealed two distinct alleles for black color, and these were denoted E^{D1} (present in the Meishan and Large Black breeds) and E^{D2} (present in the Hampshire breed). A fourth receptor variant was identified that showed complete association with the *e* allele of uniform red animals.

MATERIALS AND METHODS

Animals: A three-generation pedigree comprising two European wild boar and eight Large White founders, 26 F_1 and 200 F_2 animals (see Marklund *et al.* 1996b), was used for linkage analysis. In addition, samples from unrelated animals representing six different breeds were analyzed (Table 1; Figure 1). Large Black, Meishan, and Hampshire all have a black basic color and are assumed to be E^D/E^D; the white belt in

the Hampshire animals is controlled by another locus (Ollivier and Sellier 1982). Large White and Pietrain pigs are both assumed to be E^P/E^P homozygotes, and the lack of black spots in the Large White animals is due to the epistatic action of the dominant white locus (Johansson *et al.* 1992; Mariani *et al.* 1996). The red coat color in Duroc animals is due to homozygosity for the bottom recessive *e* allele at the *Extension* locus.

PCR amplification and sequence analysis of pig *MC1R*: Three primer pairs were used to amplify the majority of the single exon *MC1R* gene. The first pair MERL1 (5′-RGTGC CTGGAGGTGTCCAT forward) and EPIG2 (5′-CGCCCAGAT GGCCGCGATGGACCG reverse) amplify a 428-bp product from the 5′ half of the gene, whereas EPIG1 (5′-CGGCCATCT GGGCGGGCAGCGTGC forward) and EPIG3 (5′-GGAAGGC GTAGATGAGGGGGTCCA reverse) amplify a 405-bp product from the 3′ half. The two products are nonoverlapping, and a third pair EXT3 (5′-TGCGCTACCACAGCATCGTGACCC TGC forward) and EXT4 (5′-GTAGTAGGCGATGAAGAG CGTGCT reverse) was used to amplify across the gap in the middle segment of the gene. In total, 758 bp of nonoverlapping sequence was amplified (excluding the outer primer sequences). PCR was carried out on a DNA thermal cycler [Perkin Elmer (Norwalk, CT) 9600] in a total volume of 20 μl containing 25 ng genomic DNA, 1.0 mm $MgCl_2$, 50 mm KCl, 10 mm Tris-HCl, pH 8.3, 200 μm dNTPs, 0.5 units AmpliTaq Gold (Perkin Elmer), and 10 pmol of both forward and reverse primer. To activate AmpliTaq Gold, initial heat denaturation was carried out at 94° for 10 min followed by 32 cycles each consisting of 45 sec at 94°, 45 sec at 53°, and 45 sec at 72°. The final extension lasted for 7 min at 72°. PCR products were cloned into vector pUC18 using the SureClone ligation kit (Pharmacia, Uppsala, Sweden). For each animal and each gene segment, two independent clones were sequenced using dye primer chemistry on a ABI377 Prism DNA sequencer (Perkin Elmer). Comparisons of independent clone sequences within each breed identified sequence errors introduced during the PCR process. All variable positions were confirmed by direct sequencing of PCR products of genomic DNA using dye terminator chemistry.

PCR-RFLP and SSCP analysis: The PCR was performed as described above using primers MERL1 and EPIG2 followed by digestion of 10 μl PCR product using 3 units *Bsp*HI (Amersham) for 60 min at 37° in a 15 μl reaction to determine the genotype at position 121. The genotype at position 240 was determined by *Acc*II (Amersham, Arlington Heights, IL) digestions of PCR products amplified using EPIG1 and EPIG3. The entire reaction volume was subsequently electrophoresed in an 8% native polyacrylamide gel (Protogel, 37.5:1 acrylamide:bisacrylamide; National Diagnostics, Atlanta) in 0.5× TBE (44.5 mm Tris pH 8.0, 44.5 mm boric acid, and 0.5 mm EDTA) for 3 hr at 200V in a vertical slab unit (SE600; Hoefer Scientific Instruments, San Francisco). Products were visualized by ethidium bromide staining. Single-stranded conformation polymorphism (SSCP) analysis was conducted using fragments amplified by EPIG1 and EPIG3 as described by Marklund *et al.* (1996a).

Linkage analysis: *MC1R* was mapped using the single nucleotide polymorphism at position 121 using the *Bsp*HI PCR-RFLP as described above. The microsatellite marker S0099 was typed as previously described (Rohrer *et al.* 1996). Two-point and multipoint linkage analyses were performed with the CRIMAP program, version 2.4 (Green *et al.* 1990). A lod score above three was set as the significance threshold, and new data were added to the existing map (Marklund *et al.* 1996b) using the BUILD option. The CHROMPIC option was used to examine the number and location of recombination events within each individual and FLIPS was used to determine the statistical support for the obtained marker order.

***In situ* hybridization:** Bacterial artificial chromosome (BAC) clone 978E4 containing *MC1R* was isolated by PCR screening of a recently constructed porcine BAC library (P. Chardon, unpublished results). BAC DNA was isolated according to standard procedures and labeled with biotin using the GIBCO-BRL Bionick labeling system (BRL 18246-015). Unincorporated nucleotides were separated through a Sephadex G-50 column. The size of the labeled probe (~500 bp) was confirmed on a 1% agarose gel. Pokeweed stimulated porcine lymphocyte cultures were obtained using standard techniques. The metaphase slides were aged for 2 days at room temperature and kept at −20° until used. The FISH technique used has been described in detail elsewhere (Chowdhary *et al.* 1995). Approximately 200 ng of the labeled probe was hybridized in a 15-μl hybridization mix to porcine metaphase chromosomes. Hybridization signals were detected under an Olympus (Lake Success, NY) BX-60 microscope equipped with a charge coupled devise camera connected to a computer system driven by ISIS-3 software.

RESULTS

***MC1R* cloning and sequence similarities to other species:** PCR cloning of porcine *MC1R* from genomic DNA was achieved using primers designed from an alignment of human, mouse, and horse *MC1R* sequences and resulted in analysis of 758 bp of the predicted 948-bp single-coding exon. The pig *MC1R* sequence showed high sequence identity with the homologous sequences in a variety of mammals (Figure 2). The sequence identity across 252 amino-acid positions was highest with the sequenced receptor of fox (86%), followed by horse and cattle (each 83%), human (80%), and mouse (75%).

Wild and domestic pigs carry different alleles at the *MC1R*/*E* locus: Sequence data were collected from seven breeds of pig (Table 1). The sequence comparison revealed a total of seven polymorphic sites in the *MC1R* coding region, and all polymorphisms were single-base substitutions. Translation revealed that five of these seven were nonsynonymous (Figures 2 and 3). A total of four distinct *MC1R* alleles associated with different color phenotypes were identified. The wild-type coat color (E^+/E^+) of the European wild boar was associated with a distinct *MC1R* receptor variant not found in any of the domestic breeds included in this study. This allele (denoted *MC1R*1*) was at each polymorphic position consistent with the wild-type E^+ sequence in other species (Figure 2).

The dominant black phenotype is genetically heterogeneous: Sequence analysis of *MC1R* from Large Black, Meishan, and Hampshire, three breeds ascribed the dominant *Extension* allele E^D, revealed two different alleles. Large Black and Meishan pigs shared an allele (denoted *MC1R*2*) differing by two nonsynonymous and two synonymous substitutions from the wild-type allele (Figure 3). The dominant black phenotype in the Hampshire breed was associated with another allele (denoted *MC1R*3*) differing by a single missense mutation from the wild-type sequence (Figure 3). We suggest that the *Extension* alleles for dominant black color corre-

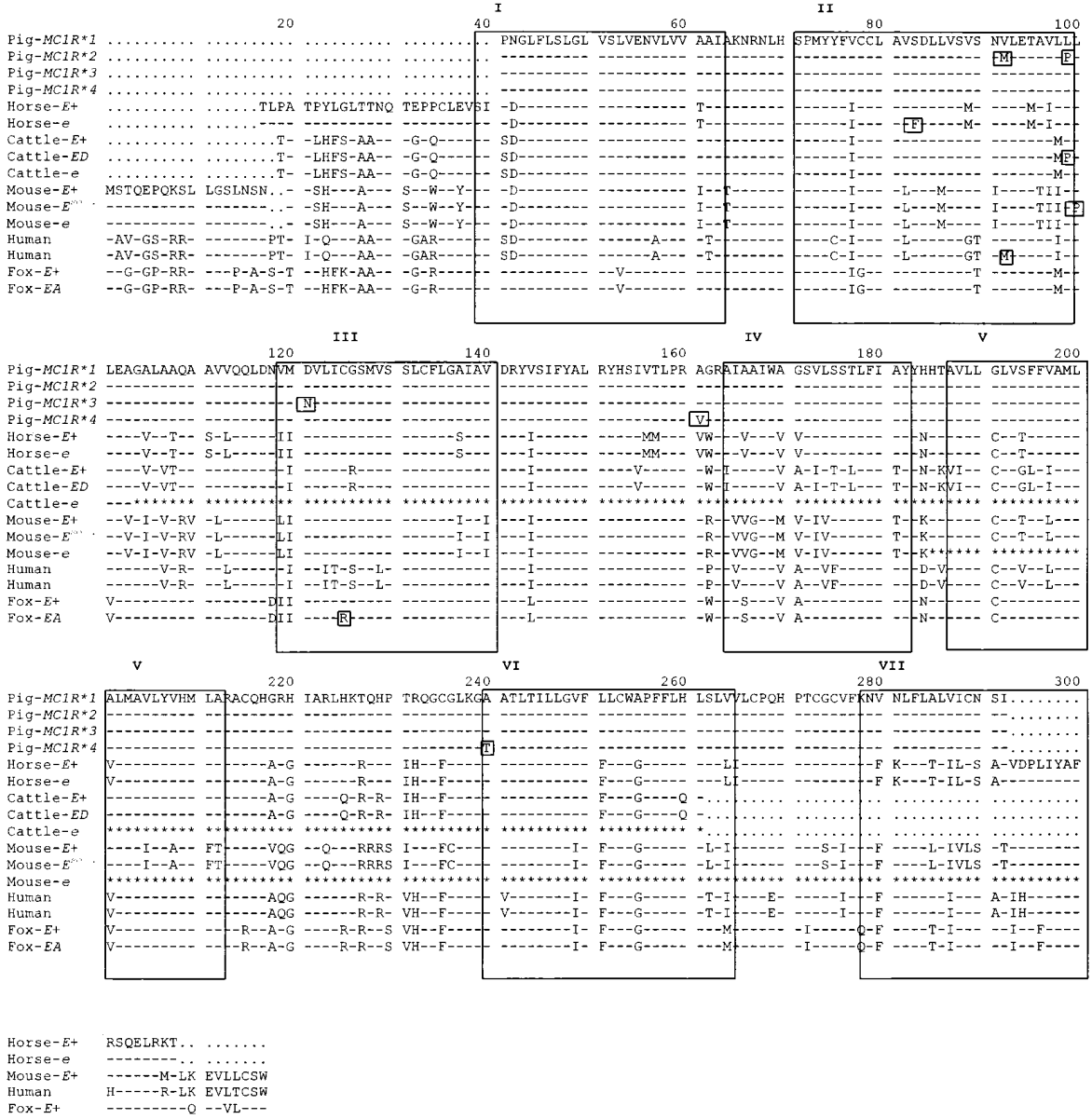

Figure 2.—Amino acid sequence alignment of *MC1R* variants in pig (present study), horse (Marklund *et al.* 1996a), cattle (Klungland *et al.* 1995), mouse (Robbins *et al.* 1993), human (Valverde *et al.* 1995), and fox (Våge *et al.* 1997). Only two alleles in humans are shown; however, more have been reported (Box *et al.* 1997). The seven transmembrane domains are indicated in large boxes and denoted with roman numerals; intraspecies substitutions are indicated by small boxes. (- - -) Sequence identity to the master sequence used; (***) positions following the 1-bp deletions within the *e* allele of cattle and mouse; (· · ·) positions where no sequence information is available. Codons are numbered according to the human sequence. DNA sequences of the porcine *MC1R* alleles have been deposited in GenBank as follows: *MC1R*1*, accession number AF082487; *MC1R*2*, accession number AF082488; *MC1R*3*, accession number AF082489; *MC1R*4*, accession number AF082490.

sponding to *MC1R* alleles *2* and *3* are denoted E^{D1} and E^{D2}, respectively.

SSCP analysis of a 428-bp MERL1-EPIG2 PCR fragment revealed a unique pattern type associated with the *MC1R*2* sequence. The results of an SSCP analysis of Large Black (E^{D1}/E^{D1}), Duroc (e/e), and wild boar (E^{+}/E^{+}) DNA are displayed in Figure 4. SSCP examination of two Large Black and a total of nine Meishan individuals showed each to have the same banding pattern thereby confirming homozygosity for the *MC1R*2* allele (Table 1). A simple PCR-RFLP test for the presence of *MC1R*3*/E^{D2} was possible because the G to A substitution at codon 121 creates a recognition site for *Bsp*HI. Thirteen out of 16 Hampshire animals were found to be homozygous for the D121N mutation, whereas the remaining 3 Hampshire animals had genotype *MC1R*3*/*MC1R*4* (Table 1). The presence of the *MC1R*4* allele, associated with the recessive red color in Duroc, was not surprising as some cross-breeding occurs between Swedish Hampshire and Duroc.

TABLE 1

Coat color phenotype and *MC1R* genotypes among 87 unrelated pigs representing seven breeds

Breed	Coat color phenotype	*Extension* genotype[a]	*MC1R* genotype[b]					
			1/1	*2/2*	*3/3*	*3/4*	*4/4*	Total
Wild boar	Wild type	E^+/E^+	3					3
Large Black	Black	E^{D1}/E^{D1}		2				2
Meishan	Black	E^{D1}/E^{D1}		9				9
Large White	White	E^p/E^p			23			23
Pietrain	White with black spots	E^p/E^p			10			10
Hampshire	Black with white belt	E^{D2}/E^{D2}			13	3		16
Duroc	Red	*e/e*					24	24
								87

[a] Previous studies of coat color in the pig did not distinguish alleles E^{D1} from E^{D2}.
[b] *MC1R* genotypes were determined using the three tests illustrated in Figure 4.

E^p for black spotting and E^{D2} for dominant black are associated with the same *MC1R* allele: Sequencing *MC1R* from a Large White animal homozygous for the E^p allele surprisingly revealed the same sequence as associated with E^{D2} for dominant black in the Hampshire pigs (Figure 3). PCR-RFLP analysis of 23 Large White and 10 Pietrain pigs showed that they were homozygous for the point mutation causing the D121N substitution (Figure 4).

Mutation A240T is associated with the recessive allele *e* for red color in the Duroc breed: Alignment of the *MC1R* coding sequence from a recessive red animal (*e*/*e*) with the wild-type receptor (E^+) identified two nucleotide substitutions resulting in amino acid substitutions A161V and A240T; the allele was designated *MC1R*4* (Figures 2 and 3). The A161V substitution is unlikely to be causative and was not further investigated as it is a conservative one, and the variant amino acid (valine) is present in the functional receptor of horse (Figure 2). The second mutation occurs at position 240, which is highly conserved and present within the sixth transmembrane domain of the receptor (Figures 2 and 3). The mutation disrupts an *Acc*II recognition site, which we used in a PCR-RFLP test (Figure 4). Analysis of 87 unrelated animals with a range of color phenotypes using this *Acc*II test found that only the 24 red animals from the Swedish Duroc breed were homozygous for the A240T mutation whereas none of the remaining 63 nonred animals had this genotype (Table 1).

***MC1R* maps to the distal part of pig chromosome *6p*:** A three-generation intercross pedigree previously used to construct a comprehensive porcine linkage map (Marklund *et al.* 1996b) was used for linkage assignment of *MC1R*. The *Bsp*HI PCR-RFLP test revealed that the two wild boar (E^+/E^+) and eight Large White (E^p/E^p) founder animals were homozygous for the *MC1R*1* and **3* alleles, respectively. Two-point linkage analysis found highly significant lod score values between *MC1R* and six chromosome *6* markers, the highest being with S0035 ($Z = 39.6$, $\theta = 0.03$) and S0479 ($Z = 38.5$, $\theta = 0.02$). The most likely order of loci including *E*/*MC1R* at the distal portion of chromosome *6p*, as revealed by multipoint analysis, is given in Figure 5. The lod score support for this order against the next most likely order, placing *E*/*MC1R* proximal to S0035, was 1.2. All other possible orders of the five loci were disfavored by lod scores exceeding 2.2.

A total of 51 of the 200 F_2 animals in the pedigree

	92	99	118 119 120 121	161	239 240
			*Bsp*HI		*Acc*II
*MC1R*1* Wild Boar (E^+)	GTG	CTG	AAT GTC ATG GAC	GCG	GGC GCG
*MC1R*2* Large Black (E^{D1})	A..	.C.	..C	...	... ..A
*MC1R*2* Meishan (E^{D1})	A..	.C.	..C	...	... ..A
*MC1R*3* Hampshire (E^{D2})	...	...	... ... A..	...	... ...
*MC1R*3* Large White (E^P)	...	...	... ... A..	...	... ...
*MC1R*3* Pietrain (E^P)	...	...	... ... A..	...	... ...
*MC1R*4* Duroc (*e*)	...	...	... ...	.T.	... A..
*MC1R*1* Wild Boar (E^+)	Val	Leu	Asn Val Met Asp	Ala	Gly Ala
*MC1R*2* Large Black (E^{D1})	Met	Pro	... ...	...	... ...
*MC1R*2* Meishan (E^{D1})	Met	Pro	... ...	...	... ...
*MC1R*3* Hampshire (E^{D2})	...	...	... ... Asn	...	... ...
*MC1R*3* Large White (E^P)	...	...	... ... Asn	...	... ...
*MC1R*3* Pietrain (E^P)	...	...	... ... Asn	...	... ...
*MC1R*4* Duroc (*e*)	...	...	... ...	Val	... Thr

Figure 3.—Mutations in the pig *MC1R* gene defining four sequence variants. The *Extension* allele associated with each *MC1R* variant is indicated in parentheses. Codon positions are numbered as in Figure 2. The sequence obtained from the wild boar is shown at the top (*MC1R*1*). The polymorphic *Bsp*HI (TCATGA) and *Acc*II (CGCG) recognition sites are indicated. Note that two different point mutations in *MC1R*2* and **4* abolish the latter recognition site.

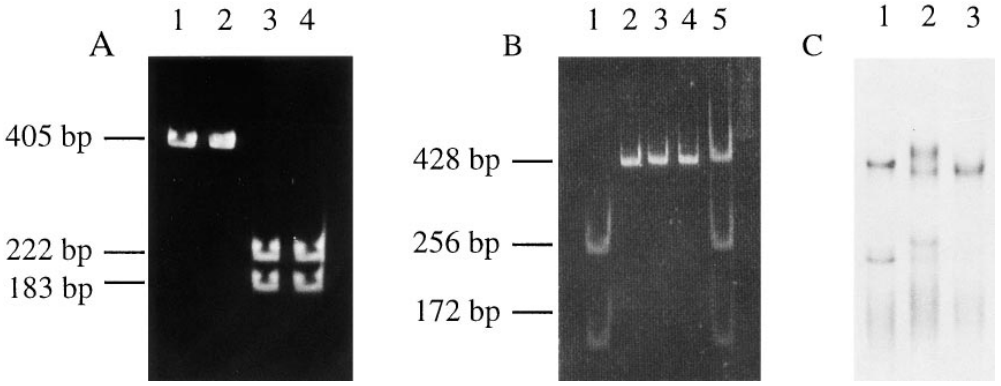

Figure 4.—PCR-RFLP and SSCP analysis used to distinguish the four porcine *MC1R* variants. (A) *Acc*II RFLP of PCR products obtained with primer pair EPIG1/EPIG3 reflecting sequence polymorphism at codon 240. The test distinguished DNA amplified from Duroc (*MC1R*4/4*, lane 1) and Meishan (*MC1R*2/2*, lane 2) from that of wild boar (*MC1R*1/1*, lane 3) and Large White (*MC1R*3/3*, lane 4). (B) *Bsp*HI RFLP of PCR products obtained with primer pair MERL1/EPIG2 reflecting sequence polymorphism at codon 121. The test distinguished DNA amplified from Large White (*MC1R*3/3*, lane 1) from the other three variants amplified from wild boar (*MC1R*1/1*, lane 2), Meishan (*MC1R*2/2*, lane 3), and Duroc (*MC1R*4/4*, lane 4). Lane 5 represents a heterozygous individual from the wild boar intercross (*MC1R*1/3*). (C) SSCP analysis of PCR products obtained with the primer pair EPIG1 and EPIG3 distinguishing DNA amplified from Meishan (*MC1R*2/2*, lane 1) from that of Duroc (*MC1R*4/4*, lane 2) and wild boar (*MC1R*1/1*, lane 3).

are colored, being either wild type or black spotted (Mariani *et al.* 1996); the remaining animals are white due to the presence of the dominant white allele and thus not informative for *MC1R/E* segregation. *MC1R* analysis of the colored F_2 animals corresponded with expectation for both the phenotype classes. All of the wild-type animals had *MC1R* variants associated with either E^+/E^+ (*MC1R*1/1*) or E^+/E^p (*MC1R*1/3*), whereas all of the black spotted phenotype class animals were homozygous for the E^p/*MC1R* allele (**3/3*). *MC1R* mapped to the same location as *Extension*, as no recombinants were identified ($Z = 10.2$, $\theta = 0.00$).

MC1R was also physically mapped by fluorescence *in situ* hybridization. BAC clone 978E4 was used to examine around 20 metaphase preparations, and in all cases, strong signals were seen on the terminal part of the p-arm of pig chromosome *6* (not shown). This chromosome has a characteristic size and shape, which makes it readily identifiable without using any banding technique (Committee for the Standardized Karyotype of the Domestic Pig 1988).

DISCUSSION

This study advances the knowledge of coat color genetics in the pig and provides strong evidence that the classical *Extension* coat color locus is equivalent to the *MC1R* locus. This was previously suggested on the basis of comparative mapping data because pig chromosome *6p* shares homology with the region harboring the *MC1R/E* locus in mice, humans, and cattle (Mariani *et al.* 1996). Most of the *MC1R* coding sequence was determined from pig genomic DNA representing seven breeds of pig. This revealed four *MC1R* alleles corresponding to five *E* alleles. The difference in number is due to the fact that two *E* alleles, E^{D2} for dominant black and E^p for black spotting, were both associated with the *MC1R*3* sequence. The European wild boar *MC1R* allele was distinct from those found among the six different domestic breeds included in this study. We assume that the presence of *MC1R*1*, or a functionally equivalent allele, is necessary (but of course not sufficient) for the development of the wild-type color and normal interaction with melanocyte stimulating hormone and the *Agouti* product. The results of this study suggest that the *MC1R*1*/E^+ allele is rare or absent among the major breeds of domestic pigs in the world and therefore presents *MC1R* as an exellent marker for distinguishing European wild boar from domestic breeds. This may have a practical application, as wild boars are bred in captivity for game or meat production. *MC1R* genotyping may be used to reveal European wild boars that are not purebred and to test meat products marketed as derived from European wild boar.

The genetic basis for black color in domestic pigs: It has been generally assumed that most domestic pig breeds have a black color because they carry a recessive nonagouti (*a*) allele at the *Agouti* locus and a normal/wild-type allele (*E*) at the *Extension* locus (Ollivier and Sellier 1982). According to this hypothesis, our Large White and wild boar founders should have the following genotypes at the *Dominant white*, *Agouti*, and *Extension* loci: *I/I*, *a/a*, and E^p/E^p and *i/i*, *A/A*, and E^+/E^+, respectively. Consequently, we should expect that about 1/16 of our 200 F_2 animals should be black nonagouti (*i/i*, *a/a*). However, no black, nonagouti animals were observed, and we could refute this model ($P < 0.001$). It is possible that there was a genetic difference at the *Agouti* locus between our wild boar and Large White founders, as we observed some variability among those F_2 animals classified as wild-type colored. However, all *i/i*, E^+/E^+ piglets showed a wild-type-like color with the typical longitudinal yellow stripes. On the basis of this result and the fact that we found *E/MC1R* mutations in

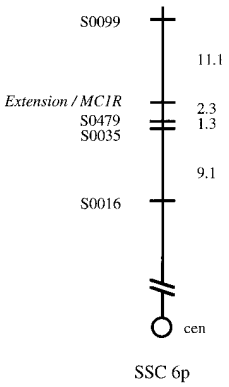

Figure 5.—Multipoint map of the distal portion of pig chromosome *6p*. Marker names and the sex-averaged Kosambi map distances between markers are given.

breeds with a black basic color, we postulate that black color in domestic pig breeds is often caused by dominant mutations at the *E*/*MC1R* locus rather than recessive nonagouti alleles at the *A* locus.

The genetic basis for the recessive red color (*e*/*e*): Nonfunctional *MC1R* products are expected to result in recessive red/yellow-coated animals. The *MC1R*4* sequence associated with the recessive *e* allele for red color in the Duroc breed involved two nonsynonymous substitutions compared with the wild-type receptor. In the absence of functional studies, the relative influence of the A161V and A240T mutations on receptor function cannot be formally confirmed; however, the second of these represents a much stronger candidate to cause disruption of receptor activity. The A240T mutation occurs at a highly conserved position in transmembrane domain (TM) 6. Residue 240 is conserved among the six mammalian species in which *MC1R* has been examined and also across all five members of the melanocortin receptor family (Prusis *et al.* 1995). In contrast, the A161V substitution is observed in the functionally normal equine *MC1R* and occurs at a residue that is variable among melanocortin receptors. This led us to investigate only the A240T mutation with PCR-RFLP analysis, which showed complete concordance between presence of the mutation and the red coat color (Table 1).

Nonfunctional MC1R in both mouse (Robbins *et al.* 1993) and cattle (Klungland *et al.* 1995) result from 1-bp deletions; however, in both the horse (Marklund *et al.* 1996a) and pig, loss of function appears to be due to missense mutations. Deletions and frameshift mutations offer no insight into receptor function; however, naturally occurring substitutions that influence receptor function pinpoint those regions of the molecule most likely to be involved in ligand binding and effector function. The equine substitution occurs within TM2, which is a region already identified as important due to the presence of numerous mutations. The porcine mutation, within TM6, represents the first case of a natural mutation in this region of the mammalian receptor. The nonconservative replacement of alanine (hydrophobic) with threonine (polar) may alter the α-helical structure of a transmembrane domain identified by homology modeling to be one of three components of the MC1R binding pocket (Prusis *et al.* 1995).

The genetic basis for dominant black color in the Meishan and Large Black breeds: The Meishan and Large Black breeds are both characterized by a uniform black coat, and the same *MC1R*2* allele was present in both breeds. $MC1R*2/E^{D1}$ differs from the wild-type allele $MC1R*1/E^{+}$ by two synonymous and two nonsynonymous substitutions. One of the amino acid substitutions, L99P, is identical to that identified in dominant black cattle (Klungland *et al.* 1995) and occurs at the neighboring position to the sombre mutation (E^{so}) in black mice. In all three species, the substitution is of leucine with proline, and pharmacological characterization demonstrated this to result in a constitutively active receptor in the mouse (Robbins *et al.* 1993). These observations strongly suggest that the pig L99P mutation acts in the same manner to drive synthesis only of black pigment. The role of the second amino acid substitution, V92M, is less clear. Substitution of valine with methionine at position 92 was one of a number of *MC1R* variants observed within red-haired human individuals (Valverde *et al.* 1995), and the mutation was shown to decrease the potency of αMSH (Xu *et al.* 1996). However, subsequent studies revealed no association between V92M and human hair color (Box *et al.* 1997). The suggestion that it may be a neutral polymorphism was strengthened by expression of a V92M-mutated *MC1R* allele followed by functional analysis. Koppula *et al.* (1997) reported that the introduced V92M mutation conferred no difference to receptor activity, and it appears likely that this conservative amino acid substitution has no major effect on receptor function.

Meishan is a Chinese breed, whereas the Large Black breed was developed in England. Therefore, we were surprised to find that they shared the same *MC1R* allele for black color differing by as many as four nucleotide substitutions from the wild-type allele, two of which are synonymous. In contrast, black color in another European breed (Hampshire) was due to a different *MC1R* allele with a single missense mutation. The finding does, however, support an old tale that Asian pigs contributed to the foundation of the Large Black breed (Porter and Tebbit 1993); the tale claims that "during the 18th century a slow boat from China docked at Plymouth and unloaded a few black Asian pigs which had managed to escape the on-voyage pork barrel" and these were then used for breeding.

The genetic basis for dominant black color in the Hampshire breeds: The black color in this breed was associated with the *MC1R*3* sequence containing a single missense mutation (D121N). This position in TM3 is very close to a causal mutation for the semidominant E^{A} allele in fox (C125R; Våge *et al.* 1997). The fox E^{A} allele shows a number of interesting and perhaps relevant characteristics to the D121N mutation identified in the pig *MC1R*3*. The mutation in the fox, C125R,

is postulated to disrupt an intramolecular bond involving aspartic acid residues at either position 117 or 121, which serves to constrain the fox receptor in its inactive form (Våge *et al.* 1997). Disruption of this intramolecular hydrogen bond, either by substitution of a neighboring residue, as in the fox E^A allele, or of D121 directly, as in the case of the pig, dictates that only the active form of the receptor will be present. Unlike the L98P-activated sombre receptor in the mouse, heterozygosity for the fox C125R mutation is not sufficient to override inhibition of *MC1R* by the *Agouti* product.

What is the genetic basis for the black spotted (E^p) phenotype? The black spotted phenotype (E^p/E^p) may appear as red with black spots or white with black spots (Figure 1). Our previous analyses of the coat color inheritance in the wild boar intercross clearly showed that the difference between these two classes is not controlled by the *E* locus, as siblings sharing the same *E* genotype differed in this respect (Mariani *et al.* 1996). Thus, one or more additional loci must control the dilution of red (phaeomelanin) but not black (eumelanin) pigment in the pigs that are white with black spots.

This study includes two breeds that are fixed for the E^p allele. Both Pietrain and Large White animals were homozygous for the *MC1R*3* allele also associated with the black color in the Hampshire attributed to the E^{D2} allele. Thus, there must be a genetic difference outside the sequenced region explaining the phenotypic difference between black and black spotted pigs. The complete cosegregation between the black spotted phenotype and *MC1R*3*/E^p in the wild boar intercross shows that this mutation must occur at the *MC1R* locus or at a closely linked gene. It is not clear how mutations at *MC1R* may cause a spotted pattern as found in the pig. *MC1R* mutations causing a constitutively active receptor are expected to lead to a uniform black coat, whereas mutations causing a defect receptor will lead to a recessive red coat. However, a partial distribution of pigmentation is in some cases thought to be controlled by alleles at the *Extension* locus such as brindling in cattle, tortoiseshell in the guinea pig, Japanese brindling in rabbit, and black mask in dogs (Searle 1968). The functional status of *MC1R* in these animals is currently unknown. The phenotype of black spotted pigs suggests that MC1R is nonexpressed or inactive in white/red areas, whereas it is overactive in the black spots. This suggests that E^p may be associated with a *MC1R* regulatory mutation. One quantitative trait locus affecting the size of white spots in mice is located in the *MC1R* region, but it is still an open question whether this is due to *MC1R*/*E* itself or a linked locus (Pavan *et al.* 1995). Further characterization of genetic variation in the *MC1R* flanking region in the pig may shed light on this enigma.

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