

Recessive black Is Allelic to the yellow Plumage Locus in Japanese Quail and Associated With a Frameshift Deletion in the *ASIP* Gene

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Manuscript received June 3, 2007
Accepted for publication December 9, 2007

ABSTRACT

The recessive black plumage mutation in the Japanese quail (*Coturnix japonica*) is controlled by an autosomal recessive gene (*rb*) and displays a blackish-brown phenotype in the recessive homozygous state (*rb/rb*). A similar black coat color phenotype in nonagouti mice is caused by an autosomal recessive mutation at the *agouti* locus. An allelism test showed that wild type and mutations for *yellow*, *fawn-2*, and *recessive black* in Japanese quail were multiple alleles (**N*, **Y*, **F2*, and **RB*) at the same locus *Y* and that the dominance relationship was $Y^*F2 > Y^*Y > Y^*N > Y^*RB$. A deletion of 8 bases was found in the *ASIP* gene in the *Y^*RB* allele, causing a frameshift that changed the last six amino acids, including a cysteine residue, and removed the normal stop codon. Since the cysteine residues at the C terminus are important for disulphide bond formation and tertiary structure of the agouti signaling protein, the deletion is expected to cause a dysfunction of *ASIP* as an antagonist of α -MSH in the *Y^*RB* allele. This is the first evidence that the *ASIP* gene, known to be involved in coat color variation in mammals, is functional and has a similar effect on plumage color in birds.

THE *yellow* mutation in the Japanese quail (*Coturnix japonica*) is controlled by an autosomal incomplete dominant gene (*Y*). Adult heterozygotes (*Y/+*, *yellow*) display a straw plumage color but the dominant homozygotes (*Y/Y*) display an early embryonic lethality (HOMMA *et al.* 1967). The *fawn-2* mutation at the *Y* locus is also controlled by an autosomal incomplete dominant gene (*Y²*), which is allelic to and incompletely dominant over the *Y* gene. Adult homozygotes (*Y²/Y²*, *fawn-2*) show a whitish light-brown color in males and a creamier plumage color in females than in males. The heterozygotes (*Y²/+*, *dark fawn-2*) show a deeper brown color than the homozygotes in each sex (TSUDZUKI *et al.* 1996). The recessive black plumage color in the Japanese quail is controlled by an autosomal recessive gene (*rb*) and gives a blackish-brown plumage to homozygotes (*rb/rb*, *recessive black*) (FUJIWARA *et al.* 2005), which is very similar to that of homozygotes for the *extended brown* plumage mutation controlled by an autosomal incomplete dominant gene (*E*) (SOMES 1979) at the *MC1R* locus (NADEAU *et al.* 2006).

There are interesting similarities between the phenotypic effects and dominance relationships of mutations at the *agouti* locus in the house mouse (*Mus musculus*). In nonagouti mice, a black coat color phenotype (*a/a*) is caused by an autosomal recessive mutation at the *agouti* (*ASIP*) locus (SILVERS 1979). Embryonic lethality of a dominant homozygous *yellow* mutation (*A^y/A^y*) is associated with a deletion upstream of *agouti* that removes the coding exons of the *Raly* (hnRNP protein that is associated with the lethal yellow) gene (MICHAUD *et al.* 1993). The plumage color of the *yellow* (*Y/+*) Japanese quail is similar to the coat color of the *yellow* (*A^y/+*) mouse. The *Y* locus in the Japanese quail was mapped on the QL10 linkage group homologous to GGA20 in chicken (*Gallus gallus*) (MIWA *et al.* 2005), where an *ASIP*-like sequence was found (KLOVINS and SCHIÖTH 2005). Recently we studied the growth of yellow quails and found that they shared similar phenotypic characteristics (increased body fat, decreased body temperature) with lethal yellow mice (MINVIELLE *et al.* 2007). Because of these points, the *ASIP* gene was considered to be a candidate gene for the *yellow* mutation in the Japanese quail.

Mutations in the *ASIP* gene have been shown to cause a wide variety of coat colors in mammals. For instance, standard silver color in red foxes (*Vulpes vulpes*), non-agouti black in Norway rats (*Rattus norvegicus*), recessive black in horses (*Equus caballus*), and black coloration in

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AB304509 (wild type), AB304510 (*Fawn-2*), and AB304511 (*Recessive black*).

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TABLE 1
Segregation data on mating experiments between yellow or dark fawn-2 and recessive black plumage color

Mating type ^a			No. of matings	No. of newly hatched chicks				Expected ratio ^b				χ^2	d.f. = 1
				Y (Y/rb)	DFA2 (Y ² /rb)	WT (+/rb)	RB (rb/rb)	Y	DFA2	WT	RB		
Hen	×	Cock											
Y (Y/+)	×	RB (rb/rb)	2	9	—	17	—	1	:	1	2.46	0.20	> P > 0.10
DFA2 (Y ² /+)	×	RB (rb/rb)	1	—	10	12	—	1	:	1	0.18	0.70	> P > 0.60
Y (F ₁) (Y/rb)	×	RB (rb/rb)	4	31	—	—	46	1	:	1	2.92	0.10	> P > 0.05
RB (rb/rb)	×	Y (F ₁) (Y/rb)	2	28	—	—	24	1	:	1	0.31	0.60	> P > 0.50
	Total		6	59	—	—	70	1	:	1	0.94	0.40	> P > 0.30
DFA2 (F ₁) (Y ² /rb)	×	RB (rb/rb)	3	—	43	—	33	1	:	1	1.32	0.30	> P > 0.20
RB (rb/rb)	×	DFA2 (F ₁) (Y ² /rb)	2	—	11	—	11	1	:	1	0		
	Total		5	—	54	—	44	1	:	1	1.02	0.40	> P > 0.30

^aY, yellow; DFA2, dark fawn-2 (see text); WT, wild type; RB, recessive black.

^bUnder the hypothesis that yellow or dark fawn-2 and recessive black are controlled by alleles Y, Y², and rb, with Y and Y² being dominant over rb.

cats (*Felis catus*) are all associated with deletions in exon 2 of *ASIP* that cause a loss of the agouti function (VÅGE *et al.* 1997; KURAMOTO *et al.* 2001; RIEDER *et al.* 2001; EIZIRIK *et al.* 2003). In dogs (*Canis familiaris*), separate substitutions in exon 3 are associated with nonagouti black (*a*) and fawn or sable (*a'*) colors (KERNs *et al.* 2004; BERRYERE *et al.* 2005). In the chicken, however, no plumage color variation was found to be associated with *ASIP* up to now, which has led to the hypothesis that birds had no functional *agouti* gene (BOSWELL and TAKEUCHI 2005).

In this study, we investigated whether *rb* was a fourth allele at the *Y* locus by segregation analysis and whether variation in the coding sequence of *ASIP* was associated with the Y, Y², and *rb* mutations. The Glu92Lys substitution (c.272G > A SNP) in *MC1R*, the specific missense mutation for the extended brown plumage, was also genotyped to confirm that this mutation was not involved in the recessive black phenotype.

MATERIALS AND METHODS

Allelism test: Under the hypothesis that the *yellow*, *fawn-2*, and *recessive black* mutations were caused by different alleles at

the same locus, single-pair mating experiments were performed to test the allelism between *yellow* and *recessive black* mutations and between *fawn-2* and *recessive black* mutations, in two successive generations (Table 1). The observed segregation data on down colors of newly hatched chicks were analyzed by a chi-square test.

Cloning and sequencing of *ASIP* cDNA: Complementary DNA (cDNA) sequences of *ASIP* from wild-type (+/+), yellow (Y/+), *fawn-2* (Y²/Y²), and recessive black (rb/rb) neonatal chicks of the Japanese quail maintained at Gifu University were compared. Total RNA was purified from homogenized dorsal skin samples by using the PureLink Micro-to-Midi total RNA purification system (Invitrogen, Carlsbad, CA).

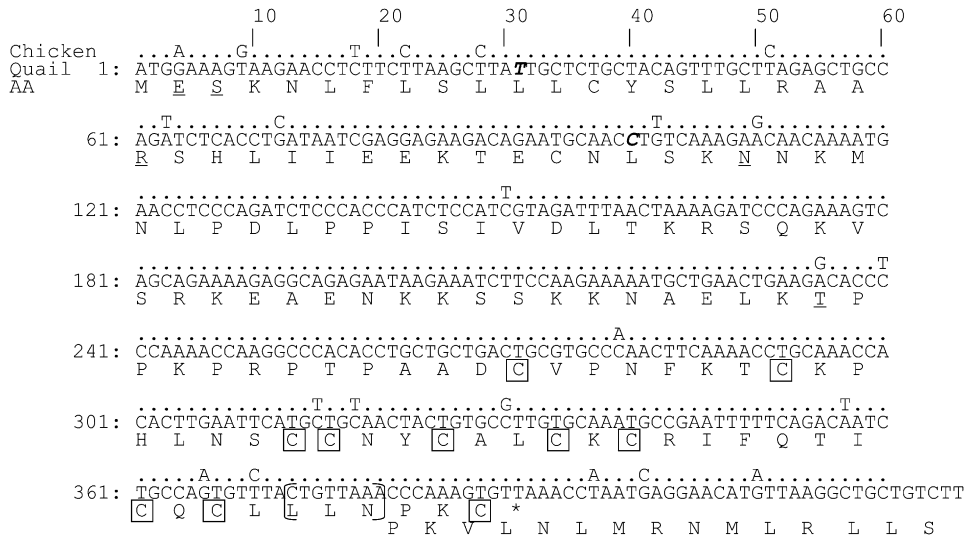
A reverse-transcription (RT) reaction was performed, using the first-standard cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ) and a poly(T) primer with an anchor region (5'-AACTGGAAGAATTCGCGGCCGAGGAAT₁₈-3'). The chicken expressed sequence tags (ESTs) homologous to the *ASIP* and *Raly* genes have already been sequenced (BBSRC ChickEST database: <http://www.chick.manchester.ac.uk/>) and were mapped on GGA20 by BLAST search (<http://www.ncbi.nlm.nih.gov/projects/genome/guide/chicken/>). To sequence the whole coding region, PCR primers were designed against the 5'- and 3'-untranslated regions (UTR) of the *ASIP*-like gene in chicken (Table 2). Sequence reactions for RT-PCR products were performed with *ASIP_F* and *ASIP_R*, using an ABI Prism 3100 DNA sequencer (PE Applied Biosystems, Foster City, CA).

Genotyping: The specific mutation for *rb* was genotyped in 34 recessive black mutants (rb/rb; e⁺/e⁺), seven wild-type quail

TABLE 2
Primer sequence and position

Primer name	Sequence (5'-3')	Nucleotide no.		Position in the chicken sequence
		5'-terminal	3'-terminal	
ASIP_F	atcttcacagcagtgaggatt	-33	-14	5'-UTR (exon 3)
ASIP_R	acacttgagaagcactactga	+445	+427	3'-UTR (exon 5)
ASIP_F2	aaatgctgaactgaagacac	+219	+238	Exon 5
MC1R_F3	tggggcgcacgggggcttt	+368	+386	Exon 1
MC1R_92Glu	gcagcatgaagagcgtctc	+750	+732	Exon 1
MC1R_92Lys	gcagcatgaagagcgtctt	+750	+732	Exon 1

+1 corresponds to the A at the start codon of each mRNA.



(*Rb⁺/Rb⁺*; *e⁻/e⁺*), and 7 extended brown mutants (*Rb⁺/Rb⁺*; *E/E*). DNA was extracted from the peripheral blood, using an AquaPure genomic DNA blood kit (Bio-Rad, Hercules, CA). DNA samples were amplified by ASIP_F2 and ASIP_R primers, using AmpliTaq Gold. PCR products were electrophoresed on an ABI Prism 3100 DNA sequencer and were analyzed using GeneScan 3.7 and Genotyper 3.7 (PE Applied Biosystems).

The Glu92Lys substitution (c.272G > A SNP) in *MC1R*, a specific missense mutation for *E*, was also genotyped in those samples, using allele-specific primers (Table 2). DNA samples were amplified by allele-specific primers named MC1R_F3-MC1R_92Glu and MC1R_F3-MC1R_92Lys. PCR products were analyzed by 1.5% agarose gel electrophoresis.

Quantitative RT-PCR: Gene expression levels in the skin were compared among four phenotypes (wild type, yellow, fawn-2, and recessive black). cDNA of three chicks from each of the four phenotypes was amplified using the SYBR ExScript RT-PCR kit, including SYBR *Premix Ex Taq* with 0.2 μ M each of ASIP_F2 and ASIP_R primers. The *GAPDH* gene was used as a control with GAPDH_F (5'-GGAGAAACCAGCCAAGTATGATG-3') and GAPDH_R (5'-AAAGGTGGAGGAATGGCTGTCA-3') primers. Three replications were carried out.

RESULTS

Allelism test: Table 1 shows the segregation data obtained in the F₁ and backcross generations, respectively. In Table 1, from matings between yellow (*Y/+*) or dark fawn-2 (*Y²/+*) hens and recessive black cocks, both yellow or dark fawn-2 and wild-type F₁ chicks were obtained, and the observed segregation did not differ from that of the null hypothesis (with similar expectations, however, under one- and two-locus modes of inheritance of the *Y* and *Y²* alleles and the *rb* gene). Only yellow or dark fawn-2 and recessive black backcross chicks, in equal proportions, were obtained from reciprocal matings of yellow or dark fawn-2 F₁ with recessive black quails. This segregation and the fact that no quail with

FIGURE 1.—Nucleotide and deduced amino acid sequence of Japanese quail *ASIP*. Nucleotide and deduced amino acid sequences in the wild-type allele (*Y^{*}N*) are shown. Nucleotide substitutions in chicken are shown at the tops of the sequences. Two synonymous nucleotide substitutions (c.31T > C SNP and c.100C > T SNP) observed in the *fawn-2* allele (*Y^{*}F2*) are italicized. Amino acid substitutions compared with chicken sequence are underlined. They are KRSSA in the chicken sequence. C-terminal cysteine residues conserved in a wide range of species are boxed. Nucleotides deleted in the *recessive black* allele (*Y^{*}RB*) resulting in a frameshift mutation are indicated in brackets and changed amino acids in *recessive black* are shown at the bottoms of the sequences.

wild-type plumage were obtained among a total of 227 backcross chicks indicate that the pattern of segregation is satisfactorily explained by a one-locus mode of inheritance and that *Y*, *Y²*, and *rb* are alleles.

Sequencing of ASIP cDNA: cDNA of *ASIP* was successfully amplified from mRNA derived from skin samples of the Japanese quail using primers designed from the chicken *ASIP* ortholog. The cDNA was sequenced starting 13 bp upstream of the start codon (-13) and finishing 33 bp downstream of the stop codon (+426) for a total of 439 bp. Sequence identity between the Japanese quail and chicken was 95% for nucleotides of the coding region and 96% for the deduced 130 amino acids. From the comparison of sequences from one bird each of wild-type, yellow, fawn-2, and recessive black phenotype, no SNP was detected in yellow quail, two synonymous SNPs (c.31T > C and c.100C > T) were detected in fawn-2 quail, and an 8-bp deletion was detected in recessive black quail in the coding region (Figure 1). No amino acid substitutions corresponding to the yellow and fawn-2 phenotypes were found. The frameshift mutation (c.373_380del) specific to the *recessive black* mutation resulted in changes in amino acids from 125 to the end, located at the C-terminal cysteine residues domain, which is conserved in mammalian *ASIP*.

Quantitative RT-PCR: The average level of relative expression of *ASIP* in the recessive black, yellow, and fawn-2 quail compared to wild-type quail is shown in Table 3. The relative expression level in the recessive black quail was significantly lower than that of the wild-type quail ($P = 0.004$). Fawn-2 quail showed ~13 times higher relative expression, whereas yellow and wild-type quail had similar relative expression.

Genotyping: For the c.373_380del in *ASIP*, we confirmed that all 7 wild-type and 7 extended brown quail

TABLE 3
Relative expression of *ASIP*

Wild type (mean ± SD)	Mutants (mean ± SD)	<i>F</i>	<i>P</i>
1.000 ± 0.348	<i>recessive black</i> 0.075 ± 0.056	20.7	0.004
	<i>yellow</i> 1.159 ± 0.219	0.4	0.528
	<i>fawn-2</i> 12.987 ± 9.488	4.8	0.071

Total RNA was purified from homogenized dorsal skin of three chicks from each of four phenotypes (wild type, *yellow*, *fawn-2*, and *recessive black*). Complementary DNA (cDNA) was amplified using *ASIP_F2* and *ASIP_R* primers. The *GAPDH* gene was used as a control. Three replications were carried out.

showed no deletion, whereas all 34 recessive black quail had the 8-bp deletion genotype.

For the c.272G > A SNP in *MC1R*, all 7 extended brown quail showed the A/A SNP genotype, whereas the 7 wild-type and 34 recessive black quail had the G/G SNP genotype. Thus the *recessive black* mutation was associated with the deletion in *ASIP*, and it was not affected by *MC1R*.

DISCUSSION

The allelism test showed that *recessive black* (*rb*) is a fourth allele at the *Y* locus. Therefore, alleles for wild type, *yellow*, *fawn-2*, and *recessive black* were named *Y*N*, *Y*Y*, *Y*F2*, and *Y*RB*, respectively, according to the gene nomenclature of CRITTENDEN *et al.* (1996), and the dominance relationship was *Y*F2* > *Y*Y* > *Y*N* > *Y*RB*.

A deletion of 8 bases was found in *ASIP* in the *recessive black* allele (*Y*RB*). Genotyping from a large population confirmed that the deletion is associated with the *recessive black* allele. The frameshift caused by this mutation changed the last six amino acids and the stop codon. Among the 10 cysteine residues conserved in a wide range of animals, the last one was missing in the *recessive black* allele because of this mutation. Since the cysteine residues are important to build the disulphide bond, the deletion might cause dysfunction of *ASIP* as an antagonist of α -MSH in the *Y*RB* allele. Several studies of black coat color mutations in mammals have found an association of the coat color with mutations in the coding regions of *ASIP*. Among them, the black coat color of German shepherd dogs was associated with a SNP to cause an arginine to cysteine substitution at the C-terminal cysteine-rich residues (KERNS *et al.* 2004). In mice, several amino acid substitutions at cysteine-rich residues are known to be related to the black phenotype (MILTENBERGER *et al.*

2002), and an insertion in the first intron was reported in nonagouti mice (BULTMAN *et al.* 1994), but no deletion in the coding sequence has been reported yet.

The result of RT-PCR in this study showed that gene expression of recessive black quail was remarkably lower than that of wild-type quail, which was 13.3 times higher. Although this result might be an indication that there is another mutation at the promoter region of the *Y*RB* allele, it is rather unlikely that a second loss-of-function mutation would have accumulated in the same gene. It is much more likely that the suppression of the termination codon in the 3'-UTR of *rb-ASIP* due to the frameshift mutation was associated with mRNA decay and, consequently, low expression of *rb-ASIP*. Indeed, the existence of a mechanism to degrade mRNA lacking a termination codon was described by VAN HOOF *et al.* (2002), and nonstop mRNA decay was further confirmed as one way to safeguard the cell from abnormal mRNA function (ISKEN and MAQUAT 2007; ITO-HARASHIMA *et al.* 2007). Moreover, the absence of mutation in the *ASIP* sequence of yellow quail is consistent with the recent finding in an accompanying article by NADEAU *et al.* (2008, this issue) that the *yellow* mutation in quail was associated with a large deletion upstream of *ASIP*, placing its expression under the control of the *Raly* promoter, as is the case for the yellow coat color in *A^y/+* mice (MICHAUD *et al.* 1993). Finally, the synonymous SNPs identified in all *fawn-2* quail might indicate that they are in linkage disequilibrium with a still unknown regulatory mutation causing higher *ASIP* mRNA expression and therefore are dominant over the *yellow* mutation. In any case, this study has shown that the recessive black plumage color in quail was closely associated with the *ASIP* gene, and the results on the three alleles *yellow*, *fawn-2*, and *recessive black* are consistent with the existence of regulatory or structural mutations for this gene. Together with the study by NADEAU *et al.* (2008), we have given here the first evidence that *ASIP* is functional in birds and that its effects on plumage color parallel that of *ASIP* on mouse coat color.

This study was partly funded by 2004 and 2005 grants from Gifu University and by a 2006–2007 grant from the Japan Livestock Technology Association.

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Communicating editor: L. SIRACUSA