Sequence Variation in the Melanocortin-1 Receptor (MC1R) Does Not Explain Continent-wide Plumage Color Differences in the Australian Magpie (Cracticus tibicen)

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Abstract

The genetic basis of plumage color variation has already been determined for many model species; however, the genetic mechanisms responsible for intraspecific color variation in the majority of wild-bird species are yet to be uncovered. The Australian magpie (Cracticus tibicen) is a large black and white passerine which is widely distributed across the Australian continent. The proportion of melanized back plumage varies between regionally delineated subspecies; where back-color forms overlap, intermediate color phenotypes are produced. This study examined the majority (861 bp) of the coding region of the melanocortin-1 receptor (MC1R), a candidate gene for plumage color differentiation in 98 magpies from across the Australian continent, to determine if the gene is associated with magpie back-color variation and explore phylogeographic signal within the gene. Neutrality and selection tests (Tajima’s D, Fu’s Fs, MKT) indicate the gene is unlikely to be currently under selection pressure and, together with other lines of evidence, suggest a past demographic expansion event within the species congruent with the results of previous mitochondrial phylogeographic work on this species. None of the 15 synonymous and four nonsynonymous substitutions within MC1R were found to be associated with plumage variation. Our results suggest that genes or regulatory elements other than MC1R may determine back-color variation in C. tibicen.

Key words: Candidate gene, melanism, pigmentation, population expansion

Plumage color variation in bird species has long fascinated both scientists and amateurs alike. These often striking, but sometimes subtle differences in plumage color and pattern provide a natural experimental design ideal for investigating questions about morphological diversity and the forces that generate and shape this diversity.

Understanding the underlying genetic and regulatory mechanisms responsible for such variation is a first step towards addressing these important questions. However until recently, the construction of large pedigrees of domesticated and laboratory animals was the only way to investigate the genetics of plumage variation. As technology has advanced, the ways in which evolutionary questions about intraspecific variation have been approached have changed dramatically; genetic sequencing advances have now made it possible to screen well-characterized candidate genes relatively affordably and efficiently (Piertney and Webster 2010).

To date, investigations of color variation within animal species have focused primarily on structural coding regions and these have revealed a number of different candidate genes that are thought to be involved in color variation within different species. Studies of the genetic basis of pigmentation have been dominated by one gene in particular over the last 20 years: the melanocortin-1-receptor (MC1R), a gene that encodes a seven-transmembrane domain G-protein coupled receptor (Mountjoy et al. 1992). This protein is expressed in specialized pigmentation cells known as melanocytes, where it plays a role in the dispersal of melanostomes through cells...
and/or initiation of the melanin-production process (Jackson 1997). Activation of the receptor by melanocyte-stimulating hormone (MSH) has been shown to lead to an increase in the production of black and brown eumelanin in melanomas (Robbins et al. 1993). Mutations in the receptor that lead to activation of MC1R and increased synthesis of eumelanin are known as gain-of-function mutations, while loss-of-function mutations in MC1R often are associated with the production of red or yellow phaeomelanin (Robbins et al. 1993).

MC1R has been implicated in intraspecific pigmentation variation across a wide range of mammal and reptile species, including the horse (Marklund et al. 1996), fox (Vage et al. 1997), arctic fox (Vage et al. 2005), pig (Kijas et al. 1998), sheep (Vage et al. 1999), dog (Newton et al. 2000), black bear (Ritland et al. 2001), cow (Klungland et al. 1995), jaguar and jaguarundis (Eizirik et al. 2003), pocket mouse (Nachman et al. 2003), domestic rabbit (Fontanesi et al. 2006), human (Vulverde et al. 1995), and lesser earless lizard and little striped whiptail (Rosenblum et al. 2004). Birds are no exception to this widespread MC1R association with color phenotype, and specific mutations within the MC1R coding region have been found to be associated with plumage color in chickens (Takeuchi et al. 1996; Andersson 2003), Japanese quails (Nadeau et al. 2006), red-footed boobies (Biao et al. 2007), lesser snow geese (Mundy et al. 2004), arctic skuas (Mundy et al. 2004) chestnut-bellied monarch (Yu et al. 2009), and hanaquaits (Theron et al. 2001), although several species with plumage variation in the form of fine-scale patterning have been shown to have no association with variation in this gene, including old-world leaf warblers (MacDougall-Shackleton et al. 2003), blue-crowned manakins (Cheviron et al. 2006), rosy finches (Drovetski et al. 2008), and carrion/hooded crows (Haas et al. 2008).

A candidate-gene approach has been successfully used to identify associations between phenotypes and genotypes across a wide range of species and traits (see Hockstra and Coyne 2007). Although this approach is more often used to investigate simple Mendelian traits, studies of complex, multigene-based traits may also benefit by using this approach to investigate the possible underlying pathways that link genetic variants to complex traits (Tabor et al. 2002).

The Australian magpie Cracticus tibicen (formerly Gymnorhina tibicen) is a sedentary, group-living passerine belonging to the family Artamidae (Order Passeriformes). Eight subspecies of magpie are currently recognized based on morphological traits including size, bill length, wing-span, and plumage color, which varies considerably across its distribution (Schodde and Mason 1999); see Figure 1 for illustrations and distribution. Three of these subspecies (C. t. telonocua, C. t. tyrannica, and C. t. hypoleuca) are white-backed (WB) plumage forms and are restricted to southeastern Australia and Tasmania. These WB plumage forms have a white back which joins their white nape and extends down to their black rump (Schodde and Mason 1999). Black-backed (BB) plumage forms dominate northern parts of the continent and comprise four different subspecies: C. t. longirostris in the north-west, C. t. eylandtensis in a belt across the central north of the continent, C. t. terraereginae throughout most of Queensland and New South Wales, and C. t. hibiscus along the New South Wales coastline. Instead of a white saddle, these BB birds have a black saddle extending from their white nape down to their rumps. All of the seven subspecies of black- and white-backed forms exhibit a sexually dimorphic plumage pattern in which the white plumage areas on males are grey or partially grey on females, although heavy parasite loads can make males appear somewhat grey in the nape area (J. Hughes, personal communication).

A third plumage group exists in the form of the varied magpie, C. t. dorsalis, a subspecies found only in the extreme south-west of the continent. The males of this subspecies resemble male WB forms in terms of plumage. Females of this subspecies have a black back which begins at their shortened white nape and extends down to the rump, but these black feathers are all edged in white, giving a “scaled” or “mottled” appearance to their back (Schodde and Mason 1999). All plumage forms seem to interbreed where their distributions overlap (Burton and Martin 1976; Hughes 1982), producing a range of different intermediate forms. In eastern Australia, BB and WB plumage forms intergrade in a 200 km–wide belt across the south-east (Burton and Martin 1976). The area over which varied western magpies intergrade with western BBs is even larger and was estimated by Schodde and Mason (1999) to be up to 500 km wide. It is predominately the length of the colors in the saddle which mark intermediate forms; half way between a black-back and a white-back male produces a bird in which the top half of the back saddle is black while the bottom half of the saddle is white. The size of these “bands” of black and white on the saddle seem to be a continuous trait, and patterns of inheritance of this trait are currently being investigated in a long-term study site in the eastern hybrid zone.

Recent molecular phylogenies based on several nuclear genes indicate the species is more closely related to Currawongs (Strepera) and Butcherbirds (Cracticus) than any other bird species (Barker et al. 2004). Magpies are widely distributed across the Australian mainland (Schodde and Mason 1999). They inhabit a range of woodland environments and are uncommon in dense forest or extremely arid regions.

In approaching the investigation of the genetic basis of back-color variation in magpies, the nature of the phenotypic variation was considered. A number of studies have implied that back-color seems to be a heritable, nonplastic genetic trait within this species (Hughes 1982; Hughes et al. 2001). Observations at a long-term study site indicate that individuals seem to maintain the same back-color throughout their adult lifetime and do not exhibit seasonal or dietary variation in plumage color (unpublished data). It seems likely that only a small number of genes are involved in this back-color variation (Hughes and Mather 1980 in Hughes 1982), and this indicates that a candidate-gene approach should be well-suited to this study, especially as a number of candidate color genes have been successfully associated with plumage variation in a number of bird species in recent decades.
Two studies of magpie phylogeography have demonstrated that genetic variation in the mtDNA control region is not concordant with back color across the eastern (Hughes et al. 2001) and western (Toon et al. 2003) areas of their distribution, inclusive of intermediate zones. Hughes et al. (2001) have suggested a mechanism, similar to that first outlined in Kallioinen et al. (1995), in which natural selection for different back colors in different habitats counteracts effects of gene flow between BB and WB populations to explain the lack of partitioning of neutral gene markers between back colors. Hughes et al. (2001) suggested that assortative mating or a preference for a particular back color in a mate, together with differential success across different habitats could drive divergent selection on either side of back-color hybrid zones, such that natural selection may favor black backs, while sexual selection may favor white-backed forms.

Black-backed magpie populations tend to inhabit relatively open woodlands, while southern white-backed populations are generally found in more thickly vegetated areas. White backs are more conspicuous in open woodland, especially in the UV spectrum, and it has been suggested they are more vulnerable to predation in such environments than black-backed individuals (Hughes et al. 2001). A study by Hughes et al. (2002) showed that in territories with nests in forested areas, white-backed males produced more fledglings than black-backed males, while black-backed males produced more fledglings in territories where nests were located in more open woodland environments. The same paper found no evidence for assortative mating, however, and relied only on social parentage observations. A more recent study which genetically determined parentage also found little evidence for assortative mating and no significant preference for brighter white-backed males, as well as no discernible increase or decrease in extra-pair fledglings produced by females with high relatedness to their social males (Hughes et al. 2011). In this paper, Hughes et al. (2011) propose an alternative hypothesis to sexual selection: that the higher bacterial resistance of black feathers in the hotter and more humid northern parts of the continent may give these individuals an advantage in these regions, and white backs occur in the south simply as the weather renders this higher bacterial resistance unnecessary and melanin may be costly to produce (Hughes et al. 2011).

In direct contrast with the strongly northern and southern groupings of plumage forms, magpie populations from eastern Australia and western Australia have been found to be strongly divergent from one another, both in the south (Baker et al. 2000; Hughes et al. 2001; Toon et al. 2003) and the north (Toon 2007) based on mtDNA data. Molecular clock estimates place the divergence of eastern and western clades during the Pleistocene, approximately 36 000 years ago (Toon et al. 2007). These genetic clades geographically and temporally correspond with arid barriers which may have restricted dispersal between eastern and western populations.

Figure 1. Magpie plumage variants and their distribution across the Australian continent. Black-backed forms inhabit black-shaded areas, white-backed forms pale-grey areas, and intermediate forms are found in the medium grey–shaded areas. The varied plumage form inhabits the shaded south–west region of the continent. White areas of the continent are not necessarily limits of the distribution and may simply represent regions of very low magpie density. Letters indicate subspecies: (A) C.t. longirostris, (B) C.t. eylandtensis, (C) C.t. terraereginae, (D) C.t. thibsen, (E) Hybrid C.t. terraereginae and C.t. tyrannica, (F) C. t. tyrannica, (G) C.t. hypoleuca, (H) C.t. telonocua, (I) C.t. dorsalis, (J) Hybrid C.t. dorsalis and C.t. longirostris. Map adapted from Toon and Hughes (2008).
and/or restricted populations to refugia areas during these periods (Toon et al. 2007). Of these arid barriers, the northern Carpentarian and Canning seem to be implicated in population structuring in the north, while in the south of the continent, the Nullabor-Eyrean arid barrier is likely to have significantly restricted gene flow and dispersal between eastern and western populations of the magpie (Toon et al. 2007).

Tasmanian populations seem to have diverged only relatively recently from mainland populations, well after the western and eastern populations had begun to diverge (Hughes et al. 2001; Toon et al. 2007). It is estimated that the isolation of Tasmanian from mainland populations occurred approximately 16 000 years ago, and this timing neatly dovetails with known geological changes which may explain this isolation—it was approximately around this time when sea levels rose and filled Bass Strait, cutting off the land bridge which had temporarily linked Tasmania to the mainland during the last glacial cycle (Chappell and Shackleton 1986; Toon et al. 2007).

Mitochondrial DNA also indicates that eastern magpie populations may have undergone an expansion more recently than the east–west divergence, with eastern populations spreading further inland and north (Toon et al. 2007). While mtDNA data has consistently supported an east–west split between lineages, nuclear DNA reveals a slightly different and interesting story: microsatellite analysis has indicated secondary recontact between eastern and western populations in northern Australia (Toon et al. 2007). Male-biased dispersal, such as that observed in magpies by Veltman and Carrick (1990) should theoretically result in nuclear gene flow preceding mtDNA gene flow; this would account for nuclear markers detecting an event of secondary recontact before the signature is detected in mtDNA. The mtDNA divide of eastern and western populations was only weakly supported by this microsatellite data, indicating that further investigation of these groupings is desirable (Toon et al. 2007).

Investigating whether selection or historical forces are more important in shaping variation within the MC1R gene underlies the main thrust of this study. Genetic structure is generally considered as the distribution of genetic variance that results from a range of factors, including genetic drift, mutation, migration, and selection. Phylogeographic analyses examining the magpie’s distribution have, to date, used only putatively neutral genes including the mitochondrial control region and a number of microsatellite loci (Hughes et al. 2001; Toon et al. 2003, 2007). These types of markers are useful for inferring demographic processes and history which may have led to the current distribution of a particular species. However, utilizing a gene that is putatively under some form of selection pressure offers the added opportunity of exploring a marker that may be responsible for an adaptive response to differing environments in the form of plumage coloration (Hoffmann and Willi 2008). Different haplotype sets, allele frequencies, or some form of genetic structure might be expected in a given candidate-gene subject to selection pressures for different variants of a phenotypic trait, and the phylogeographic metrics from variation in such a gene can be compared to neutral markers to scrutinize selection processes further (Piertney and Webster 2010).

It is expected that patterns of major genetic groupings inferred from gene regions of disparate evolutionary histories (maternal, neutral, and functional) ought to have significantly different topologies. The MC1R gene is so intricately involved in pigmentation pathways that regardless of whether or not structural changes in the coding region of the gene directly determine plumage morphs in the magpie, phylogeographic analyses of this gene have the potential to unearth genetic structure that may expose patterns of pigmentation selection history in this species.

In this study, we examined sequence variation in the coding region of the MC1R gene to test the hypothesis that back-color variation is associated with MC1R variation in C. tibicen, in that an amino acid change(s) may lead to further melanization of certain patches on their back, as well as characterize the gene in magpies, and answer questions about phylogeographic structure in a nuclear gene potentially under selection pressure. It was hypothesized that MC1R variation would be associated with magpie back-color variation, and if so, this mutation or set of mutations may be similar to others found to be associated with pigmentation changes in other animal species in terms of their location within the MC1R gene.

Material and Methods

Sampling

Magpie DNA samples used in this project were collected by a number of different researchers over the last few decades. These samples are both whole blood and blood in a range of different buffers and are frozen at –80 °C. The samples have been collected from across most of the Australian continent, and include individuals of each subspecies, back-color, and sex from most of the Australian magpies’ current distribution. Both forward and reverse MC1R sequences from 100 magpie individuals were sequenced and included approximately even numbers of representatives of all back-color variants and subspecies, from 20 sites around Australia (Table 1).

MC1R Genotyping and Analysis

Whole genomic DNA was extracted from blood in buffer using a modified version of the simplified Rapid Method (RM) described in Lahiri and Schnabel (1993) using NP-40 as the detergent and a MgCl2 concentration of 4 mM. A reduced volume of 50 μL of blood in buffer yielded an adequate volume of DNA for subsequent analysis.

An 861 bp fragment of the MC1R gene that encompassed all sites known to be associated with color variation in birds was amplified using the primer MSHR72 (ATGCCAGTGAGGGCAACCA) (Mundy et al. 2004) and a reverse primer designed specifically for C. tibicen, Ana-R (TGTAGAGCACCAGCATGAGG) developed to overcome the problem of nonspecific amplification.

PCR reactions were carried out in 10 μL reaction volumes of 0.3 mM each of forward and reverse primers, 0.2 mM of 772
These reactants were subjected to a thermal cycling protocol in Sequencher 4.1 (Gene Codes Corporation 2000) and automated sequencing machine (Applied Biosystems 3130x1). PCR primers using BigDye 3.1 terminator chemistry on an Applied Biosystems 3130xl vacuum bell. Both strands were then directly sequenced with exon-sap (Fermentas).

Purified PCR products were used in a sequencing reaction of 10 μL volume which contained: 2.0 μL of 5× sequencing buffer (Applied Biosystems), 2.0 μL Big Dye Terminator Mix 3.1 (Applied Biosystems), 0.32 mM of the respective primer, and 0.1 μL of purified PCR product. These reactants were subjected to a thermal cycling program of consisting of a hold at 96 °C for 1 min, followed by 30 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, for each individual were randomly picked and amplified with M13F and M13R primers (Invitrogen), then run on 0.8% agarose gels. Colonies were cultured overnight in LB broth, 24 colonies of each individual were randomly picked and amplified with M13F and M13R primers (Invitrogen), then run on 0.8% agarose gels to detect successful MC1R inserts. Eight of these inserts were then sequenced in both directions for each individual. Multiple inserts were sequenced for each individual as PCR error is common when amplifying from a single clone, and both the misincorporation of nucleotides and PCR recombination can lead to inaccuracies in resultant sequences (Paabo and Wilson 1988). Comparison of sequences of multiple inserts and directly sequenced genomic DNA, as in Harrigan et al. (2008), enabled distinction of misincorporated nucleotides and sites of PCR recombination.

Magpie sequences were aligned to bird MC1R sequences from 11 additional species obtained on Genbank (details and GenBank accession numbers in Table 2) and SNPs found to be associated with melanism in other bird species were scrutinized in magpies. This alignment also enabled sequences to be translated into amino acid sequences and checked for amino acid substitutions that might explain the similarity shown to other species. Misincorporated nucleotides and stop codons that often flag the misincorporated nucleotides and stop codons that often flag the sequence products were also manually inspected and discarded.

### Table 1. Number of sampled haplotypes according to subspecies, site location, and back color.

<table>
<thead>
<tr>
<th>Site name</th>
<th>Sub-species</th>
<th>Back color morph</th>
<th>Number haplotypes sampled</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charters Towers</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>10</td>
<td>20°04'S 146°15'E</td>
</tr>
<tr>
<td>Hydeaway Bay</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>10</td>
<td>20°05'S 148°29'E</td>
</tr>
<tr>
<td>Dubbo</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>8</td>
<td>32°14'S 148°36'E</td>
</tr>
<tr>
<td>Brisbane</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>8</td>
<td>27°28'S 153°01'E</td>
</tr>
<tr>
<td>Grafton</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>8</td>
<td>29°40'S 152°56'E</td>
</tr>
<tr>
<td>Ouyen</td>
<td><em>C. tybien terraereginae</em></td>
<td>White back/ Hybrid</td>
<td>16</td>
<td>35°04'S 142°21'E</td>
</tr>
<tr>
<td>Seymour</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back/ White back/ Hybrid</td>
<td>6</td>
<td>37°01'S 145°09'E</td>
</tr>
<tr>
<td>Horsham</td>
<td><em>C. tybien terraereginae</em></td>
<td>Hybrid</td>
<td>4</td>
<td>36°42'S 142°13'E</td>
</tr>
<tr>
<td>Rowsley</td>
<td><em>C. tybien terraereginae</em></td>
<td>White back</td>
<td>14</td>
<td>37°43'S 144°24'E</td>
</tr>
<tr>
<td>Phillip Island</td>
<td><em>C. tybien terraereginae</em></td>
<td>White back</td>
<td>10</td>
<td>38°27'S 145°15'E</td>
</tr>
<tr>
<td>Tasmania</td>
<td><em>C. tybien terraereginae</em></td>
<td>White back</td>
<td>8</td>
<td>41°26'S 147°06'E</td>
</tr>
<tr>
<td>Nullabor</td>
<td><em>C. tybien terraereginae</em></td>
<td>White back</td>
<td>12</td>
<td>32°07'S 133°40'E</td>
</tr>
<tr>
<td>Esperance</td>
<td><em>C. tybien terraereginae</em></td>
<td>Varied form</td>
<td>16</td>
<td>33°51'S 121°53'E</td>
</tr>
<tr>
<td>Albury</td>
<td><em>C. tybien terraereginae</em></td>
<td>Varied form</td>
<td>10</td>
<td>35°00'S 117°53'E</td>
</tr>
<tr>
<td>Busselton</td>
<td><em>C. tybien terraereginae</em></td>
<td>Varied form</td>
<td>10</td>
<td>33°39'S 115°21'E</td>
</tr>
<tr>
<td>Mandurah</td>
<td><em>C. tybien terraereginae</em></td>
<td>Varied form</td>
<td>8</td>
<td>32°31'S 115°44'E</td>
</tr>
<tr>
<td>Pilbara</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back/ Varied</td>
<td>16</td>
<td>27°05'S 116°09'E &amp; 26°53'S 115°57'E</td>
</tr>
<tr>
<td></td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>6</td>
<td>27°26'S 117°39'E &amp; 22°41'S 117°74'E &amp; 24°18'S 116°54'E</td>
</tr>
<tr>
<td>Nth. WA</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>6</td>
<td>16°49'S 124°55'E</td>
</tr>
<tr>
<td>Kimberley</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>6</td>
<td>14°55'S 133°04'E &amp; 19°42'S 135°49'E</td>
</tr>
<tr>
<td>Northern Territory</td>
<td><em>C. tybien terraereginae</em></td>
<td>Black back</td>
<td>10</td>
<td>13°48'S 124°55'E</td>
</tr>
</tbody>
</table>

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**dNTPs (Bioline), 2 mM MgCl₂ (Fisher Biotech), 2 μL 10× reaction buffer (Fisher), 3.76 μL dH₂O₂, 3.0 μL of extracted template DNA and 0.2U Thermus aquaticus DNA Taq polymerase (Fisher).** Cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 42 cycles of 30 s at 94 °C, 45 s at 67 °C, and 45 s at 72 °C, followed by a final extension step of 30 min at 72 °C before samples were held at 4 °C. The amplified product was then purified with exon-sap (Fermentas).

Comparison of sequences of multiple inserts and directly sequenced genomic DNA, as in Harrigan et al. (2008), enabled distinction of misincorporated nucleotides and sites of PCR recombination.

Magpie sequences were aligned to bird MC1R sequences from 11 additional species obtained on Genbank (details and GenBank accession numbers in Table 2) and SNPs found to be associated with melanism in other bird species were scrutinized in magpies. This alignment also enabled sequences to be translated into amino acid sequences and checked for amino acid substitutions that might explain the similarity shown to other species. Misincorporated nucleotides and stop codons that often flag the sequence products were also manually inspected and discarded.
unintentional amplification of pseudogenes, using DNASTAR version 4.50 (Rozas et al. 2003).

Putative transmembrane helices were calculated in TMHMM 2.0 and drawn to map the magpie MC1R gene and the relative position of amino acid changes (Figure 2), and a haplotype network was constructed in TCS (Clement et al. 2000). A number of separate AMOVAs were carried out to test which factors best explained the genetic variation observed: back-color, populations, or the historical east–west divide suggested by mitochondrial data of previous studies (Baker et al. 2000; Toon et al. 2003, 2007). Fu’s Fs and Tajima’s D were calculated to examine the possibility of selection at the MC1R locus. A McDonald–Kreitman test for selection was also carried out utilizing MC1R sequences of another passerine bird, the carrion crow (Corvus corone corone) for comparison (GenBank Accession no. EU348721-630).

### Results

#### Analysis of Genetic Variation at MC1R

A total of 861 base pairs of the MC1R gene were sequenced for 100 magpies, from which 83 individuals were initially able to be confidently assigned haplotypes using the statistical Bayesian method implemented in Phase 2.1.1. The haplotypes of a further 11 individuals were determined experimentally by cloning and the addition of these known haplotypes into the Phase analysis improved the confidence in the estimation of haplotypes of the remaining 6 individuals to the extent that an additional 4 of these were then at sufficient confidence levels (>75%) to be included, yielding a total of 98 individuals utilized in downstream analyses. No unexpected indels or stop codons were encountered; it is therefore likely MC1R sequences were not confounded by the amplification of pseudogenes. Within these 196 haplotype sequences, 19 sites were variable, of these, 15 were synonymous substitutions and 4 were nonsynonymous substitutions.

#### Phylogeography

The nature of selection at MC1R was examined using several different tests of neutrality and selection. Tajima’s D value was significantly negative at −1.78 (P = 0.016). A negative Tajima’s D can be indicative of purifying selection or population-size expansions, both of which characteristically produce an excess of low-frequency polymorphisms. The Fs value for magpie MC1R was also negative (−24.22) and highly significant (P < 0.0001). Fu’s Fs has been shown to have a great deal of power to detect recent demographic expansions (Ramos-Ordóñez and Rozas 2002).

A McDonald–Kreitman test comparing MC1R sequences of 10 haplotypes of carrion crow (Corvus corone corone) with magpie haplotypes was not statistically significant (P = 0.18), and the Neutrality Index score of 0.45 sat firmly in the range expected under neutrality, providing no evidence of selection on MC1R in magpies. This test uses the ratio of synonymous to nonsynonymous mutations within a species of interest and between that species and another closely related species at the same loci to evaluate the loci’s selective neutrality (McDonald and Kreitman 1991; Ee et al. 2008 McDonald and Kreitman 1991).

The 861 bp fragment of MC1R screened across 196 magpie alleles identified 25 unique haplotypes (Figure 3).
The haplotype network revealed a star-shaped phylogeny, featuring a dominant central haplotype. This central and common haplotype (57% of individuals) occurred in 19 of the 20 sampled sites from all around the Australian continent, and was also the most abundant haplotype at all but three of these sites. This dominant haplotype was common to every subspecies and back-color sampled.

The haplotype network (Figure 3) of MCIR illustrates no readily apparent geographic structure: no particular population, region, or geographically bound subspecies was restricted to a specific group of haplotypes. Global AMOVAs testing for structure between both populations and eastern and western groups found a statistically significant level of genetic structure among geographically delineated groups. \( F_{ST} \) values of these global AMOVAs indicated differences between populations and east/west accounted for 8.11% and 4.81% of genetic variation in MCIR, respectively (Table 3). These results indicate that the MCIR is weakly geographically structured in magpies, and this geographical structure accounts for slightly more of the genetic variance in this gene than plumage differences or subspecies designations (2.67% and 4.05%, respectively), although these were also found to have statistically significant levels of genetic structure at the 1% level.

**MCIR as a Candidate Gene for Magpie Plumage Differences**

The 861 base pairs sequenced represent the majority of the MCIR gene, which varies slightly in length between species but is generally close to 954 bp in vertebrates (Wlasiuk and Nachman 2007). The magpie MCIR gene is highly similar to MCIR regions characterized in other birds, with 7 trans-membrane domains. The 861 base pairs correspond to amino acids 21–306 of the *Gallus gallus* MCIR gene, and all amino acids discussed in this study are numbered after this model species for comparability and convenience.
Amino acid substitutions within **MC1R** that are known to be associated with plumage variation in other bird species, including the well-known Glu92Lys change linked to plumage changes in chickens, quails, and bananaquits were scrutinized in magpie sequences (Table 2). Across all of these “candidate amino acids,” all magpies assayed were invariable. The amino acids present in magpies at these positions in the gene were a mix of those associated with both melanic types and nonmelanic types in other bird species. **MC1R** haplotype(s) did not show a perfect or strong association with back-color phenotype. Instead, all back-colors were dominated by the two most common haplotypes in similar frequencies.

The results of a number of separate AMOVAs suggest that more **MC1R** variation was explained by variation within individual populations than any other geographical, taxonomic, or phenotypic grouping (Table 3). In pairwise estimates among back colors, only 2.67% of variance was explained by differences between different plumage types, while differences between populations, subspecies, and eastern and western mtDNA clades accounted for 8.11%, 4.05%, and 4.81%, respectively.

The 4 nonsynonymous substitutions identified (Gly30Ser, Asn40Ser, Gly192Ser, Ala227Thr) were mapped onto the haplotype network (Figure 3), as they have the potential to be of functional significance, regardless of their lack of association with back color. These changes do not seem to have particular associations with subspecies or geographic location (excepting the singletons).

**Discussion**

**MC1R** and Plumage Variation

Although variation in the **MC1R** gene has been linked with plumage variability across many other bird species (Takeuchi et al. 1996; Theron et al. 2001; Andersson 2003; Doucet et al. 2004; Mundy et al. 2004; Nadeau et al. 2006; Baião et al. 2007; Uy et al. 2009), back-color plumage in Australian magpies does not seem likely to be determined by variation in the coding region of this gene. No single allele or set of alleles was found to be exclusive to any plumage type; likewise no geographically defined group or subspecies could be delineated based on **MC1R** variation.
A Glu92Lys mutation has been implicated in pigmentation changes in bananaquits (Theron et al. 2001), Japanese quail (Nadeau et al. 2006), chickens (Takeuchi et al. 1996; Kerje et al. 2003) and has been shown to lead to constitutive activation of MC1R during in vitro experiments of both chickens and mice (Robbins et al. 1993; Ling et al. 2003). At this position, none of the sampled magpies had this mutation, but rather both alleles in all individuals coded for Glutamic acid, the amino acid of less melanic forms of Japanese quail and bananaquits. Other mutations in MC1R posited to lead to plumage changes in other bird species were all nonvariable across all magpie individuals and the amino acid present in magpies at each of these sites was not consistently either the supposed melanic or nonmelanic form (Table 2).

The sequenced fragment encompassed all but one of the sites that have been associated with color variation in birds and includes all transmembrane domains (Figure 2). As the entire length of the MC1R gene has not been sequenced, it remains possible that structural variation within these unsequenced portions of MC1R may be associated with the back color of magpies. However, this is less likely, given most SNPs associated with color variation in birds have been mapped to transmembrane and cytoplasmic regions of MC1R (Cheviron et al. 2006).

This finding is interesting in the context of the numerous other studies of MC1R and color variation in bird species. A large number of bird species have been found to have associations between MC1R variation and color phenotype (Andersson 2003; Takeuchi et al. 1996; Theron et al. 2001; Andersson 2003; Mundy et al. 2004; Nadeau et al. 2006; Baiao et al. 2007; Uy et al. 2009). However, as more wild species of birds with variable plumage colors are now being screened for segregating mutations in the MC1R gene, a number of species have been identified in which no association between phenotype and MC1R genotype has been detected. These include old world leaf warblers (MacDougall-Shackleton et al. 2003), blue-crowned manakins (Cheviron et al. 2006), rosy finches (Drovetski et al. 2008), and carnation/hooded crows (Haas et al. 2008). Plumage variants within the white-winged fairy wren were initially thought to be associated with MC1R variants (Doucet et al. 2004); however, a more recent and much broader study conclusively demonstrated the variation in question in the gene was not correlated with color variation in this and several other fairy-wren species (Driskell et al. 2010). All of these studies suggest that further investigation of other structural candidate color genes is needed, and some also propose that regulatory processes affecting the expression of MC1R and other color genes are deserving of further research, especially when plumage colors are variable seasonally or across life-history stages of a species (MacDougall-Shackleton et al. 2003; Cheviron et al. 2006; Drovetski et al. 2008).

Haas et al. (2008) sequenced MC1R in the hooded crow, carrion crow, and a range of hybrids that occur along a long corridor where their distributions overlap in Europe, and found no association between MC1R variation and plumage variation. The plumage of the carrion crow is entirely black, while the hooded crow only has a black head, neck, wings, thighs, and tail feathers, the remainder of their feathers are a slate grey, resulting in a sharp contrast between areas of grey and black. Plumage of hybrids between the two species varies along a continuum between the two extremes (Haas et al. 2008). Both Haas et al. (2008) and MacDougall-Shackleton et al. (2003) discuss the nature of the color variation as a likely important factor; they suggest that MC1R is less likely to be the genetic basis for fine-scale patterning changes and/or discrete plumage patterning, as is the case in magpies. Rather, variation in MC1R seems to be associated with whole-body color changes or gradational patterning of color. Australian magpies bear a striking resemblance to hooded crows in terms of the discrete nature of the color variation on their bodies and the lack of association between MC1R variation and back-color in magpies found in this study may add further weight to this hypothesis.

A number of other structural and regulatory candidate-color genes, such as TYRP1, ENDRB2, PMEL17, ASIP, and SLC45A2 have also been linked to plumage color variation in other bird species (Kerje et al. 2004; Gunnarsson et al. 2007; Miwa et al. 2007; Nadeau et al. 2007; Hiragaki et al. 2008), and it is suggested future studies of magpie pigmentation will benefit from further investigation of such genes.

**Phylogeography and Selection at MC1R**

Phylogeographic structure ought to be substantially weaker in nuclear genes than mitochondrial markers, a consequence of the lower effective population size (Birky et al. 1989; Moore 1995), thus it is not remarkable that MC1R shows weak structuring, despite the fact that high levels of structure have been found in the mitochondrial control region of this species (Toon et al. 2007). In the same study, nuclear microsatellite markers revealed only weak structuring, a level of magnitude below that observed in the mitochondrial control region, although there was enough power to detect the divergence between eastern and western haplotypes first delineated by mtDNA (Toon et al. 2007). Structural coding regions such as MC1R might be expected to have inherently

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<td><strong>AMOVA tests</strong></td>
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less variability and evolve more slowly than microsatellites, as they are constrained by selective pressures; mutations in such regions can be lethal if gene function is disrupted, for example, lethal yellow mutation in mice (Michaud et al. 1993), whereas the majority of mutations in microsatellite regions should have no functional consequences and be selectively neutral (Schlotterer 2000).

Star-like haplotype networks are generally recognized as predictive of either an expansion event following a bottleneck or a selective sweep in the recent evolutionary history of the species (Slatkin and Hudson 1991). Thus, the star-like MC1R network observed (Figure 3) may indicate one or both of these processes have influenced the trajectory of Australian magpie populations.

The significant negative Tajima’s D value, indicating an excess of low frequency polymorphisms compared to neutral expectations, is also suggestive of either a demographic population expansion or past selective sweeps (Tajima 1989), or alternatively may indicate that purifying selection has acted on the MC1R gene. This negative Tajima’s D value also indicates the gene is unlikely to have undergone a recent bottleneck event for which a significantly positive value of this test would be expected. Fu’s F_S value was also highly significant, a result strongly suggestive of recent population expansion or genetic hitchhiking (Fu 1997).

The weight of evidence indicates magpies experienced a population expansion at some point in the species’ recent past. The significant Tajima’s D value, highly significant Fu’s F_S value and star-shaped haplotype network all indicate either a population expansion or selective process has occurred in the recent evolutionary history of magpies. Mitochondrial DNA markers also show some evidence of this past expansion (Toon et al. 2007). However, as the results of a McDonald–Kreitman test could not reject neutrality of MC1R, it seems this gene is unlikely to be currently under selection. In addition, mismatch distributions (not presented here) at MC1R more closely resemble the distribution simulated for a population influenced by growth or decline than one of constant population size.

The balance of evidence indicates that magpies within Australia seem to have undergone a change in population size which is more likely to have been an expansion than a contraction. A number of authors have suggested that European settlement may have led to an increase and/or expansion of magpies as the creation of pastoral and urban lands transformed large tracts of Australia into high-quality magpie habitat (Campbell 1929; Schodde and Mason 1999). Toon (2007) found evidence to support this contemporary gene flow in north–western Australia with microsatellites, which are rapidly evolving nuclear markers (Ellegren 2003). However, the patterns observed in MC1R gene sequences will pre-date this anthropogenic influence significantly. Climatic fluctuations during the Pleistocene have been linked to geographically delineated genetic structure between magpie populations, for example, the eastern and western divergence dated at c. 36 000 years ago (Toon et al. 2007), putatively through periodic restrictions to dispersal and gene flow between groups posed by arid barriers such as the Nullarbor-Eyrean in South Australia.

It is suggested that the signature of population expansion found in both this study of a nuclear gene and (although less significant) in a mitochondrial marker Toon et al. (2007) seems likely to be related to the increase in available habitat suitable for magpies following the Last Glacial Maximum in the late Pleistocene, and the subsequent expansion of magpies out of refugia habitats.

Our findings indicate that MC1R is not likely to be under selection in the Australian magpie and implicate an expansion event in the recent evolutionary history of the species. Sequence variation in the majority of coding region of the MC1R gene screened was not found to be associated with back-color variation and it is anticipated that future studies which screen other likely candidate-color genes and regulatory elements will help further our understanding of plumage color evolution in this species.

**Funding**

This work was supported by Griffith University. A.D. was supported by a Griffith School of Environment Postgraduate Research Scholarship.

**Acknowledgments**

Sampling was carried out by Jane Hughes, Peter Mather, Alicia Toon, Andrew Baker, Kate Durrant, and a large number of volunteers. We would like to thank Steve Smith for his contribution to laboratory work, and two anonymous reviewers for their helpful comments on this manuscript. Samples were collected under Western Australian Department of Conservation and Land Management permit SP000903; Queensland Parks and Wildlife permit W4/002670/01/SA, Northern Territory Parks and Wildlife Commission permit 18145, Victorian Wildlife permit 10003058, and animal ethics protocol AES/16/04/AEC.

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Received June 30, 2011; Revised June 7, 2012; Accepted June 8, 2012

Corresponding Editor: Robert C. Fleischer