POSTER

THE USE OF *MC1R* AND *KIT* GENOTYPES FOR BREED CHARACTERISATION

EL USO DE LOS GENOTIPOS *MC1R* Y *KIT* PARA LA CARACTERIZACIÓN RACIAL

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PALABRAS CLAVE ADICIONALES

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SUMMARY

We describe the characterisation of the two main coat colour determining loci (*MC1R* and *KIT*) for pigs and demonstrate how this information can be utilized for breed identification. These approaches can be useful components of quality assurance and traceability schemes which are increasingly demanded by consumers.

RESUMEN

Describimos la caracterización de dos *loci* determinantes del color de la capa en cerdos y demostramos cómo esta información puede ser utilizada para la identificación racial. Estos avances pueden ser útiles en esquemas para asegurar la calidad y la trazabilidad cuya demanda se está incrementando en los consumidores.

INTRODUCTION

Pig breeds have been developed to satisfy particular market or production requirements. For example, in the UK the Large White and particularly the Landrace breeds were developed for bacon whilst the Berkshire was known as a *pork* pig. In Spain, the Iberian pig has been developed for outdoor rearing with acorns for the production of specialist ham. Traditionally breeds were classified by their colour or type so that over time a *Standard of Excellence* (phenotypic conformity) was developed that defined phenotype and supplemented the pedigree. One of the main distinguishing features for breed in pigs is coat colour and pattern. Two loci, *Extension* and *Dominant White* control much of the variation in coat colour. The genes involved have been identified (*MC1R* and *KIT* respectively), variants have been described and associations with breed and colour determined

(Giuffra *et al.*, 2002, Johansson Moller *et al.*, 1996, Kijas *et al.*, 1998, 2001, Marklund *et al.*, 1998, Pielberg *et al.*, 2002). These polymorphisms provide a simple means of verifying the breed of pig from which products such as semen, pork or hams originate. In this way they can play an important role in Quality Assurance programmes and maintenance of brand identity. In this paper we describe two clear examples of the use of these markers for breed identification and preliminary results for Iberian pigs.

MATERIALS AND METHODS

Simple PCR tests were developed for the *MC1R* and *KIT* genes (for example see Kijas *et al.*, 1998, 2001, Marklund *et al.*, 1998 and Pielberg *et al.*, 2002). Genotype and/or haplotype scores were then developed to characterise different breeds (see **table I** for *MC1R*). Four polymorphisms were analysed for the *MC1R* gene. These consisted of three RFLPs as well as a two base pair insertion at the 5' end of the coding sequence (Kijas *et al.*, 1998, 2001). Several alleles of *KIT* have now been identified. The gene is duplicated in breeds such as Pietrain, Large White and Landrace and the presence of the duplication can be detected with a simple PCR test (Giuffra *et al.*, 2002). The dominant white allele found in white breeds is associated with a polymorphism at a splice site within one of the *KIT* sequences in the duplicated allele. This polymorphism can also be detected using a simple PCR-RFLP (Marklund *et al.*, 1998). These two tests can only be scored dominantly for presence of the duplication and/or the splice site. An additional three intronic polymorphisms detected by sequencing from Exon 16 to Exon 19 of *KIT* (Okumura *et al.*, 2000) were used in the development of haplotypes for the

Table I. MC1R *Haplotypes.* (Haplotipos *MC1R*).

Allele	Polymorphism[1] Nt67insCC[2]	L99P	D121N	A240T	Colour	Breed Examples
E^{+}[3]	-	L	D	A	Brown	Wild Boar
E^{D1}	-	P	D	A	Black	Meishan/large Black
E^{D2}	-	L	N	A	Black	Hampshire
E^{p}	+	L	N	A	Red and/ or Black spots	Pietrain/LW/LR/ Berkshire/Tamworth
E	-	L	D	T	Red	Duroc
e^{Ib}	+	L	D	A	Red?	Iberian

[1]Nucleotide or amino acid position, e.g. amino acid 99 is either Leucine or Proline.

[2]-/+ indicates presence or absence of the 2bp insertion.

[3]Two sequences (*MC1R**1 or *5) have been identified for wild boar, which may differentiate Asian and European types. Both are the same at these positions.

differentiation of the Berkshire breed from other pig populations. These polymorphisms are detected by PCR-RFLP as follows:

Primers were developed to span five polymorphic regions of interest, situated in two intronic regions of the *KIT* gene.

Primer pair Forward (5'-ACATG CAAAATGAGTTTTCC-3') and Reverse (5'-ACTCACAAAAAACAATAC TTA-3') were designed to study the SNP's situated at positions 862 (C or T) and 863 (A or G) of Intron 16 of *KIT*.

Primer pair Forward (5'-TGGGAG GAAGAATGAGTAT-3') and Reverse (5'-TCAGGAGTTTGCTTGTGGT-3') were designed to study the SNPs at positions 1001 (C or T), 1002 (C or A) and 1288 (G or A) of Intron 17 of *KIT*. PCR conditions were 94°C for 45 secs, 55°C for 45 secs, 72°C for 60 secs, and 32 cycles for both sets of primers, producing PCR fragments of 543 and 460 bp in length respectively. To study the SNPs at positions 958 and 959 of *KIT*, the PCR amplicon was digested with the enzyme Csp45I (BstBI) and electrophoresed on a 4 percent agarose gel. Two alleles were detected: allele 1 - fragment 543 bp (not cut) and allele 2 - fragments 432 and 111 bp. To study the three SNPs situated at positions 2313, 2314 and 2600 of *KIT*, the PCR amplicon was split into two separate reactions and digested with either MseI or SmaI enzymes. Two alleles were detected by electrophoresis on a 4 percent agarose gel for both enzymes: MseI allele 1 - fragments 241, 189 and 30 bp and allele 2 - fragments 189, 168, 73 and 30 bp; SmaI allele 1 - fragment 460 bp (not cut) and allele 2 - fragments 359 and 101 bp.

Samples were obtained from different breeds (Berkshire, Duroc, Landrace, Large White Meishan and Pietrain) maintained by PIC in Europe and the US supplemented with additional samples from Europe, Japan and the US (Berkshire, Meishan, Large Black, Tamworth and Wild boar, see Kijas *et al.*, 1998, 2001). In addition, the British Wild Boar Association provided samples from members' herds. Samples of pork (UK) or Iberian hams (Spain) were collected from retail stores. DNA was prepared from tissue (e.g. meat samples) or hair samples using a simple proteinase K protocol to lyse cells and release the DNA. Samples of this crude DNA lysate were used for PCR amplification.

RESULTS

MC1R

Haplotypes were determined for each of the genes following analysis of samples from different pig populations, including Berkshire, Duroc, Iberian, Landrace, Large Black, Large White, Meishan, Pietrain, Tamworth and European and Japanese Wild Boar. To date we have identified six haplotypes for *MC1R* using the four polymorphisms described here **(table I)**. An additional polymorphism was identified that distinguishes the two wild boar populations. These haplotypes can be used to distinguish several of the common breeds, although the Ep haplotype is present in several of the Western breeds including Berkshire, Pietrain, Landrace, Large White and Tamworth (Kijas *et al.*, 2001). Even, so this information can be used to

identify breeds such as European wild boar and Duroc. In the case of the Duroc, the distinctive red coat is due to a mutation at amino acid 240 of *MC1R* that changes an alanine to a tyrosine **(table I)** (Kijas *et al.*, 1998). Thus, only pigs with Duroc ancestry will contain a T at this position in the *MC1R* gene. Interestingly this polymorphism is probably not present in other red breeds such as Tamworth and Hereford, where the red coat is the result of a frameshift caused by the insertion at nucleotide 67 (Kijas *et al.*, 2001). It should be noted that the Duroc allele has been found in some Tamworth samples, where it is thought to be the result of crossing with the Duroc breed.

UTILISATION OF *MC1R* IN A QUALITY ASSURANCE SCHEME FOR WILD BOAR

In the UK, the British Wild Boar Association has been established to develop the commercial farming of this breed. In order to protect their investment and assure the validity of products labelled as wild boar they have established a registration scheme under the aegis of Assured British Pigs and the UK's Meat and Livestock Commission (MLC). This scheme includes the use of DNA testing to verify the status of the herds. The test is based on genetic variation in the *MC1R* locus. Wild boar have a variant producing a brown colour not found in commercial pigs (the two sequence variants identified to date in Wild boar give the same coat colour). Therefore, any animal that does not carry two copies of the wild boar allele cannot be of pure wild boar ancestry. In this study over 300 samples, most of them purchased from shops across the UK but including some from live animals, were tested for this diagnostic marker. **figure 1** shows the percentage of those samples that purported to be wild boar and those that purported to be commercial pork with various genotypes at the *MC1R* locus. Most wild boar samples carried two copies of the wild boar allele and this allele was never found in samples of commercial pork. The class *Wild boar* samples that were not homozygous for the wild boar allele includes some controls used to test the system (the method detected all of

Table II. *Polymorphisms in the* KIT locus *and their association with breed.* (Polimorfismos en el *locus KIT* y su asociación con la raza).

Allele	Polymorphism Duplication	Splice variant	Intron haplotype	Breed Examples
i	-	-	1	Meishan, Large Black
i	-	-	1, 2, 3	Berkshire (Japan)
i	-	-	4	Duroc, Tamworth
I^{Be} (Belt)	-	-	4	Hampshire
I^{Ro} (Roan)	-	-	Not known	
I^{p} (Patch)	+	-	4	Pietrain
I (Dominant white)	+	+	4	Landrace, Large White

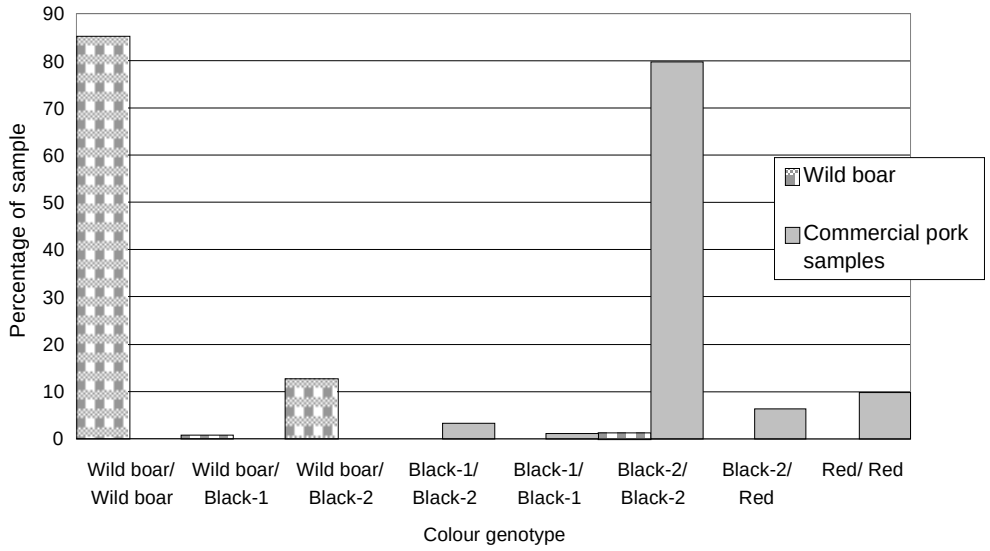

Figure 1. *A survey of MC1R status in UK wild boar and commercial pork. The chart shows the percentage of samples that purported to be either wild boar or commercial pork in each of the MC1R genotype classes. (Wild boar* - $E+$*; Black-1* - E^{D1}*; Black 2* - E^{p}/E^{D2}*; Red - e. Note* E^{p}/E^{D2} *were not distinguished in this survey).* (Una revision del *status* de MC1R en el jabalí y los cerdos comerciales del Reino Unido. El gráfico muestra el porcentaje de muestras que se asignan a Jabalí o al cerdo comercial en cada una de las clases de genotipos MC1R. Jabalí - *E*+; Black-1 - E^{D1}; Black 2 - E^{p}/E^{D2}; Red - e. Note E^{p}/E^{D2} no fueron distinguibles en esta revisón).

these). Commercial pork samples all carried alleles for black or red colouration (although in this case E^{D2} was not separated from Ep, which would be present from the use of white breeds in this sample). These results demonstrate that insisting wild boar have two copies of the wild boar allele is both tractable and a powerful adjunct to an accreditation scheme.

KIT

Several alleles have been identified at the *KIT* locus including roan, patch and belt as well as dominant white (Giuffra *et al.*, 1999, Johansson Moller *et al.*, 1996, Marklund *et al.*, 1998). Although the detection of these types and the associated alleles can be complicated by the *masking* of alleles by the dominant white allele (see Pielberg *et al.*, 2002), and also in some situations the interaction with additional loci (Hirooka *et al.*, 2002), it is possible to establish breed identification rules based on the common alleles and especially the presence of the duplication of *KIT* sequences and or the splice variant (see **table II**). The effectiveness of these breed classification criteria is further strengthened by combining *MC1R* and *KIT* haplotypes. In addition, Mitsuhashi and Plastow and their colleagues found that they

could identify four *KIT* haplotypes using three intronic polymorphisms (see Okomura *et al.*, 2000). Most interestingly one haplotype seemed to be associated with the majority of Western breeds, but not those of Asian origin including the Berkshire breed which was developed in the UK but included genes from imported Asian stock (see Porter 1993). However, it should be noted that the *Western* haplotype is detected in samples from the current US Berkshire population. This division into Western and Asian haplotypes is consistent with the separate domestication of Asian and European breeds as proposed based on analysis of mitochondrial sequences (see Giuffra *et al.*, 2000).

VERIFICATION OF KURO BUTA IN JAPAN

In Japan *Black Pork* or *Kuro Buta* is produced from the Berkshire breed. This pork is highly prized and is typically two or three times the price of domestically produced pork from other breeds. This premium pricing led to a situation where there was more *Kuro Buta* being sold than could be produced from the Berkshire herds in Japan. We therefore established a test regime that would enable Japanese MAFF, retailers and consumers to verify the origin of *Kuro Buta*. The test utilises a *Berkshire* haplotype developed using both *MC1R* and *KIT* polymorphisms. Berkshire meat must carry two copies of the wild type allele (i) at *KIT* and the Asian haplotype at this locus (Mitsuhashi and Plastow unpublished) as well as two copies of the E^p allele at *MC1R*. This test has already been provided on a commercial basis by two licensed laboratories in Japan.

THE POTENTIAL USE OF *MC1R/KIT* TESTING FOR IBERIAN PIG PRODUCTION

Iberian products have recently been differentiated in Spain as a component of a sustainable system supporting biodiversity and delivering products of the highest quality with special sensory properties. As a result, products such as the Iberian Cured Ham have acquired an excellent reputation among consumers and may be as much as ten times the price of normal cured ham. As for Wild Boar and Kuro Buta, the ability to attract a premium price for these products led to an indiscriminate use of the term *Iberico*, resulting in a detrimental impact on authentic Iberian products. For these reasons, new regulations have been put in place describing Iberian ham, shoulder, and loin products (Real Decreto 1083/2001). The breed specification for these products is ≥ 50 percent Pure Iberian with Duroc representing the rest of the genetic makeup. The Iberian breeds vary in their coat colour from *blonde* to black which immediately complicates the situation in relation to the use of the *MC1R* and *KIT* loci. In order to determine whether the colour genes can be incorporated into a verification scheme we have begun to sample cured hams labelled as Iberian to establish the frequency of the different alleles. As expected, most Iberian hams carry two copies of the wild type allele *(i)* at *KIT* and a selection of *MC1R* alleles **(table III)** including the *e* allele associated with Duroc. However, some of the hams we sampled also carried the *KIT* duplication associated with Pietrain. Interestingly, in this study we found evidence for the existence of a new *MC1R* haplotype (e^{Ib}). Some of the

Iberian samples contained the 2bp insertion that is associated with the E^p allele, but they did not have the polymorphism associated with the dominant black mutation (E^{D2}), which is in complete linkage disequilibrium with the 2bp insertion in, for example, the white breeds, Pietrain and Tamworth. We would anticipate that the new allele e^{Ib} would produce a non-functional product such that homozygotes would be red (similar to Tamworth). This new allele may be the origin of the red or chestnut types of the Iberian and it may be a useful marker for this type. We have previously shown that the black spotting in pigs carrying E^p was due to somatic reversion of the insertion (Kijas *et al.*, 2001), and so it will be interesting to see if this is observed for this allele. The existence of this new allele also suggests the possibility that the E^{D2} allele arose from a germline reversion of E^p rather than the accumulation of the insertion on an E^{D2} allele. In order to clarify this situation, we are undertaking a substantial survey of pure Iberian pigs (including individual phenotypic information), in addition to commercial products labelled as *Iberico*. In this way we hope to determine whether these tests can form part of a specification to verify the production of branded products.

DISCUSSION

Variation at the two main coat colour loci (*MC1R* and *KIT*) provides a relatively simple DNA testing regime that can be used to verify the breed origin of pig products. However, in some situations - for example for Iberian products - it may be necessary to develop the specification based on additional markers or systems. We are undertaking a survey of commercial products and pigs in order to determine whether these tests can form part of a specification to verify the production of specific labelled products.

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Table III. MC1R *allele frequencies in a sample of Iberian hams.* (Frecuencias de los alelos *MC1R* en una muestra de jamones Ibéricos).

Allele	Frequency	Note
e	0.31	Recessive red (Duroc)
E^{D1}	0.02	Dominant black (Asian)
E^{D2}	0.08	Dominant black (Hampshire)
e^{Ib}	0.19	Recessive red (Iberian)
E^p	0.41	Red/Black/Spot (Pietrain, Berkshire, Tamworth, LW, LR)

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