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Rupali Ugrankar
University of Arkansas, Fayetteville

Kim Cheng
University of Arkansas, Fayetteville

Ronald Okimoto
University of Arkansas, Fayetteville

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DNA sequence of melanocortin 1-receptor gene in *Coturnix japonica*: correlation with three *E* locus alleles, *E*, e^{+}, and e^{rh}

Rupali B. Ugrankar, Kim Cheng†, and Ronald Okimoto§*

ABSTRACT

The melanocortin 1-receptor (MC1-R) gene plays a key role in the expression of fur and feather color in mammals and birds by regulating the distribution of two melanin pigments: eumelanin (black/brown) and pheomelanin (red/yellow). MC1-R corresponds to the classical *Extension (E)* locus in mice, pigs, dogs, horses, and chickens. Three *E* locus alleles, the wild-type (e^{+}), brown (*E*), and redhead (e^{rh}) have been identified in Japanese quail (*Coturnix japonica*). To determine if the quail *E* locus phenotypes were due to variation in the MC1-R gene, the coding region of the MC1-R gene was PCR amplified and DNA sequenced using genomic DNA isolated from individuals exhibiting the phenotypes of the three quail alleles. The DNA sequence comparison revealed two missense mutations that differentiated the brown from the wild-type and redhead quail. A single-base substitution resulted in a Val58Ile change, and another single-base substitution produced a Glu92Lys change in the brown quail. The redhead quail sequence carried a seven-base deletion extending from nucleotide position 682 to 688, resulting in a reading frame shift and premature termination of the MC1-R gene after amino acid position 231. The Glu92Lys change in the brown allele created a *Msc* I restriction fragment length polymorphism (RFLP). A PCR-*Msc* I RFLP test was developed and a direct correspondence between phenotype and genotype was found by testing the DNA of a population segregating for the brown and wild-type alleles. The DNA sequence and segregation data indicate that the quail *E* locus is homologous to the *E* locus identified in other birds and mammals.

* Rupali Ugrankar graduated in May 2003 with a degree in environmental, soil and water sciences

† Kim Cheng, collaborator, is a faculty mentor with the Department of Animal Science at the University of British Columbia, Vancouver.

§ Ronald Okimoto, faculty sponsor, is an assistant professor in the Department of Poultry Science

MEET THE STUDENT-AUTHOR

Rupali B. Ugrankar

I am an international student from Bombay, India, where I completed my high school education at Jai Hind College. In May 2003 I completed my B.S. degree in environmental, soil, and water sciences with honors, graduating Summa Cum Laude and Senior Scholar. I was named Presidential Scholar for 2002-2003 and have received numerous other scholarships including International Student Scholarship, C. Roy Adair Scholarship, Fontaine Earle Crop Science Scholarship, RP and Mildred Bartholomew Memorial Scholarship, and Gamma Sigma Delta Scholarship. I was a Bumpers College Ambassador during 2001-2002 and am a member of Gamma Sigma Delta agricultural honors society and Golden Key Honors Society.

In fulfillment of my Honors Program requirements, I selected a genetics project, completely unrelated to my major. During the course of my research I learned valuable molecular techniques, and my interest in genetics grew. I will be pursuing this interest further when I embark on a Ph.D. degree program in molecular genetics and microbiology at the University of Texas at Austin, in Fall 2003.

INTRODUCTION

The melanocortin 1-receptor or MC1-R gene corresponds to the *E* or *Extension* locus in mice, pigs, dogs, horses, sheep, fox, cows, and chickens (Kijas et al., 1998; Lu et al., 1996; Takeuchi et al., 1996). Polymorphisms in the MC1-R gene affect the distribution of the two melanin pigments- black/brown eumelanin and red/yellow pheomelanin in the fur of these mammals and in the feathers of chickens. Dominant mutations at the *E* locus result in a constitutively active MC1-R generating a uniformly black coat or feather color, while recessive mutations at this locus usually eliminate receptor activity, producing more red and yellow pigmentation (Kijas et al., 1998).

Relative to the above animals, few feather pigmentation studies have been conducted on the Japanese quail (*Coturnix japonica*). In fact it is not known if the *E* locus in Japanese quail is homologous to what is called the *E* locus in chickens. But considering that the Japanese quail and chickens are close relatives, it is expected that the same genetic mechanisms, with regard to feather color, may apply to both species (Cheng and Kimura, 1990). Three quail *E* locus alleles, the wild-type (e^{+}), brown *(E)*, and redhead (e^{rh}) have been identified by the efforts of Truax (1979), Truax et al. (1979), and Truax and Siegel (1981), but specific polymorphisms giving rise to these phenotypes have not been studied. Fig. 1 shows the phenotypes of the brown, wild-type, redhead, and shafted quail (E/e^{+}).

There are several reasons why the Japanese quail has recently become a subject of biological research. Domesticated in the 11th century, it was used as a songbird and later as a source of meat and eggs. Amateur bird hobbyists and fanciers find them easy to raise because they are hardy and easy to manage. Due to the Japanese quail's short reproductive cycle of only 35 days, it is ideal as a model species for comparative studies (Ernst, 2000). In spite of its small body size and cryptic coloration, it is widely consumed in the Middle East, Asia, and Europe, but its consumption in North America and Australia has remained low as it has been considered more of a specialty item (Cheng and Kimura, 1990). However, recently its popularity as a delicious food source has increased, and many people enjoy pickled hard-boiled quail eggs (Ernst, 2000). Considering that feather pigmentation potentially affects tissue/meat coloration and, therefore, affects the consumers' perceptions of quail meat as an appealing food source, it is important to understand the molecular genetics of melanin production in quail feathers.

Through this study we sought to identify the polymorphisms in the *E* locus that are associated with the different feather-color phenotypes in Japanese quail.

MATERIALS AND METHODS

Polymerase chain reaction and DNA sequencing

Blood was extracted from the wing veins of six Japanese quail: two brown (*E/E*), two wild-type (e^+/e^+), and two shafted (E/e^+) birds. Blood samples of two redhead (e^{rh}/e^{rh}) were shipped from Canada. DNA was isolated from the whole blood using a Qiagen Blood Minikit (Qiagen, Valencia, CA), quantitated, and diluted to 20 ng/µl. We initially employed chicken MC1-R primers for polymerase chain reaction (PCR) amplification using Promega (Promega, Madison, WI) or Biolase (Bioline, Canton, MA) Taq polymerase. Thermocycler conditions for PCR were 94°C 30 s denaturation, 55°C 1 min annealing, and 72°C 1 min elongation for 35 cycles. A 0.8 or 1.0% TAE agarose gel electrophoresis of the PCR product separated the DNA bands, which were extracted using dialysis-filter paper method of Girvitz et al. (1980). The DNA isolated was sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The oligonucleotide primer sequences used in this study are listed in Table 1. Chicken MC1-R primers +MC977 and –MC1132 amplified the 3' end of only wild-type MC1-R. From this initial sequence data, we designed the –QMC1-1522 quail MC1-R primer that was coupled with +MC977 to amplify the 3' end of the brown sequence. Using chicken and the new-quail MC1-R sequences, we designed several specific quail MC1-R primers for the 5' and 3' ends of the coding region of the MC1-R gene. Other notable primers used were +MC1R-340 and –QMC1087 for wild-type sequencing and +QMC-77 and –QMC1087 for brown sequencing. Relative to the wild-type, we faced considerable difficulty in amplifying the brown MC1-R coding region. On one occasion, we had to resort to a nested PCR technique to obtain the brown sequence (i.e. we re-amplified the DNA isolated from the agarose gel using the same primers +QMC-102 and –QMC1087, re-isolated the DNA from the agarose gel, and then sequenced the DNA using primers +QMC-77 and –QMC1087). The same steps as above were repeated for the redhead. Notable redhead MC1-R primers were +MC1R600 and –QMC-1535, and +MC1R-340 and –MCR11005. All DNA sequencing was done at the DNA Resource Center of the University of Arkansas, Fayetteville. The brown, wild-type, and redhead MC1-R sequences were assembled and analyzed using the DNAstar software package (DNAstar, Madison, WI).

PCR-RFLP segregation study

Four quail matings were set up in separate cages. Two cages housed one wild-type male with two shafted females, and the other two had one shafted male with two wild-type females. Eggs were collected for 2 weeks before they were incubated in petersime incubators. One of the wild-type male-shafted female mating was infertile and did not yield any chicks. A second round of matings was set up for the three that worked. Chicks obtained from both matings were typed as wild-type or shafted at time of hatch and typed again as adults. Ratio of wild-type progeny to shafted progeny was determined. Blood samples were taken from the parents and the progeny, and DNA was isolated from whole blood using Qiagen blood minikit. A PCR-RFLP (RFLP = Restriction Fragment Length Polymorphism) test was performed on the DNA from all the wild-type and shafted parents and progeny. A segment of the MC1-R was amplified by PCR using primers +QMC1Rq401 and –MCR11005. The PCR product was treated with restriction enzymes *Msc* I at 37°C for one hour, and then run on 1.5% TAE ethidium bromide-stained (0.15mg/ml) agarose gel. The arrangement/separation of the DNA bands on the gel for the wild-type and shafted progeny was examined under ultra-violet light, and a picture of the ethidium bromide stained gel was taken using the fluorescence setting on the Fujifilm Intelligent Dark Box II (Fujifilm, Edison, NJ). The entire MC1-R coding sequences of the brown, wild-type, and redhead quail were assembled using DNAstar software package

RESULTS AND DISCUSSION

Examination of the sequence data of the brown, wild-type, and redhead quail revealed some notable differences between the three. The wild-type and redhead quail had amino acid valine at position 58, while the brown quail had isoleucine at the same position owing to a single-base substitution of adenine (A) for guanine (G) at nucleotide position 172. In addition, the wild-type and redhead quail had glutamic acid at position 92, while the brown quail had lysine resulting from a single-base substitution of A for G at nucleotide position 274. Interestingly, a similar Glu92Lys substitution is found in chickens and somber-3J black mice. Apparently this single-nucleotide polymorphism results in a constitutively active MC1-R in both species and produces a solid black phenotype in these species (Takeuchi et al., 1996). In quail this mutation is associated with the extended-brown phenotype. In chickens, the *E* allele referred to as extended black is almost completely dominant over all other *E* locus chicken alleles, viz., birchen (E^R); dominant wheaten (e^{Wh}); wild-type (e^+); brown (e^b); speckled (e^s); buttercup (e^{bc}); and recessive wheaten (e^y) (Smyth, 1990). Similarly, the *E* allele in Japanese quail, conferring the brown phenotype, is incompletely dominant over the wild-type e^+ allele and completely dominant

over the redhead e^{rh} (Truax and Siegel, 1981).

Other than these missense single-nucleotide polymorphisms, three silent single-base substitutions were found. At nucleotide position 177, the wild-type and brown had thymine (T), while the redhead had cytosine (C). The brown and redhead had T at nucleotide position 513, and the wild-type at C instead. Finally, at nucleotide position 627 the brown and redhead had C, while the wild-type had T.

We discovered a significant mutation in the redhead sequence. A seven-base deletion extending from nucleotide position 682 to 688 resulted in the shifting of the reading frame and premature termination of the MC1-R gene after amino acid 231 (Fig. 2). This is expected to reduce the activity of the MC1-R because the shorter gene will fail to produce a full-length polypeptide and may be responsible for the dilution of color in the redhead plumage. In cattle a frameshift mutation in the dominant allele *ED* causes premature truncation of the receptor and produces red coat color (Klungland et al., 1995).

Truax (1979) crossed the brown and wild-type quail, which yielded all shafted progeny in the F1 generation. He concluded that the brown *E* allele was incompletely dominant over the wild-type (e^+) allele. We set up four test crosses between wild-type (e^+/e^+) and shafted (E/e^+) parents, as described in the materials and methods section. According to Mendelian ratios, we expected that the progeny would segregate 1:1 for the wild-type and shafted. Our results were skewed, but the difference was not significant by chi-square analysis ($\chi^2 = 2.8$): of the total 70 progeny obtained, 28 were wild-type and 42 were shafted, giving instead a wild-type:shafted ratio of 1:1.5.

Analysis of the brown, wild-type, and redhead MC1-R sequences revealed that the A to G substitution at nucleotide position 274 responsible for the Glu92Lys change in the brown allele creates a palindromic sequence TGGCCA, recognized by the restriction enzyme *Msc* I. The *Msc* I enzyme was expected to cleave the brown allele, but not the wild-type or redhead allele. The +QMC1Rq401 and -MC1R1005 primers were used to amplify a 433 base-pair (bp) segment of the MC1-R gene of the test cross parents and the 70 progeny. The *Msc* I was expected to cleave the 433 bp fragment twice. The brown DNA fragment would be cut into 56 bp, 158 bp and 219 bp fragments. The wild-type allele is cleaved once and produces a 377 bp and 56 bp fragment. The shafted quail carry one wild-type allele and one brown allele, hence three bands were observed for the shafted parents and progeny: an uncut wild-type band at 377 bp, and the additional two bands at 158 bp and 219 bp. The 56 bp fragment was difficult to observe on the gel (Fig. 3). The PCR-RFLP test confirmed that there was a 1:1 correspondence of the Lys92 mutation with the dominant brown phenotype, indicating that this mutation in the MC1-R gene was associated with the *E* locus phenotype.

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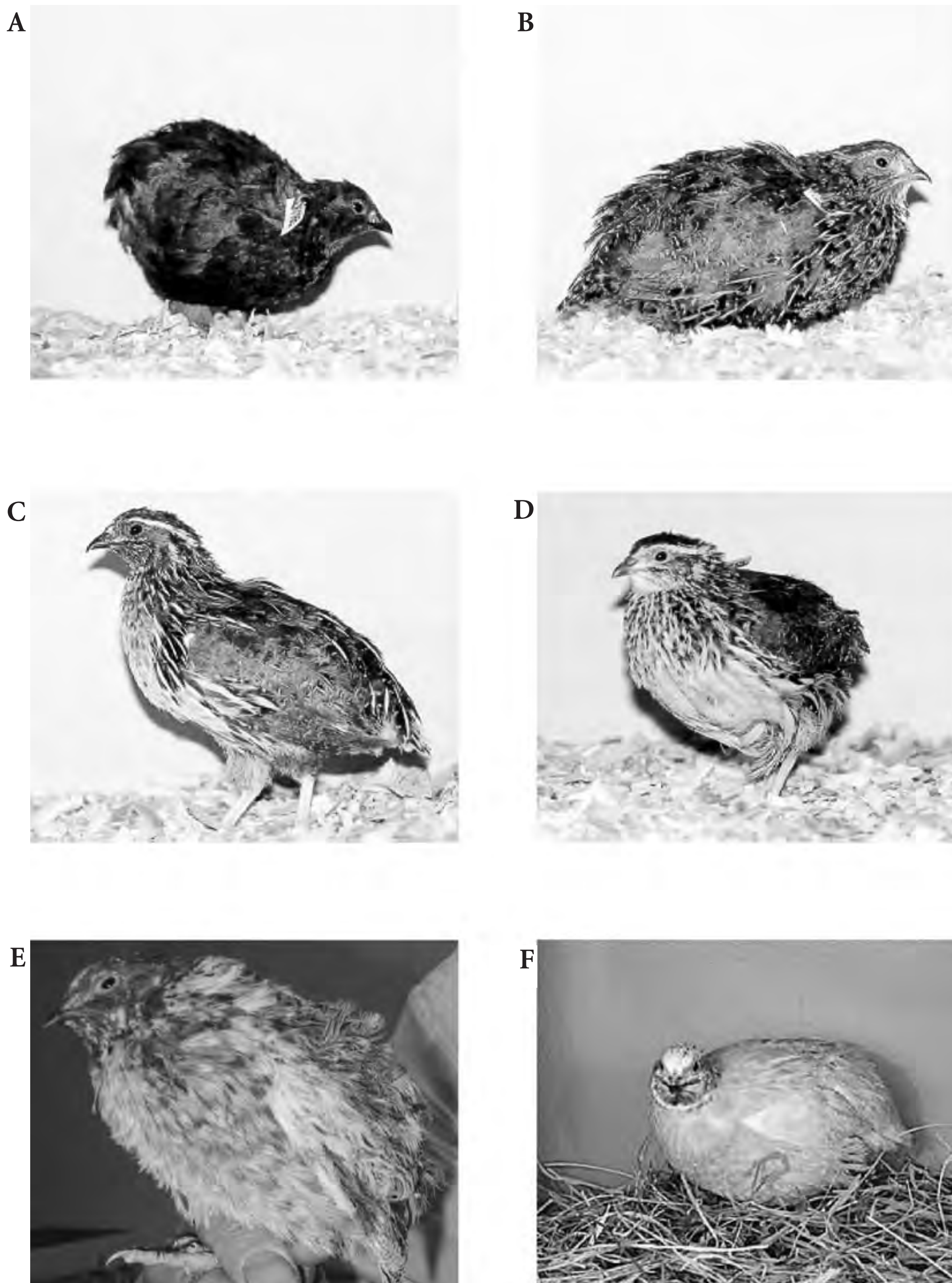

Fig. 1. A represents a male brown mutant (*E/E*) having rust brown feathers with varying degrees of dark markings along with dark brown legs, toes, and beaks, but the bottoms of the feet are light (Truax and Siegel, 1981). Fig. 1B is a female shafted quail (E/e^+) with brown feathers similar to the brown mutant in addition to light yellow shafting on the body plumage (Truax, 1979). Fig. 1C and 1D represent a male and female adult wild-type (e^+/e^+), respectively. Their plumage is a mix of several colors, but black and numerous shades of brown predominate on the dorsal side. Brown plumage is crossed by a black bar, and wheat-straw colored shafting occurs on the back and hackle feathers (Somes, 1979). Fig. 1E and 1F represent male and female redhead mutants (e^{rh}/e^{rh}), respectively. Phenotype is predominantly white with irregular black and rust colored markings, and beak color may range from a mixture of pink and black to solid black; the toes and legs are pink with black markings (Truax and Siegel, 1981).

Table 1. Oligonucleotide primer sequences used in this study

Primer name	Sequence (5'-3')
+MC977	CAGCACCGTCTTAATCACCTACTA
+MC-117	TGCTGCGGGAGCACTGGT
+MC805	ATGTCATCGACATGCTCATCTG
+MC1R-77	GGCTTTGTAGGTGCTGCAGTTG
+MC1R-400	GGGCACAGGCTGTCATGTG
+MC1R-340	GCCAGCTTTAAATCAGGACAGAG
+MC1R600	CTGGTGAGCCTGGTGGAGAAC
+MC1R-153	GCAGAGGTGCCCACATCC
+QMC1-71	GTAGGTGCTGTGGTTGTGCTC
+QMC1-495	CCCTGGAACGCCACTGAG
+QMC-102	GCTGGCAGGGCTGATAGG
+QMC-77	GGGGCTTTGTAGGTGCTG
+QMC1Rq401	CCCCAATGAGCTGTTCCTGAC
-MC1132	AGCCTTTATTTGGGAGCGCGA
-MCR11005	GCGGTAGTAGGTGATTAAGAC
-QMC1087	AACATGTGGATGTAGAGCACCAG
-QMC1135	CTGCTTCTGCTGGCTGGAGAT
-QMC1-1535	CCCACAGTGCGTCCGTC
-QMC1-1089	GCGCAAACATGTGGATGTAG
-QMC1-1522	CACAGTGCGTCCGTCTGTC

Allele	Amino Acid Positions									
e^+	225	226	227	228	229	230	231	232	233	234
	CAG	CCC	ACC	ATC	TAC	CGC	ACC	AGC	AGC	CTG
	Gln	Pro	Thr	Ile	Tyr	Arg	Thr	Ser	Ser	Arg
e^{rh}	225	226	227			228	229	230	231	232
	CAG	CCC	ACC	---	---	-GCA	CCA	GCA	GCC	TGA
	Gln	Pro	Thr			Ala	Pro	Ala	Ala	TER

Fig. 2. Comparison of the DNA sequence of the wild-type (e^+) and the redhead alleles (e^{rh}), where a seven-base deletion in the redhead sequence produces a stop codon at amino acid position 232, causing premature truncation of the coding region of the MC1-R gene.

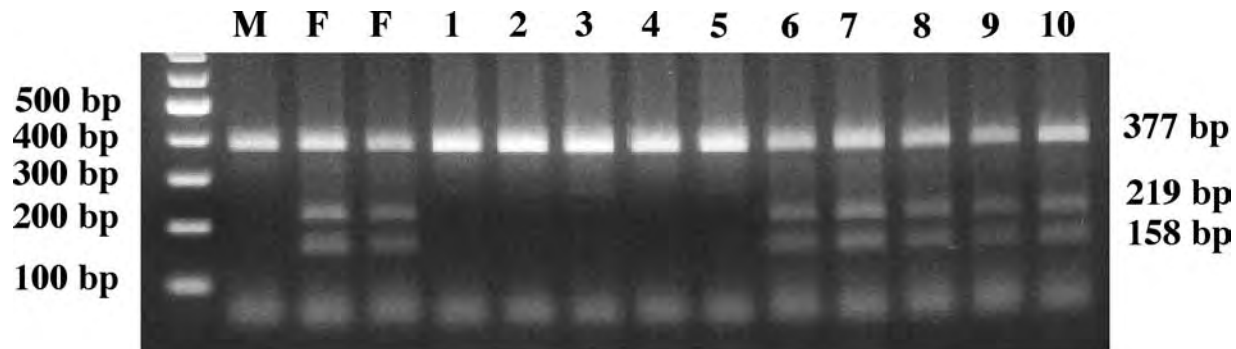

Fig. 3. The ethidium bromide stained 1.5% agarose gel shows the arrangement of *Msc* I digested MC1-R DNA fragments of one wild-type male (M) and two shafted female (F) parents and their five wild-type (1 to 5) and five shafted (6 to 10) progeny.