Evolution of an avian pigmentation gene correlates with a measure of sexual selection

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The extravagant plumage traits of male birds are a favourite example of sexual selection. However, to date the units that selection is acting upon, the genes themselves have been a 'black box'. Here, we report evidence of change driven by sexual selection at a pigmentation gene locus in the galliform birds. Across species, we find a correlation between the rate of amino acid change (dN/dS) at this locus (*MC1R*) and the degree of sexual dichromatism, which we use as a measure of the strength of sexual selection. There is no evidence for a similar pattern in any of five other loci (four candidate and one control locus). This is consistent with previous work on colour polymorphisms and suggests that *MC1R* may be a key target for selection acting on plumage colour. The pattern of selection at *MC1R* seems to be consistent with the continuous or cyclical evolution of traits and preferences that is the outcome of several Fisherian and good-genes models of sexual selection. In contrast, we found no support for models of sexual selection that predict an increase in purifying selection as a result of purging of deleterious mutations or for models that predict an increased rate of mutation in association with stronger sexual selection.

Keywords: plumage colour; sexual selection; secondary sexual trait; galliform; *MC1R*; melanin

1. INTRODUCTION

The extravagant plumage traits of many male birds are a classic example of sexual selection (Darwin 1871; Hamilton & Zuk 1982). Many empirical (e.g. Zuk *et al.* 1992; Owens & Hartley 1998) and theoretical (e.g. Kirkpatrick 1982; Mead & Arnold 2004) studies have greatly improved our understanding of how these apparently costly secondary sexual traits can arise and be maintained. However, the proximate mechanisms linking selection to the resulting male plumage traits have largely been treated as a 'black box'. An understanding of the genes involved would allow us to fill this gap and investigate the individual units on which selection is acting.

There are many hypotheses about sexual selection and how it acts to bring about the traits seen in nature. Many of these are hard to evaluate using conventional comparative data on the phenotypes of extant taxa (Bennett & Owens 2002). Although neither few-locus models nor quantitative genetic models make specific predictions about the accumulation of mutations at a single locus, the evolutionary scenarios produced by different quantitative genetic models can be investigated at the single-locus level. For example, certain (particularly 'good-genes') models predict evolution leading to a stable outcome or equilibrium, while others predict ongoing or cyclical evolution (Mead & Arnold 2004). This second scenario has been found under Fisherian, sexual conflict and some good-genes models and would lead to prolonged or continual change at a locus.

Most good-genes models of sexual selection, in which females select male traits because they indicate condition

or viability, rely on mutation–selection balance to maintain genetic variance in a trait under sexual selection (Pomiankowski & Møller 1995; Rowe & Houle 1996; Houle & Kondrashov 2002). This would predict higher degrees of purifying selection to be associated with higher levels of sexual selection. In what could perhaps be seen as an extension of this idea are models that predict an increased rate of mutation in taxa with higher levels of sexual selection (Møller & Cuervo 2003). This could be due to higher levels of sperm competition leading to more meiosis and so a higher rate of mutation (Bartosch-Harlid *et al.* 2003) or to specific mutator alleles (Petrie & Roberts 2006).

The galliform birds (pheasants, partridges and allies) show extraordinary diversity in ornamental male plumage colour, and there is evidence that these traits can be used in female choice (Petrie *et al.* 1991; Zuk *et al.* 1992) or male–male competition (Mateos & Carranza 1997). Plumage colour in this group of birds is largely due to melanins and fine feather structures, while carotenoids are generally considered not to be used. Further, melanin granules are an essential part of all known structural colours in galliform plumage (Prum 2006).

In this study, we investigate the effect of sexual selection on a set of genes involved in melanin synthesis in the Galliformes. *MC1R* (melanocortin-1-receptor) is involved in the regulation of melanogenesis. In birds, the relative activity level of this receptor acts as a switch from synthesis of red/yellow pheomelanin (chicken, Takeuchi *et al.* 1998) or no melanin synthesis (bananaquit, Theron *et al.* 2001), to synthesis of black/brown eumelanin. *TYR* (tyrosinase), *TYRP1* (tyrosinase-related protein-1) and *DCT* (DOPA-chrome tautomerase, also known as tyrosinase-related protein-2, *TYRP2*) are all in the tyrosinase gene family, coding for melanogenesis enzymes. *AGRP* (agouti-related protein) is an endogenous antagonist of the melanocortin

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system in the central nervous system, with possible involvement in the peripheral system but is treated primarily as a control for the purposes of this study. *TYRP1* is located on the Z chromosome in chickens; all other nuclear loci investigated were autosomal. Additionally, as a control, we included *CYTB*, a mitochondrial gene not involved in pigmentation.

2. MATERIAL AND METHODS

(a) *Laboratory methods*
Samples of soft tissue, blood or feather were obtained from 36 galliform species in 25 genera (for list of taxa sampled, see table 1 of electronic supplementary material). Species were selected based on the availability of samples while trying to sample species that spanned the galliform phylogeny and were as representative of the observed variation in sexual plumage dichromatism as possible. Genomic DNA was extracted from samples using standard methods. We sequenced 859 bp of the *MC1R* exon, 772 bp of *TYR* exon 1, 267 bp of *TYRP1* exon 1, 233 bp of *DCT* exon 2 and 432 bp of the *AGRP* coding sequence (comprising exon 2 and parts of exons 1 and 3). We also sequenced the entire *AGRP* introns 1 and 2. PCR primers are given in table 2 of electronic supplementary material. PCR products were directly sequenced on both strands. *CYTB* sequences were downloaded from GenBank or sequenced as described previously (Kimball *et al.* 1999).

PCRs were performed in a 50 μl total reaction containing 1 unit *Taq* polymerase (Thermoprime plus DNA polymerase), $1 \times$ reaction buffer, 1.5 mM $MgCl_2$, 0.1 mM each dNTP, 0.4 μM each primer and 50–200 ng DNA. PCRs were performed in a DNA Engine (MJ Research), with the following cycling parameters: 94°C for 2 min; $35 \times (94$°C for 30 s, 55–65°C for 45 s, 72°C for 1 min); and 72°C for 5 min. Cycle sequencing on both strands was carried out using Bɪɢ Dʏᴇ v. 3.1 (PE Biosystems) under standard conditions. Sequences were edited in Sᴇǫᴍᴀɴ v. 5.05 (DNASTAR Inc.). Sequences were aligned using Cʟᴜsᴛᴀʟ W and adjusted manually. Sequences have been deposited in GenBank (accession numbers EF569209 and EF57103– EF571223, see table 1 of electronic supplementary material).

(b) *Phylogenetic analysis*
The phylogeny we used was based on *AGRP* intron, *CYTB* and the coding sequences as described above. Two combined (partitioned) datasets, one of *AGRP* introns and *CYTB*, and one containing these plus the coding sequences for the five nuclear genes, were analysed using Mʀ Bᴀʏᴇs v. 3.0 (Huelsenbeck & Ronquist 2001) with 550 000 generations, discarding the first 50 000 as burn-in and sampling every 100 generations. Fifty per cent consensus trees for these datasets were similar (see figure 1 of electronic supplementary material), although with improved resolution for the larger dataset. These phylogenies were largely consistent with previously published phylogenies (Kimball *et al.* 1999; Kaiser *et al.* 2006), but with improved resolution among many of the Phasianidae.

The consensus tree from the larger dataset was used as the main phylogeny for the analysis. To check that this phylogeny did not affect the results, the *MC1R* dataset was also analysed using six additional phylogenies with noticeably different topologies arbitrarily sampled from within the set of trees generated by Mʀ Bᴀʏᴇs (figure 2 of electronic supplementary material), and also analysed using the consensus tree from the

analysis of *AGRP* introns and *CYTB* only, to check that using a phylogeny based on the pigmentation gene-coding sequences did not bias the results. The two outgroup taxa (curassow and megapode) were not included in the comparative analysis, as the long branches leading to them created uncertainty in dichromatism reconstruction.

Most genera in the study were found to be unambiguously monophyletic in all phylogenetic reconstructions. The only exception was the francolins (*Francolinus*), in which the two sampled species under some reconstructions were polyphyletic with respect to the jungle fowl (*Gallus*). The monophyly of the francolins has previously been questioned (Bloomer & Crowe 1998) and these two species assigned to separate genera (*Scleroptila* and *Francolinus*). Our phylogenies show some support for the latter classification and therefore it was adopted for the present analysis.

(c) *Character state scoring and reconstruction*
Sexual plumage dichromatism was scored from field guide information and illustrations (Madge & McGowan 2002) on a scale of 0–6. This was based on three body regions (head and neck; back, wings and tail; chest, belly and legs). Each region was scored from 0 to 2, where 0 was no difference in plumage colour between males and females; 1 was a difference of shade or feather patterning; and 2 was a difference in colour. Scoring was performed by two independent observers. Scores between observers were found to have high repeatability ($r = -0.89$, $p < 0.01$, intraclass correlation coefficient). The mean of the two scores was used. This scoring system was based on that of Owens & Bennett (1994), which has been widely used and found to correlate with other measures of the strength of sexual selection (Dunn *et al.* 2001). Ideally, mating system might have been used to measure the potential for sexual selection but this information is only sparsely available for the species in this study. Sexual size dimorphism was also measured and found to correlate with dichromatism under a correction for phylogenetic non-independence ($r^2 = 0.17$, $p = 0.02$), suggesting that these traits are both evolving in response to sexual selection. Measurement of UV reflectance was considered unnecessary in this case, given that galliforms are less sensitive to UV than other bird taxa and their 'violet' cone has a peak sensitivity similar to that of the 'blue' cone of humans (Bowmaker *et al.* 1997; Ödeen & Håstad 2003).

Dichromatism scores were reconstructed over the galliform phylogeny (figure 1) using Mᴀᴄ Cʟᴀᴅᴇ v. 4 (Maddison & Maddison 2000). Branches with equivocal states were resolved by equivocal cycling. Branches on which changes in dichromatism occurred were scored as three categories (increase, no change or decrease).

(d) *Main analysis*
dN/dS ratios were obtained by maximum likelihood using a codon-based substitution model in Pᴀᴍʟ v. 3.14 (Yang 1997). Branch-specific models were implemented in which dN/dS ratios were estimated separately for lineages grouped by the reconstructed levels of dichromatism (seven branch categories, one for each level of dichromatism, see figure 1). Heterogeneity in dN/dS among these lineages was tested using likelihood ratio tests comparing these models to null models with a single dN/dS ratio across the phylogeny. Linear regressions were performed with dN/dS as the dependent and dichromatism as the independent variables. If sexual selection were having a consistent effect on any of the pigmentation genes, we would
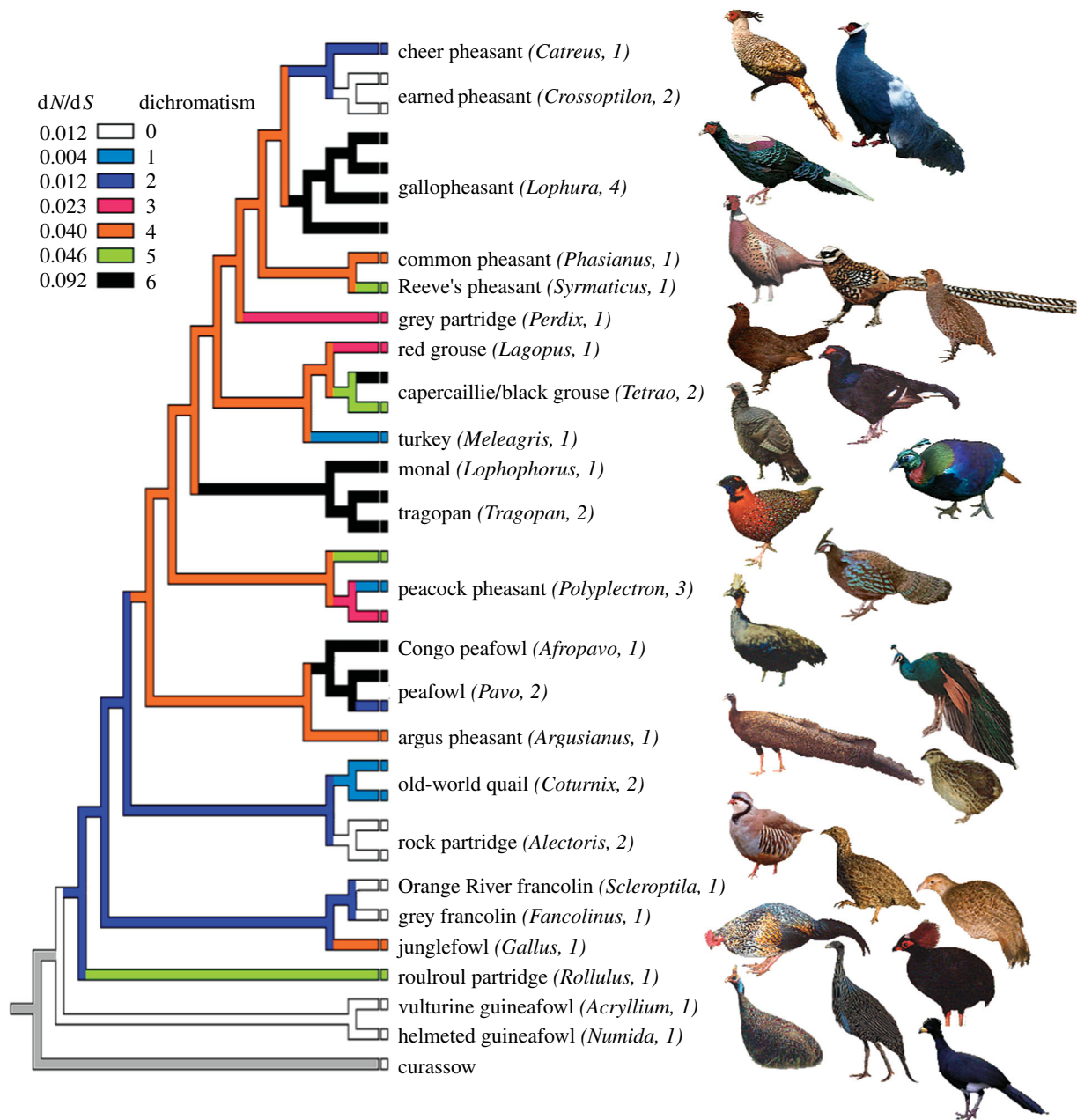
Figure 1. Reconstruction of plumage dichromatism evolution on the phylogeny of the galliform species used in this study. dN/dS ratios at *MC1R* are estimated for each dichromatism category, excluding grey branches genera are listed as: common name (scientific name, number of species sampled). An image of a male from each genus is shown to the right.

expect both of these tests to be significant. Within this hypothesis, if sexual selection were causing ongoing or cyclical evolution, we would expect a positive regression between dN/dS and dichromatism, but if it were increasing purifying selection, as under the good-genes models, we would expect a negative regression. The same analysis was performed with dN/dS ratios estimated for lineages grouped by the reconstructed change in dichromatism.

Several different site-specific models in PAML were also implemented (M1, M2, M3, M7 and M8, see Yang *et al.* (2000) for details), which allow individual codons to be under different selective pressures but assume that the selective pressure on each codon remains constant along all lineages.

(e) *Genus-level analysis*

As further, independent tests of the above hypotheses, the relationships between dN/dS and dichromatism were also analysed at the genus level. Free-ratio models were

implemented in PAML, which estimate a dN/dS ratio for every branch on the phylogeny. From this, mean dN/dS ratios (weighted by branch length) were calculated for each genus (including the branch leading to the common ancestor of all species within the genus and all branches leading from this level to the tips). Dichromatism scores averaged at the genus level and dN/dS values were analysed as traits in the software package CONTINUOUS (Pagel 1999). For all of the loci, dN/dS values based on three or fewer nucleotide changes were excluded from the analysis since these do not provide enough information to estimate dN/dS. Covariance between dN/dS and dichromatism was tested using a likelihood ratio test (LRT) to compare a model where covariance between traits was set to zero with one where covariance was allowed. Covariance between dS and dichromatism was also analysed using this approach to test the hypothesis that sexual selection is associated with higher rates of mutation.
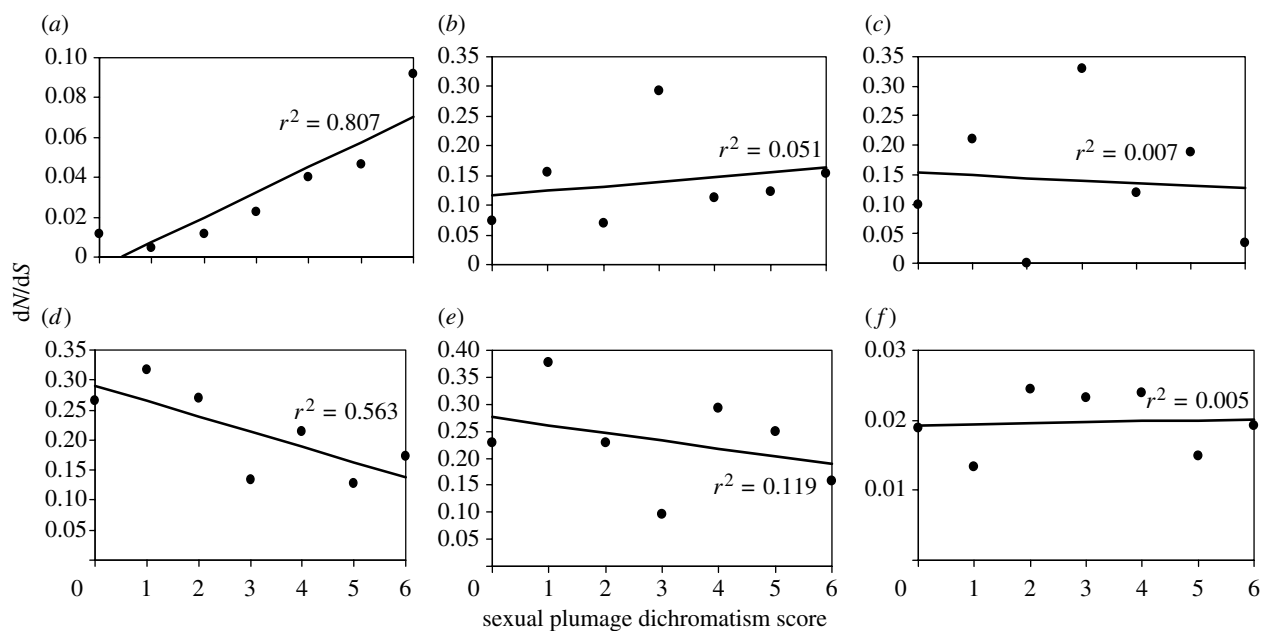
Figure 2. Relationships between sexual plumage dichromatism and dN/dS for different categories of dichromatism at each of the six loci. For clarity, y-axes are on different scales. (*a*) *MC1R*, likelihood ratio test for heterogeneity: LRS$=26.82$, d.f.$=6$, $p<0.001$; (*b*) *TYR*: LRS$=7.38$, d.f.$=6$, $p=0.287$; (*c*) *TYRP1*: LRS$=8.90$, d.f.$=6$, $p=0.179$; (*d*) *DCT*: LRS$=1.96$, d.f.$=6$, $p=0.923$; (*e*) *AGRP*: LRS$=3.74$, d.f.$=6$, $p=0.712$; and (*f*) *CYTB*: LRS$=7.68$, d.f.$=6$, $p=0.263$.

3. RESULTS AND DISCUSSION

There was significant heterogeneity in dN/dS ratios at the melanocortin-1 receptor locus (*MC1R*) among lineages with different levels of dichromatism (likelihood ratio statistic, LRS$=26.8$, d.f.$=6$, $p<0.001$). Furthermore, a strong and significant positive regression was found between dichromatism and dN/dS ratios at *MC1R* ($r^2=0.81$, $p=0.006$; figures 1 and 2). These results were significant under a Bonferroni correction for multiple tests (corrected α-level$=0.008$ for six tests). When this analysis was performed at the other loci, no significant heterogeneity or regressions between dichromatism and dN/dS were found (figure 2). At *DCT*, there was a tendency towards a negative relationship ($r^2=0.56$, $p=0.05$), but this was far from significant in a sequential Bonferroni test (corrected α-level$=0.01$) and the absence of heterogeneity in dN/dS ($p=0.923$) makes any trend highly unlikely to be meaningful.

To verify that a relationship between dN/dS at *MC1R* and dichromatism was robust to changes in the phylogeny and alternative reconstructions of dichromatism, we performed a separate analysis concentrating at the level of the genus. This used the program CONTINUOUS, a maximum-likelihood-based implementation of a generalized least squares (GLS) model of trait evolution (Pagel 1999). This corrects for any effect of phylogenetic relationships on the observed correlation, with 'independent contrasts' (Felsenstein 1985) being a special case of this approach. The basic result was similar: there was a significant covariance between dichromatism and dN/dS at *MC1R* (LRS$=8.5$, $p=0.004$; again significant at $p<0.05$ level after Bonferroni correction), but not for any other loci (figure 3 of electronic supplementary material). Significant covariance between *MC1R* dN/dS and dichromatism was also found with seven alternative phylogenies (LRS range$\geqq6.2$, $p\leq0.013$). The absence of this pattern at any of the other loci investigated suggests that the result

at *MC1R* cannot be due to non-selective confounding factors, such as time since divergence or population size, which can affect dN/dS values (Rocha *et al.* 2006).

The pattern of higher dN/dS ratios for *MC1R* in lineages with stronger dichromatism, together with dN/dS values that do not exceed one, could be due to a higher level of positive selection acting on the *MC1R* gene, an increased rate of change due to decreased constraint and/or neutral drift, or a combination of these mechanisms. In this case, we suggest that the most probable explanation for this pattern is positive selection acting on the *MC1R* in temporal bursts, and/or in restricted parts of the gene, masked by the background of strong constraint at *MC1R* (average $dN/dS=0.03$). Site-specific analyses of *MC1R* evolution in PAML did not reveal a category of sites under positive selection (not shown), but the power of this analysis is low since a large number of sites are known to affect *MC1R* function, and different sites may have been involved in different lineages (e.g. position 92, see discussion below). It is hard to envisage a scenario under which constraint would decrease systematically with degree of dichromatism, without invoking selection, although this possibility cannot be ruled out. Sexual dichromatism is widely accepted as a measure of pre-mating sexual selection (McLain *et al.* 1999; Prinzing *et al.* 2002) and several studies have found a correlation with other measures of sexual selection such as mating system (Figuerola & Green 2000; Dunn *et al.* 2001) or extra-pair paternity (Owens & Hartley 1998). Therefore, the robust relationship that we have detected between dichromatism and evolutionary change at the *MC1R* locus is extremely interesting as a possible signature of sexual selection, particularly given what we know about the extreme mating systems and extravagant ornamental traits in male galliforms (Petrie *et al.* 1991; Zuk *et al.* 1992; Mateos & Carranza 1997).

The *MC1R* locus is well known for its association with melanic polymorphisms in several bird and other vertebrate species that affect both sexes equally (reviewed in Hoekstra 2006), raising the issue of how protein-coding changes in this locus could affect sexually dimorphic coloration, which clearly involves differential gene regulation among the sexes. In fact, there are precedents for *MC1R* affecting coloration where regulation is involved: different *MC1R* alleles have differential effects on coloration among the sexes in domestic chickens (Kerje *et al.* 2003). In addition, *MC1R* variation is associated with male-specific coloration in fairy wrens (*Malurus leucopterus*; Doucet *et al.* 2004) and seasonal variation in coloration in arctic foxes (*Alopex lagopus*; Våge *et al.* 2005). A simple model to explain the results presented here is that the basic level of sexual dichromatism in galliforms is determined by variation at other loci, with *MC1R* then having a disproportionate effect on male-specific coloration. Corroborative evidence for important functional changes in *MC1R* during galliform evolution is found in two taxa (*Polyplectron* and *Crax*) with relatively dark coloration that have a glutamate to lysine change at amino acid position 92 in *MC1R*, which is known to be associated with increased melanin deposition in several birds and mice (Robbins *et al.* 1993; Takeuchi *et al.* 1996; Nadeau *et al.* 2006).

To determine whether dN/dS at *MC1R* was responding to a constant level of dichromatism or to changes in dichromatism, we also performed an analysis using branch groupings corresponding to the change in dichromatism (decrease, dN/dS = 0.02; no change, dN/dS = 0.04; increase, dN/dS = 0.03). These categories did not significantly explain the variation in dN/dS at *MC1R* (LRS = 3.44, d.f. = 2, $p = 0.18$). This suggests that the pattern of increased dN/dS at *MC1R* is not due to changes in the degree of dichromatism (which according to our scoring scheme involve changes in the overall degree of male–female colour differences but *not* differences in the colour itself) but instead corresponds to periods of a sustained high level of dichromatism. The most plausible explanation for this seems to be that change at *MC1R* is ongoing in lineages with high sexual dichromatism. This seems feasible because changes in plumage colour, particularly in males, do appear to occur frequently between closely related lineages that have similarly high levels of sexual dichromatism. The absence of a clear relationship between *MC1R* evolution and changes in the level of dichromatism may either be because such changes occur rapidly and therefore only produce a small molecular signal and/or because selection on *MC1R* plays a limited role in evolutionary change in *the degree* of dichromatism, which would be consistent with the model outlined above.

The detectable signature of selection implies that novel functional variants of *MC1R* have become repeatedly fixed in galliform evolution. This increased rate of substitution occurs once a high level of dichromatism has been attained, as it is not associated with increases in the level of dichromatism. This is consistent with continuous or cyclical evolution in ornaments and preferences (Pomiankowski & Iwasa 1998; Mead & Arnold 2004). Furthermore, point substitutions at *MC1R* are associated with large-scale variation in melanin distribution in several bird species (Theron *et al.* 2001; Mundy *et al.* 2004). Sexual selection for such mutations would promote their rapid fixation, with further mutations at *MC1R* producing minor or no phenotypic 'improvements' under the same selective regime. Thus, under constant sexual selection, we predict that relatively few substitutions would occur in *MC1R*, and these would be hard to detect using our methods. Our results therefore strongly suggest an alternative scenario in which sexual selection has not been constant within lineages. Instead, novel *MC1R* alleles of moderate to large phenotypic effect became fixed during one regime of sexual selection, with other alleles selected under subsequent regimes. This model is consistent with the general view that galliforms are prominent examples of rapid plumage change under sexual selection (Andersson 1994; Young *et al.* 1994).

The prediction of stronger purifying selection (i.e. lower dN/dS) on branches with higher dichromatism, which is expected under most good-genes models (Rowe & Houle 1996), is clearly refuted at *MC1R* and at four out of the five other loci studied (*TYR*, *TYRP1*, *AGRP*, *CYTB*). The slope of the regression between dichromatism and dN/dS at *DCT* is negative, but neither the regression nor the LRT for heterogeneity reach significance (LRS = 1.96 $p = 0.92$). Other models have predicted a genome-wide increase in the level of background mutation in lineages under strong sexual selection (Møller & Cuervo 2003). We found no evidence for this, as there was no covariance between dS and dichromatism in a combined analysis of all five nuclear loci controlled for phylogeny (LRS = 0.02, $p = 0.88$). It has also been widely suggested that sex-linked loci, particularly on the Z chromosome, should be more easily co-opted by sexual selection than autosomal loci (Sætre *et al.* 2003; Kirkpatrick & Hall 2004; Albert & Otto 2005). However, we did not find this pattern here: *MC1R* is autosomal and we found no evidence for sexual selection acting on *TYRP1*, the only sex-linked locus in the study.

Our study is the first to our knowledge to make a link between molecular evolution of specific loci and secondary sexual traits in vertebrates. In contrast, previous work has concentrated on rapidly evolving loci that have a direct role in reproduction, particularly in males (e.g. Dorus *et al.* 2004). A surprising feature of our results is that despite the enormous diversity in male–female colour differences in galliform birds generated over their approximately 50 Myr history of diversification (van Tuinen & Dyke 2004), a proportion of this variation has a common genetic basis. These results suggest that it is possible to detect a signal of sexual selection even against a background of strong constraint, and pave the way for a far deeper mechanistic understanding of sexual selection than has so far been achieved.

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