Confirmation of sable QTL that modifies the effects of the A^y allele on yellow coat color on mouse chromosome 1

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Abstract: F_1-A^y mice between RR (aabbCC) and C57BL/6J-A^y (A^yaBBCC) have a much darker yellow coat color than do C57BL/6J-A^y. Quantitative trait locus (QTL) analysis was carried out to identify genes responsible for the darker modification of the yellow coat color (this has been traditionally termed “sable”). A significant sable QTL was identified on chromosome 1 (Dmyaq4, LOD score 15.5 for lightness, and 13.4 for color difference), in a chromosomal position similar to that of Dmyaq2, a sable QTL previously identified in C3H/HeJ. Another significant sable QTL was identified on chromosome 4 (Dmyaq5, LOD score 5.6 for lightness, and 4.3 for color difference) near the tyrosinase-related protein 1 (Tyrp1) locus. The effect of Dmyaq5 was significant only in the presence of the RR allele at Dmyaq4, suggesting that the Dmyaq4 (as well as Dmyaq2) is a novel coat color gene that may act up-stream of Tyrp1 signaling to increase eumelanin production.

Key words: Agouti; A^y allele; QTL; sable; Tyrp1.

Introduction. In mice, the agouti coat color is determined by the relative amounts of two kinds of pigments, eumelanin (black or brown pigment) and pheomelanin (yellow or reddish-yellow pigment) in the hair. The agouti hair has a layer of pigments, an apical and a basal black band with a central yellow band. In normal agouti mice, α-melanocyte stimulating hormone (αMSH) stimulates the melanocytes to produce eumelanin, and the agouti protein serves as an inverse agonist that inhibits a low level of constitutive melanocortin 1 receptor (MC1R) activity in the absence of αMSH, to produce pheomelanin transiently. Mice carrying the A^y allele at the agouti locus are predominantly veiled by yellow fur as a consequence of constitutive antagonism of the effects of the αMSH by an ectopically over-expressed agouti peptide. As a rule, a darker modification of the yellow color sometimes occurs, and this has been termed “umbrous”. Essentially, both sable and umbrous coat color phenotypes are multigenic traits, and they share genetic bases. Indeed, because sable and umbrous occurred in F_1-A^y between C3H/HeJ and C57BL/6J-A^y, both traits were analyzed in F_2. As a result, three sable quantitative trait loci (QTLs) were identified on chromosomes 1 (Dmyaq4, Dmyaq2) and 15 (Dmyaq3). The effect of Dmyaq5 was significant only in the presence of the RR allele at Dmyaq4, suggesting that the Dmyaq4 (as well as Dmyaq2) is a novel coat color gene that may act up-stream of Tyrp1 signaling to increase eumelanin production.

I carried out the present QTL study to reveal further gene loci responsible for sable (umbrous can be analyzed with the C3H, but not the RR cross) on the basis of the observation that F_2-A^y between RR (aabbCC) and C57BL/6J-A^y (A^yaBBCC) were strikingly darker than C57BL/6J-A^y. Furthermore, F_2-A^y exhibited a wide spectrum of yellow phenotypes in terms of lightness and darkness. Additional sable QTLs are expected to be identified in the RR genome. Sable QTLs may be expected to
have relations to metabolic disorders, because the $A^y$ allele is known to cause maturity onset obesity and diabetes.\textsuperscript{6,7}

**Materials and methods.** *Mice and genetic cross.* The inbred strain C57BL/6J-\textsuperscript{AaBBCC} was purchased from the Jackson Laboratory (Bar Harbor, ME), the inbred strain RR (\textit{aaBBCC}) was bred and maintained in the National Institute of Agrobiological Sciences (NIA, Tsukuba, Japan), and the inbred strain C3H/HeJ (\textit{AABBCC}) was purchased from Clea Japan (Tokyo). Hereafter, the C57BL/6J-\textsuperscript{Aa} is defined as having B alleles, the RR as having R alleles, and the C3H/HeJ as having C alleles, throughout the genome. 

$F_1$ progeny (\textit{AaBbCC} and \textit{aaBbCC}) were produced between $\sqrt[3]{2}$RR and $\sqrt[3]{2}$C57BL/6J-\textit{Aa}; thereby, the $F_1$ were intercrossed to produce $F_2$ by mating either of $\sqrt[3]{2}A^a/a \times \sqrt[3]{2}a/a$ or $\sqrt[3]{2}a/a \times \sqrt[3]{2}A^a/a$ (the \textit{Aa} allele is recessive lethal, and therefore only half of the $F_1$ and half of the $F_2$ progeny can be used for analysis). In total, 256 $F_2$-\textit{Aa} (141 males and 115 females) were produced. Mice of ages ranging from 54 to 76 days (most from 64 to 68 days) were used for this study. All mice were maintained throughout the experimental period under specific pathogen-free conditions, with a regular light cycle of 12 h light/12 h dark, with the temperature controlled at $22 \pm 3^\circ\text{C}$, and a relative humidity of 50%. They had free access to diet (rodent pellet chow, CE-2, Clea Japan) and water.

**Coat color analysis:** measurements of “lightness ($Y$ value)” and “color differences ($\Delta E$ value)”. The coat color properties were analyzed by means of colorimetry. The tri-stimulus values, CIE (Commission International de l’Eclairage) 1931 ($X$, $Y$, $Z$), were determined by a spectrophotometric procedure, and color differences ($\Delta E_{ab}$) between the specimens (coat colors) were calculated by a formula which was defined in CIELAB color space.\textsuperscript{8} The instrument used for spectrophotometry was a GRETAG SPM 50 spectrophotometer (Gretag Data and Image Systems, Switzerland). The measurement of coat colors was carried out directly on dorsal fur with the apparatus under the CIE standard illuminant $D_65$. Based on dominant wavelength ($\lambda_d$) analysis, it was confirmed that the coat colors lay in the yellow zone. The $Y$ value (lightness parameter) was adopted for evaluation of the lightness and darkness of the colors irrespective of the hues. In the calculation of $\Delta E_{ab}$, the $F_2$-\textit{Aa} mouse coat color that was judged as having the largest $Y$ value was employed as reference. For convenience, the symbol “$\Delta E_{ab}$” has been expressed simply as “$\Delta E$ value” or as “$\delta E$ value” in the following part of this paper. The unit for $Y$ value is %, and $\Delta E$ value has no numerical unit.

**QTL analysis.** Genomic DNA was isolated from the tail of each mouse with a commercial DNA extraction kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI) according to the manufacturer’s instructions. Microsatellite sequence length polymorphism was detected by electrophoresis subsequent to PCR. Microsatellite primers were purchased as MapPairs from Research Genetics Inc. (Huntsville, AL), or synthesized according to the information in Mouse Genome Informatics (MGI: http://www.informatics.jax.org/). PCR amplification was carried out in a Takara PCR thermal cycler (Takara Biomedicals, Kyoto) under the following conditions: 1 cycle at 94 °C for 5 min; 35 cycles at 94 °C for 30 sec, 55 °C for 1 min, and 72 °C for 45 sec; 1 cycle at 72 °C for 7 min. PCR products were separated on 10 % polyacrylamide gels and visualized with ethidium bromide staining. QTL analysis was carried out with Mapmaker/EXP and Mapmaker/QTL software.\textsuperscript{9} In an initial QTL analysis, 48 $F_2$-\textit{Aa} comprising the 24 lightest (judged as having the largest $Y$ value) and the 24 darkest (judged as having the smallest $Y$ value) were selected from 228 $F_2$-\textit{Aa}, and afterwards, 28 $F_2$-\textit{Aa} were added for mapping of QTLs in 256 $F_2$-\textit{Aa}. In the 256 $F_2$-\textit{Aa}, because the $Y$ values for males and females were not significantly different from each other [20.52 ± 4.9 (mean ± S.D.) in males and 20.64 ± 3.8 (mean ± S.D.) in females, $P>0.8$], trait values for males and females were analyzed together. With regard to the selected 48 $F_2$-\textit{Aa}, point-wise statistical analysis by one-way ANOVA was performed at each microsatellite marker locus. Subsequently, the remaining 208 mice were genotyped at the loci that had a nominal $P$ value less than 0.05. The genome-wide threshold for statistical significance is a LOD score of 4.3, corresponding to a $P$ value of $5.2 \times 10^{-6}$.\textsuperscript{10} The $\alpha$ level for suggestive linkage implies that there may be one false positive in a genome-wide search.

**Candidate gene sequencing.** Part of the nucleotide sequences of the ORF of X-ray repair complementing defective repair in Chinese hamster cells 5 (\textit{Xrcc5}, NM_009553) cDNA were determined in C57BL/6J, RR and C3H/HeJ, and part of the nucleotide sequences of the ORF of whn-dependent transcript 2 (\textit{Wdt2}, NM_001005423) were determined in RR and C3H/HeJ. The nucleotide sequencings were carried out by direct sequence of PCR products which were specifically amplified by oligonucleotide primers (the primer information are available upon request) by use of cDNA and genomic DNA as templates.
Results. On the basis of the comparison of dorsal fur color with the naked eye, $F_1\text{-}A^y$ was apparently darker than C57BL/6J-$A^y$ (Fig. 1). This was confirmed by means of colorimetry based on measurement of the $Y$ value (lightness, Fig. 2A) and $\Delta E$ value (color difference, Fig. 2B). The $Y$ value in $F_1\text{-}A^y$ was significantly lower than that in C57BL/6J-$A^y$ ($P=7.90 \times 10^{-8}$), and the $\Delta E$ value in $F_1\text{-}A^y$ was significantly higher than that in C57BL/6J-$A^y$ ($P=3.01 \times 10^{-6}$). Furthermore, there was a wide spectrum of coat color phenotypes in $F_2\text{-}A^y$ in terms of the $Y$ value and $\Delta E$ value (Fig. 2), suggesting that the observed sable phenotype was a quantitative trait. In $F_2\text{-}A^y$, the $Y$ value ranged from 6.58 to 30.82, and the $\Delta E$ value from 0 to 36.91. $F_2\text{-}A^y$ with a $Y$ value lower than 10.0 did not occur in (C57BL/6J × KK-$A^y$) $F_2\text{-}A^y$ or in (C3H/HeJ × C57BL/6J-$A^y$) $F_2\text{-}A^y$.

A list of the microsatellite markers used in this study is presented in Table I with their chromosomal positions retrieved from the MGI (MGI search was done on February 23, 2002). In total, 80 autosomal and two X-

| Marker   | Position (cM) | Marker   | Position (cM) | Marker   | Position (cM) | Marker   | Position (cM) |
|----------|---------------|----------|---------------|----------|---------------|----------|---------------|
| Chr 1    |               | Chr 5    |               | Chr 10   |               | Chr 15   |               |
| D1Mit211 | 15            | D5Mit257 | 24            | D10Mit188| 4             | D15Mit175| 10.1          |
| D1Mit36  | 25.7          | D5Mit359 | 50            | D10Mit42 | 44            | D15Mit63 | 29.2          |
| D1Mit68  | 34.8          | D5Mit440 | 50            | D10Mit297| 70            | D15Mit159| 51            |
| D1Mit133 | 42            | D5Mit95  | 68            | Chr 11   |               | D15Mit193| 57.9          |
| D1Mit90  | 52            | D5Mit221 | 80            | D11Mit149| 1             | Chr 16   |               |
| D1Mit217 | 63.1          | Chr 6    |               | D11Mit229| 14            | D16Mit131| 4.3           |
| D1Mit33  | 81.6          | D6Mit116 | 6             | D11Mit86 | 28            | D16Mit4  | 27.3          |
| D1Mit16  | 87.2          | D6Mit188 | 32.5          | D11Mit219| 43            | D16Mit139| 43            |
| D1Mit94  | 101.5         | D6Mit149 | 46.3          | D11Mit212| 50            | D16Mit71 | 69.2          |
| Chr 2    |               | D6Mit390 | 63.9          | D11Mit124| 61            | Chr 17   |               |
| D2Mit312 | 2.2           | D6Mit14  | 74            | Chr 12   |               | D17Mit16 | 18.15         |
| D2Mit297 | 29            | Chr 7    |               | D12Mit109| 19            | D17Mit139| 30.2          |
| D2Mit274 | 62            | D7Mit76  | 3.4           | D12Mit201| 29            | D16Mit93 | 44.5          |
| D2Mit285 | 86            | D7Mit228 | 18            | D12Nds2  | 60            | D17Mit123| 56.7          |
| Chr 3    |               | D7Mit250 | 37            | Chr 13   |               | Chr 18   |               |
| D3Mit60  | 0             | D7Mit253 | 52.8          | D13Mit139| 32            | D18Mit21 | 6             |
| D3Mit25  | 29.5          | Chr 6    |               | D13Mit110| 47            | D18Mit149| 24            |
| D3Mit230 | 38.3          | D8Mit191 | 21            | D13Mit220| 62            | D18Mit129| 31            |
| D3Mit254 | 64.1          | D8Mit248 | 43            | D13Mit35 | 75            | Chr 19   |               |
| D3Mit162 | 84.9          | D8Mit211 | 49            | Chr 14   |               | D19Mit40 | 25            |
| Chr 4    |               | D8Mit113 | 52            | D14Mit11 | 3             | D19Mit53 | 43            |
| D4Mit214 | 17.9          | Chr 9    |               | D14Mit192| 40            | D19Mit6  | 55            |
| D4Mit178 | 35.5          | D9Mit90  | 9             | D14Mit165| 52            | Chr X    |               |
| D4Mit12  | 57.5          | D9Mit191 | 26            | D14Mit64 | 45            | DXMit64  |               |
| D4Mit232 | 71            | D9Mit107 | 40            | D14Mit121| 67            | DXMit121 |               |

The underlined microsatellite markers showed $P<0.05$ in ANOVA in the selective genotyping analysis. Chr: Chromosome.
linked microsatellite markers were genotyped, so that the average marker density was 20 cM (1,600 cM/80 loci). I cannot deny the possibility that there might be overlooked QTL effects because of modest numbers of markers.

In an initial selected genotyping based on the Y value in 48 F2-Ay, it was strongly suggested that there would be a significant QTL on chromosome 1 (Table I). In addition, eight loci on chromosomes 5, 6, 8, 10, 11, 13, and 14 showed a nominal P value of less than 0.05 (Table I). With regard to these loci, genotyping was also carried out in the remaining 208 F2-Ay. As a result, a highly significant QTL was identified on chromosome 1, placing a maximum LOD score near the microsatellite marker D1Mit131 (42 cM) [LOD 15.5 for Y value (lightness) (Table II, Fig. 3) and LOD 13.4 for AE value (color difference) (Table III, Fig. 3)]. This locus explained 26.0% of the F2 phenotypic variance in terms of the Y value, and the R allele was associated with increased darkness. I named this QTL Dmyaq4, even though Dmyaq2 has already been mapped to a very similar chromosomal position (near D1Mit19, 36.9 cM) in ♀ C3H/HeJ × ♂ C57BL/6J-Av F2-Ay. Dmyaq4 was also a significant QTL when males and females were analyzed separately (maximum LOD score for Y value was 9.4 in males (n=141), and 7.1 in females (n=115)). Thus, there was no significant gender specificity as to the Dmyaq4.

On the other hand, three suggestive QTLs for Y value were identified on chromosomes 6 (D6Mit142), 10 (D10Mit42), and 11 (D11Mit124), as a result of analysis in all 256 F2-Ay (Table II). The R alleles at D10Mit42 and D11Mit124 were associated with increased darkness, whereas the R allele at D6Mit14 was associated with decreased darkness (Table II). Both loci on chromosomes 6 and 11 were also suggestive QTLs for AE value (Table III). On the basis of two-way ANOVA, there were no statistically significant interactions between Dmyaq4 and three other suggestive loci (data not shown).

For X-linked loci, if there were differences between B/B and R/B in F2-Ay females, and B and R in F2-Ay males, were examined. There were no statistically significant differences between genotypes in the two sexes. Mice with an R/R genotype on X loci could not be evaluated.

Although no significant association was found with microsatellite markers on chromosome 4 in selected 48 F2-Ay, all 256 mice were genotyped for three loci on this chromosome, because the Tyrp1 (formerly b) locus, which is known to have effect on the eumelanin production, is located at 38.0 cM. As a result, a significant QTL was identified near the microsatellite marker D4Mit178 (35.5 cM) [LOD 5.7 for the Y value (Table II, Fig. 4) and LOD 4.3 for the AE value (Table III, Fig. 4)]. This locus explained 10.6% of the F2 phenotypic variance in terms of the Y value, and the R allele was associated with increased lightness. I named this QTL Dmyaq5 for the time being, even though Tyrp1 has already been mapped to the vicinity as a strong candidate gene locus.

Next, synergisms between Dmyaq4 and Dmyaq5, and that between Dmyaq4 and a locus on chromosome

![Fig. 2A](image-url)  
**Fig. 2A** Plots of (A) Y value (lightness) and (B) AE value (color difference) in C57BL/6J-Av, F1-Av, and F2-Av mice. Each point represents an individual value. Horizontal bar indicates mean of trait values.
were examined. There was no statistically significant interaction between $Dmyaq4$ and $Dmyaq5$, nor between $Dmyaq4$ and a locus on chromosome 6; however, the effect of $Dmyaq5$ was significant only in the presence of the R allele at $Dmyaq4$ (B/R or R/R at $D1Mit131$), and the B allele was dominant to the R allele at $Dmyaq5$ (Fig. 5A). When the $D1Mit131$ genotype was B/B, there was no significant difference among three $D4Mit178$ genotypes (P>0.5). When the $D1Mit131$ genotype was B/R, there was a significant difference in...
among D4Mit178 genotypes (P<0.0002), and the R/R genotype had a significantly higher Y value than did the B/R and B/B genotypes (by Scheffe’s post hoc test). When the D1Mit131 genotype was R/R, there was also a significant difference among D6Mit14 genotypes (P<0.04), and the B/B genotype had a significantly lower Y value than did the R/R genotype (by Scheffe’s post hoc test) (Fig. 5B).

Candidate genes were searched for Dmyaq4 and Dmyaq2 on the assumption that they are allelic. When the chromosomal localization of 1.0-LOD support confidence intervals (1.0-LOD support CI) for Dmyaq4 (36–45 cM) and Dmyaq2 (35–59 cM) were compared, a chromosomal region from 36 cM to 45 cM overlapped between them. Three candidate genes that are known to be expressed in the melanocytes were located within a confidence interval for Dmyaq4 as well as Dmyaq2: fibronectin 1 (Fn1, 36.1 cM), X-ray repair complementing defective repair in Chinese hamster cells 5 (Xrcc5, 42.0 cM) and nucleolin (Ncl, 48.4 cM). Among them, Xrcc5 is located on the peak of the LOD score curve for Dmyaq4, and has been shown to have an effect on pigmentation. In addition, a known coat color gene, wbn-dependent transcript 2 (Wdt2), is also located within the 1.0-LOD support CI (36.9 cM). Therefore, parts of Xrcc5 and Wdt2 ORF sequences were determined and compared among C57BL/6J, C3H/HeJ, and RR. As seen in the Table IV, nucleotide sequences of C3H/HeJ were different from those of C57BL/6J and RR for both genes, and any differences were not found between C57BL/6J and RR. Thus, regarding polymorphisms in the candidate genes, it was not validated that Dmyaq4 and Dmyaq2 are allelic.

Discussion. The present study is a third QTL analysis concerning the darker modification of the yellow coat color (sable) caused by the A<sub>y</sub> allele at the agouti locus. The present study identified two significant sable QTLs, Dmyaq4 and Dmyaq5, on chromosomes 1 and 4, respectively. Dmyaq4 was mapped to a chromosomal position similar to that for Dmyaq2, which was identified previously in F<sub>2</sub> between C3H/HeJ and C57BL/6J-A<sup>y</sup>. The result further reinforces the probability that there is a previously unidentified gene that modifies the effects of the A<sub>y</sub> allele on yellow coat color on chromosome 1. Dmyaq5 was mapped near Tyrp1, which is known to have a significant influence on eumelanin production, but to have no demonstrable influence on pheomelanin production. In consequence, A<sub>y</sub>/B-
Table III. Identification of QTLs and results of ANOVA for $\Delta E$ values

| Marker   | Mean ± SD values (%) by marker genotype (sample size) | LOD score (variance, %) | Nominal P-value |
|----------|------------------------------------------------------|--------------------------|-----------------|
|          | R/R                                                   | R/B                      | B/B             |                 |
| D1Mit236 | 14.96 ± 7.25 (75)                                     | 10.99 ± 5.13 (115)       | 8.66 ± 2.67 (66) | 10.1 (16.6)     | 1.14 x 10^{-11} |
| D1Mit303 | 15.35 ± 7.24 (72)                                     | 10.99 ± 5.14 (118)       | 8.42 ± 2.04 (66) | 12.1 (19.6)     | 1.10 x 10^{-12}  |
| D1Mit131 | 15.69 ± 7.38 (67)                                     | 10.87 ± 5.04 (124)       | 8.58 ± 2.21 (65) | 12.6 (20.2)     | 4.01 x 10^{-12}  |
| D1Mit80  | 15.80 ± 7.83 (65)                                     | 10.76 ± 4.62 (124)       | 8.90 ± 2.86 (67) | 12.1 (19.5)     | 1.17 x 10^{-12}  |
| D4Mit178 | 9.25 ± 4.24 (74)                                      | 11.97 ± 5.94 (123)       | 13.56 ± 6.62 (59) | 4.3 (8.3)       | 6.11 x 10^{-5}   |
| D5Mit240 | 13.04 ± 6.55 (59)                                     | 11.45 ± 6.03 (120)       | 10.58 ± 4.86 (77) | 1.3 (2.3)       | 0.051            |
| D6Mit290 | 9.94 ± 3.91 (71)                                      | 11.50 ± 5.88 (121)       | 13.28 ± 7.14 (64) | 2.4 (4.3)       | 0.0004           |
| D6Mit114 | 9.54 ± 2.90 (67)                                      | 11.46 ± 5.79 (129)       | 13.99 ± 7.52 (60) | 4.1 (7.1)       | 0.0000088       |
| D8Mit113 | 9.87 ± 5.27 (59)                                      | 11.99 ± 5.80 (146)       | 12.25 ± 6.48 (51) | 1.4 (2.5)       | 0.041            |
| D10Mit42 | 12.81 ± 7.99 (59)                                     | 11.96 ± 5.33 (141)       | 9.19 ± 3.56 (56)  | 2.8 (4.9)       | 0.0018           |
| D11Mit124| 13.43 ± 6.73 (68)                                     | 11.57 ± 5.99 (121)       | 9.67 ± 3.89 (65)  | 3.0 (5.3)       | 0.0010           |
| D13Mit110| 11.75 ± 5.95 (72)                                     | 11.95 ± 6.00 (129)       | 10.55 ± 5.48 (52) | 0.5 (0.9)       | 0.34             |
| D14Mit193| 12.83 ± 6.21 (64)                                     | 11.69 ± 5.89 (138)       | 9.67 ± 5.02 (54)  | nd              | 0.013            |

Table IV. Comparison of nucleotide and amino acid sequences for ORF of Xrcc5 and Wtd2 cDNA among C57BL/6J, C3H/HeJ, and RR strains

| Nucleotide and amino acid position | C57BL/6J | C3H/HeJ | RR |
|-----------------------------------|----------|---------|----|
| **Xrcc5**                         |          |         |    |
| Nucleotide 45                     | GAT      | GAC     | GAT|
| Amino acid 15                     | Asp      | Asp     | Asp|
| Nucleotide 493                    | TTG      | CTG     | TTG|
| Amino acid 165                    | Leu      | Leu     | Leu|
| Nucleotide 891                    | CTA      | CTC     | CTA|
| Amino acid 297                    | Leu      | Leu     | Leu|
| Nucleotide 1325                   | AAG      | ATG     | AAG|
| Amino acid 442                    | Lys      | Met     | Lys|
| Nucleotide 1672                   | AAT      | CAT     | AAT|
| Amino acid 558                    | Asn      | His     | Asn|
| Nucleotide 2010                   | AAG      | AAA     | AAG|
| Amino acid 670                    | Lys      | Lys     | Lys|
| **Wtd2**                          |          |         |    |
| Nucleotide 288                    | ACC      | ACT     | ACC|
| Amino acid 96                     | Thr      | Thr     | Thr|
| Nucleotide 306                    | GAC      | GAA     | GAC|
| Amino acid 122                    | Asp      | Glu     | Asp|

Nucleotide and deduced amino acid positions are represented by the numbers of nucleotides from the start codon of reference sequences (Xrcc5: NM_009553, Wtd2: NM_001005423).
and \(A^y/\_; b/b\) animals can be distinguished by their coat colors.\(^1\) Because the mode of inheritance of the B allele at \(Dmyaq5\) was dominant to the R allele and was associated with an increased darkness, it was consistent with the fact that the B allele at \(Tyrrp1\) was dominant to the R allele and was associated with an increased eumelanin. Therefore, \(Tyrrp1\) is a strong candidate gene for \(Dmyaq5\). A suggestive, but nearly significant QTL was also mapped to chromosome 6, and this locus was also associated with increased darkness. The R allele at \(Dmyaq5\) as well as a locus on chromosome 6 behaved in the opposite direction to \(Dmyaq5\) to modify the yellow coat. There was no statistically significant interaction between \(Dmyaq4\) and \(Dmyaq5\), nor between \(Dmyaq4\) and a locus on chromosome 6; however, the effects of \(Dmyaq5\) and a locus on chromosome 6 were significant only in the presence of the R allele at \(Dmyaq4\), suggesting that \(Dmyaq4\) (as well as \(Dmyaq2\)) is a novel coat color gene that acts up-stream of \(Dmyaq5\) and a locus on chromosome 6. In particular, \(Dmyaq4\) was strongly suggested to be involved in the synthetic pathway of eumelanin rather than that of pheomelanin, if \(Tyrrp1\) was responsible for \(Dmyaq5\).

Because \(Dmyaq4\) and \(Dmyaq2\) share a confidence interval on chromosome 1, it was strongly suggested that they are allelic; however, their allelism was not demonstrated on the basis of the candidate gene sequencing. Nevertheless, it is reasonable to believe that \(Dmyaq4\) and \(Dmyaq2\) are allelic based on the following experimental results: [1] The mode of inheritance was semi-dominant in both loci. [2] The LOD score plot curves for the Y value and \(\Delta E\) value showed a similar pattern in both loci. To substantiate the putative allelism between the two loci further, it will be necessary to demonstrate that the C3H allele at \(Dmyaq2\) and the R allele at \(Dmyaq4\) interact in an additive manner. It is also worthwhile to demonstrate that the R allele at \(Dmyaq4\) serves as an umbrous factor. At any rate, there may be mutations in 5'- or 3'- untranslated sequences in candidate genes that give rise to mutant phenotype by affecting translational efficiency or mRNA stability; therefore, I cannot deny the possibilities that \(Dmyaq4\) and \(Dmyaq2\) are allelic.

Because the darker modification of the yellow coat color caused by the \(A^y\) allele occurred in all three genetic crosses examined to date, the sable seems to be a very common phenomenon. In this respect, it is not appropriate to regard the C57BL/6j background as genetically standard. Although it was not validated in the present circumstances, the C57BL/6j background may be a peculiar one in comparison with that of other mouse strains. Therefore, it is thus suggested that further sable QTLs will be identified in the \(F_2\)-\(A^y\) descendants between C57BL/6j-\(A^y\) and other inbred strains.

Several mutant loci or genes with phenotypic effects on coat color similar to sable have been known in mice. For example, mice with loss of function in MC1R (recessive yellow, \(Mc1r^{-}\)) exhibit reduced black and increased yellow pigments in the hair, whereas mice with constitutive activation in MC1R (somber, \(Mc1r^{E-tob}\), tobacco, \(Mc1r^{E-sab}\)) exhibit increased black and reduced yellow pigments.\(^10\) \(Mc1r\) is located at 68 cM on chromosome 8. Although \(D8Mit113\) (52 cM) showed a point-wise significant linkage (\(P=0.05\)) with the Y value and \(\Delta E\) value (Tables II and III), \(P\) values for both traits are far from that required for accomplishing any suggestive linkage (There were no informative microsatellite markers distal to \(D8Mit113\)). Because the R allele at \(D8Mit113\) was associated with an increased Y value (Table II), if the R allele at \(Mc1r\) is mutated, the R allele must be recessive just like \(Mc1r^{-}\); however, this is at variance with the original observation that \(F_2\)-\(A^y\) are darker than C57BL/6J-\(A^y\). Therefore, \(Mc1r\) is unlikely to be a gene that contributes to the sable phenomenon in the present case. Another example is \(Attractin\) (\(Attn\), chromosome 2, 73.9 cM). In homozygotes, \(Attn^{om}\) darkens the back, ears, and tail of agouti mice. Loss of \(Attn\) suppresses not only the normal effects of agouti on pigment production,\(^10\) but also the pleiotropic effects (e.g., obesity) produced by the ectopically expressed agouti protein as seen in \(A^y\) mice.\(^17,18\) The \(Attn\) is located approximately 15 cM proximal to the agouti (chromosome 2, 89.0 cM), and therefore it could not be examined appropriately. Although a possible contribution of \(Attn\) cannot be excluded completely, it is unlikely that \(Attn\) is causative of the sable effects observed in this study, because darker \(F_2\)-\(A^y\) mice showed an apparent tendency to be obese.

An important aspect of the sable phenomenon is that the sable can be caused by the agouti locus itself. Among alleles at the agouti locus, \(viable\ yellow\ (A^v)\) is such a case. \(A^v\) mice have a clear yellow coat that is indistinguishable from that of \(A^v\) (probably C57BL/6j-\(A^v\))\(^1\); however, many of them become sooty with successive molts. In consequence, isogenic \(A^v\) mice display a spectrum of yellow coats ranging from bright yellow to wild type agouti, with continuous degrees.\(^19\) The variability of the yellow coat in \(A^v\) mice depends on the epi-genetic state of IAP, the intracisternal A particle, inserted upstream of the agouti gene.\(^20\) A similar IAP insertion
is also found in A^{vy}, A^{yw}, and A^{yw}, but not in A^{y}. Therefore, I concluded that the sable phenotype induced by A^{y} has a different genetic basis in comparison with the sable phenotype by A^{y}.

A number of other gene loci that potentially account for the darkening of coat color phenotypes have been known, and it is believed that there are further polygenic factors that specify the sable phenotype. In fact, DK/Lm-A^{y} mice become sable at the first moult, 23 and JU/CtLm-A^{y} have an evidently darkened yellow coat in comparison with C57BL/6J-A^{y}. More specifically, microphthalmia-associated transcription factor (Mitf, chromosome 6, 40.0 cM) can serve as a sable factor, because Mitf^{MtsbA/+}, A^{y}/A^{y} is darker than +/+ , A^{y}/A^{y} on a C57BL/6J background. 24 One of the present suggestive QTLs was mapped to chromosome 6. The closest marker, D6Mit14, was located at 70.0 cM; therefore, Mitf should be excluded as a candidate for these suggestive QTLs.

Finally, according to studies on mice lacking proopiomelanocortin (Pomc), a metabolic precursor of αMSH, there is only a subtle alteration in coat color phenotype. 25 That is, Pomc null mice produce abundant eumelanin pigment in spite of the absence of MC1R ligand. 26 Slominski et al. suggested that basal MC1R activity may be sufficient to sustain eumelanin production, or that there may be redundant nonmelanocortin pathways that compensate for the αMSH deficiency. 26 With regard to the latter possibility, it seems to be interesting to test whether Dmyaq2 and/or Dmyaq4 could darken yellow coat color in the absence of MC1R activity (Mc1r/

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