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Association of a single-nucleotide substitution in *TYRP1* with *roux* in Japanese quail (*Coturnix japonica*)

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Summary

We investigated *TYRP1* as a candidate locus for the recessive, sex-linked *roux* (br^r) phenotype in Japanese quail. A screen of the entire coding sequence of *TYRP1* in *roux* and wild-type quail revealed a non-synonymous T-to-C substitution in exon 3, leading to a Phe282Ser mutation. This was perfectly associated with plumage phenotype: all *roux* birds were homozygous for Ser282. Co-segregation of the Phe282Ser mutation with the *roux* phenotype was confirmed in three $br^r/BR^+ \times br^r/-$ backcrosses. We found no significant difference in *TYRP1* expression between *roux* and wild-type birds, suggesting that this association is not due to linkage disequilibrium with an unknown regulatory mutation. In addition, the Phe282 amino acid appears to be of functional significance, as it is highly conserved across the vertebrates. This is the first demonstration that *TYRP1* has a role in pigmentation in birds.

Keywords pigmentation, quail, roux, sex-linked, *TYRP1*.

Introduction

The tyrosinase-related protein 1, encoded by the *TYRP1* gene, is a melanogenic enzyme that has been well characterized in mammals as being required for the production of eumelanin (dark black/brown pigment) but not phaeomelanin (pale yellow/red pigment) (Zdarsky *et al.* 1990). Loss or reduction of function mutations of *TYRP1* have been identified in many mammal species generally leading to a pale, brown phenotype and *TYRP1* is the classic *b* (*brown*) locus in mice (Schmutz *et al.* 2002; Berryere *et al.* 2003; Schmidt-Kuntzel *et al.* 2005). Chicken *TYRP1* has been cloned and sequenced (April *et al.* 1998) and localized to the Z chromosome. However, the role of this locus in pigmentation in birds has yet to be established, as no functional mutations have so far been described.

The genetics of avian colouration is of evolutionary interest because of its role in signalling and mate choice (Andersson 1994; Hill & McGraw 2006). Sex-linked loci have been proposed as being particularly important in the control of secondary sexual and sexually selected traits (Reinhold 1998; Sætre *et al.* 2003; Kirkpatrick & Hall 2004). Therefore, the possibility that *TYRP1* is a sex-linked pigmentation gene is interesting from an evolutionary perspective.

The Japanese quail *roux* mutation is a recessive, sex-linked mutation and has been found to be allelic to the sex-linked *brown* mutation (Minvielle *et al.* 2000) and so has been given the symbol br^r. However, the molecular genetics of this locus and the causation of *brown* and *roux* have not been determined. These mutations are of commercial interest because of their potential for auto-sexing in quail production (Minvielle *et al.* 1999). Both result in a dilution of eumelanin pigmentation with *brown* being slightly darker and dominant to *roux* (Fig. 1). Therefore, *TYRP1* seems a good candidate gene for the *BR* locus in quail.

Materials and methods

Japanese quail were maintained at the INRA Experimental Unit GFA in Nouzilly (France). Single-pair matings between roux females ($br^r/-$) and F1 wild-type males (br^r/BR^+) were carried out to obtain three families segregating for the *roux* phenotype. Dorsal skin samples were obtained from 12 male individuals from one family, six with a *roux* phenotype (br^r/br^r) and six with a wild-type phenotype (br^r/BR^+). Feathers were plucked from the region of skin that would be sampled 11 days prior to sampling, to stimulate feather

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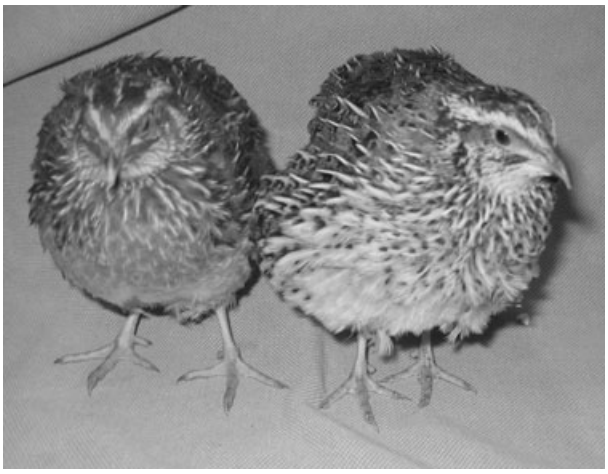

Figure 1 Japanese quail (*Coturnix japonica*) with *roux* (left) and wild-type (right) phenotypes.

growth. Skin samples were taken by dissecting a piece of skin (approximately 4 cm^2), which was snap-frozen in liquid nitrogen and stored at −80 °C.

Total RNA was extracted from these skin samples using the RNeasy mini-kit (Qiagen). RNA concentration, purity and integrity (RIN values) were checked using a BioAnalyser (Agilent). RNA was stored at −80 °C until needed. cDNA syntheses were performed in a 20-μl volume with 1–3 μg total RNA and 7.5 ng/μl N6 primer using Superscript RT II (Invitrogen). The entire 1611-bp coding region of *TYRP1* and 13-bp of flanking sequence were amplified using primers TP1F4 (5′-CAGCTCTGCTGAACCTGTTG-3′) and TP1R5 (5′-TCAGAAGGAATCTTTTGGATCTT-3′), designed to the published Japanese quail *TYRP1* mRNA sequence (AB005228). PCR reactions were performed in a 50-μl total reaction containing: 1.0 unit Taq polymerase (Advanced Biotechnologies), 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM each primer and 4 μl cDNA reaction product. PCR reactions were performed in a DNA Engine (MJ Research), with the following cycling parameters: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min; and 72 °C for 5 min. Direct sequencing was performed using the PCR primers and five internal primers: TP1F5 (5′-TCAGAAGGAATCTTTTGGATCTT-3′), TP1F6 (5′-AAATACGACCCGGCAGTTC-3′), TP1R6 (5′-GCCACTCATCAAAAACAGCA-3′), TP1e2R1 (5′-GCATGTCCCTTTCAAGTTGC-3′) and TP1e1R1 (5′-GATTTGCTGGCTACAGGTAGGTC-3′).

Genomic DNA was extracted from dorsal skin samples taken from all individuals from the remaining two families ($n = 17$ for each family) using the QIAamp DNA mini kit (Qiagen). 159 bp of exon 3 of *TYRP1* was amplified and directly sequenced with primers TP1F7 (5′-TGCTACAGGATCCCAGTTTTG-3′) and TP1R7 (5′-TGTTACAAATGGTTCCCAAGC-3′). Sequences are deposited in GenBank with accession numbers EU046599 and EU046602.

Quantitative RT-PCR for *TYRP1* was performed using primers TP1e1F3 (5′-CTCAGTTCCCTCGCCAGT-5′) and TP1e2R1 in a 25-μl total reaction containing: 1× SYBR Green master mix (Qiagen), 0.4 μM each primer and 1–2 μl of product from the cDNA reactions. Reactions were performed in an Opticon 2 DNA engine (MJ Research), with the following cycling parameters: 95 °C for 15 min; 40–55 cycles of 94 °C for 15 s, 55–68 °C for 30 s and 72 °C for 30 s; 72 °C for 10 min. Melting curves were generated between 55 and 90 °C with readings taken every 0.2 °C for each of the products to check that a single product was generated. At least one product from each set of primers was also run on a 1% agarose gel to check that a single product of the expected size was produced and the identity of the product was confirmed by direct sequencing. Results were normalized using *β-actin*, which was amplified with primers ACTF1 (5′-TGCGTGACATCAAGGAGAAG-3′) and ACTR1 (5′-CAGGTCCTTACGGATGTCCA-3′). Amplified fragments always spanned at least one intron to ensure that genomic DNA contamination could be identified. Ct values were defined as the point at which fluorescence crossed a threshold (R_{Ct}) of 10 × SD of the background fluorescence. Amplification efficiencies (E) were calculated using a dilution series of clean PCR product. Starting fluorescence, which is proportional to the starting template quantity, was calculated as $R_0 = R_{Ct}(1 + E)^{-Ct}$. Normalized values were then obtained by dividing R_0 values for the target loci by R_0 values for *β-actin*. All results were taken as averages of triplicate PCR reactions, and PCRs on target and control loci were always performed using product from the same cDNA synthesis reaction. Relative expression levels were calculated by assigning a value of 1 to the average R_0 of wild-type individuals. Statistical significance was assessed using unrelated samples two-tailed t-tests assuming unequal variance.

Results and discussion

The full coding sequence of *TYRP1* in Japanese quail is 3 bp longer than in chicken (1611 bp) and 95% similar to the chicken sequence at the nucleotide level. A single variable nucleotide site was found at *TYRP1* in the 12 individual males (six *roux* and six wild-type) sequenced from family 1. This T-to-C substitution at nucleotide position 845 (c.845T>C in EU046599, EU046602) is non-synonymous, leading to a phenylalanine-to-serine mutation at amino acid 282 (p.Phe282Ser). There was a perfect association between the homozygous presence of the p.Ser282 amino acid and the *roux* phenotype; all of the *roux* individuals were homozygous for the c.845C allele (the p.Ser282 amino acid), and the wild-type siblings were heterozygous c.845TC, which is consistent with the recessive nature of the *roux* mutation (Table 1). This significant association was maintained in the additional 34 individuals sampled from two families ($P < 0.001$, Fisher's exact test). The previously published Japanese quail *TYRP1* sequence

Table 1 Phenotypes and associated *TYRP1* genotypes of individuals sampled from three families segregating for the *roux* mutation.

Family	Phenotype (sex)	*BR* genotype	*n*	Nucleotide at position 845[1]	Putative amino acid at position 282[2]
1	Wild-type (M)	br^r/BR^+	6	c.845CT	p.Phe282, p.Ser282
	Roux (M)	br^r/br^r	6	c.845C	p.Ser282
2	Wild-type (M)	Br^r/BR^+	6	c.845CT	p.Phe282, p.Ser282
	Wild-type (F)	$BR^+/-$	3	c.845T	p.Phe282
	Roux (M)	br^r/br^r	5	c.845C	p.Ser282
	Roux (F)	$br^r/-$	3	c.845C	p.Ser282
3	Wild-type (M)	br^r/BR^+	5	c.845CT	p.Phe282, p.Ser282
	Wild-type (F)	$BR^+/-$	4	c.845T	p.Phe282
	Roux (M)	br^r/br^r	5	c.845C	p.Ser282
	Roux (F)	$br^r/-$	3	c.845C	p.Ser282

[1]Accession numbers EU046599, EU046602.
[2]Inferred from nucleotide sequence.

Table 2 *TYRP1* sites associated with diluted pigmentation in mammals and birds.

Species	Allele	3	41	110	282	290	345	420	434	Accession no.
Coturnix japonica	BR^+	L	C	C	F	C	P	A	H	AB005228, EU046600[2]
	br^r	L	C	C	**S**	C	P	A	H	EU046599[2]
Gallus gallus	BR^+	L	C	C	F	C	P	A	H	AF003631[3]
Mus musculus	B^+	S	C	C	F	C	P	A	H	NM_031202
	b	S	C	**Y**	F	C	P	A	H	[4]
Canis familiaris	B^+	A	C	C	F	C	P	A	H	AY052751[5]
	b^1	A	**S**	C	F	C	Δ	A	H	[5]
Felis catus	B^+	A	C	C	F	C	P	A	H	AY956310[6]
	b	**G**	C	C	F	C	P	A+**16/17**	H	AY965744-5[6]
Bos taurus	B^+	S	C	C	F	C	P	A	H	NM_174480[7]
	b	S	C	C	F	C	P	A	**Y**	[7]
Ovis aries (Soay)	B^+	S	C	C	F	C	P	A	H	EF102110[8]
	b	S	C	C	F	**F**	P	A	H	EF102109[8]
Homo sapiens	B^+	A	C	C	F	C	P	A	H	BC052608
Equus caballus	B^+	A	C	C	F	C	P	A	H	BK000021[9]
Sus scrofa	B^+	A	C	C	F	C	P	A	H	NM_001025226
Xenopus laevis	B^+	K	C	C	F	C	P	A	H	BC043815
Danio rerio	B^+	–	C	C	F	C	P	A	H	BC076406

Δ, deletion; $+n$ = insertion of *n* length. Positions are numbered based on the multiple alignment shown in Fig. S2.
Bold letters indicate the variant amino acids associated with the mutant allele.
[1]At this genotype, any one of the highlighted variant positions in homozygous or two in heterozygous state are sufficient to produce a pale phenotype.
[2]Sequences generated in this study.
[3]Reported by April *et al.* (1998).
[4]Reported by Zdarsky *et al.* (1990).
[5]Reported by Schmutz *et al.* (2002).
[6]Reported by Schmidt-Kuntzel *et al.* (2005).
[7]Reported by Berryere *et al.* (2003).
[8]Reported by Gratten *et al.* (2007).
[9]Reported by Rieder *et al.* (2001).

(Mochii *et al.* 1998) is homozygous for the c.845T allele (p.Phe282). There are six additional variable nucleotide positions between this individual and both the wild-type and *roux* individuals in our study. Five of these are synonymous and one leads to a p.Ala21Val substitution (Fig. S1).

The perfect association between the phenotype and non-synonymous substitution in three independent crosses strongly suggests that this mutation may be causative. However, an alternative possibility is that this mutation is tightly linked to an unknown regulatory mutation. To test this, *TYRP1* expression was compared between *roux* and wild-type birds by quantitative RT-PCR. Melting curves demonstrated that a single product was produced, which was of the expected size based on gel electrophoresis and

confirmed by direct sequencing to be *TYRP1*. We found no significant difference in expression (mean relative expression = 0.26 ± 0.24, $P = 0.29$, two-tailed *t*-test), suggesting that the phenotype is not due to a regulatory mutation. Regulatory mutations of *TYRP1* have been described in mice, which lead to *TYRP1* mRNA levels of about 1% of wild-type levels (Jackson *et al.* 1990). Heterozygotes for this mutation, which produced about half-normal levels, exhibit a wild-type phenotype. This suggests that the small, non-significant reduction in *TYRP1* expression seen in the *roux* quail is unlikely to be the cause of the change in phenotype.

TYRP1 coding sequence variants with an effect on phenotype have been found in mice, dogs, cats, cattle and Soay sheep (Zdarsky *et al.* 1990; Schmutz *et al.* 2002; Berryere *et al.* 2003; Schmidt-Kuntzel *et al.* 2005; Gratten *et al.* 2007) and none of these have involved an amino acid at the Phe282 position (Table 2). However, an alignment of *TYRP1* protein sequences from a wide range of taxa including several mammal species, the zebra fish (*Danio rerio*) and a frog (*Xenopus laevis*) revealed that this amino acid position is conserved as a phenylalanine in all these taxa, suggesting it is important for enzyme function (Fig. S2). The phenotypic effect of the *TYRP1* mutation in the *roux* quail seems more pronounced than the effect of most of the previously described point mutations at this locus in mammals. The brown mouse, chocolate cat and dun Dexter cattle phenotypes and the brown coat colour of dogs are all darker than the *roux* phenotype in quail. One possible exception is the p.Cys290Phe substitution in Soay sheep, which is associated with pronounced lightening (Gratten *et al.* 2007). This is intriguing as it is in close proximity to p.Phe282 within a cysteine-rich region thought to be important for enzyme structure.

Our results provide the first direct evidence that *TYRP1* is involved in pigmentation of birds. This adds to the growing number of quail pigmentation loci that are characterized at the molecular level (Mochii *et al.* 1998; Nadeau *et al.* 2006; Miwa *et al.* 2007). *TYRP1* is the first sex-linked pigmentation gene to be identified in birds, making it an interesting candidate for studies of sexual selection and population differentiation, for which sex-linked loci are suggested to be particularly important (Sætre *et al.* 2003). However, molecular evolution at this locus was not associated with sexually dimorphic colouration across the galliform order (Nadeau *et al.* 2007).

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Supplementary material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2007.01667.x

Figure S1 Alignment of the quail sequences generated in this study to the existing quail *TYRP1* sequence and the chicken *TYRP1* sequence.

Figure S2 Alignment of *TYRP1* amino acid sequences among taxa.

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