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Published
2012

Journal Title
Journal of Heredity

DOI
https://doi.org/10.1093/jhered/ess053

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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 sometime 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 (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 melanostains (Robbins et al. 1993). Mutations in the receptor that lead to activation of \textit{MC1R} and increased synthesis of eumelanin are known as gain-of-function mutations, while loss-of-function mutations in \textit{MC1R} often are associated with the production of red or yellow pheomelanin (Robbins et al. 1993).

\textit{MC1R} has been implicated in intraspecific pigmentation variation across a wide range of mammal and reptile species, including the horse (Merkulov et al. 1996), fox (Vage et al. 1997), arctic fox (Vage et al. 2005), pig (Kjaers 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 (Nuchman et al. 2003), domestic rabbit (Fontanesi et al. 2006), human (Valverde et al. 1995), lesser earless lizard and little striped whiptail (Rosenblum et al. 2004). Birds are no exception to this widespread \textit{MC1R} association with color phenotype, and specific mutations within the \textit{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 (Baiao et al. 2007), lesser snow geese (Mundy et al. 2004), arctic skuas (Mundy et al. 2004) chestnut-bellied monarch (Uy et al. 2009), and banaquats (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 Hoeckstra 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 \textit{Cracticus tibicen} (formerly \textit{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, wingspan, and also plumage color, which varies considerably across its distribution (Schoodde and Mason, 1999); see Figure 1 for illustrations and distribution. Three of these subspecies (\textit{C. t. telonocuca}, \textit{C. t. tyrannical}, and \textit{C. t. hypoenea}) 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 (Schoodde and Mason, 1999). Black-backed (BB) plumage forms dominate northern parts of the continent and comprise four different subspecies: \textit{C. t. longirostris} in the north-west, \textit{C. t. eylandtensis} in a belt across the central north of the continent, \textit{C. t. terraereginae} throughout most of Queensland and New South Wales, and \textit{C. t. tihiien} 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, \textit{C. d. doralis}, 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 short-ened white nape and extends down to the rump, but these black feathers are all edged in white, giving a “scalloped” or “mottled” appearance to their back (Schoodde 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 Schoodde 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 (\textit{Strepera}) and Butcherbirds (\textit{Cracticus}) than any other bird species (Barker et al. 2004). Magpies are widely distributed across the Australian mainland (Schoodde 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 \textit{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 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 \textit{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.
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 (Pierrney 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 inextricably 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 has 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 (ATGCCAGTTGAGGGCAACCA) (Mundy et al. 2004) and a reverse primer designed specifically for C. tibian, Ana-R (TGTAGAAGCCACCGATGAG) 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
These reactants were subjected to a thermal cycling protocol in Sequencher 4.1 (Gene Codes Corporation 2000) and PCR primers using BigDye 3.1 terminator chemistry on an Applied Biosystems equipment. Both strands were then directly sequenced with 70% ethanol before the pellet was dried out completely in a vacuum bell. Before being held at 4 °C until clean-up of the sequencing reaction was carried out.

30 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, 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 exo-sap (Fisher) and M13F and M13R primers (Invitrogen), then run on 0.8% agarose gels for unexpected indels and stop codons that often flag the sequence.

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), 4.68 μL ddH2O, 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 exo-sap (Fermentas).

Forward and reverse sequences were aligned and manually edited in Sequencher 4.1 (Gene Codes Corporation 2000) and sequences deposited in Genbank (Accession numbers JN172943-JN172967). Heterozygous sites were considered sufficient evidence to deem an individual heterozygous at that site. Phase thresholds >60% have been shown to be relatively robust; lowering thresholds from a highly stringent 90% to 60% has been found to reduce the number of unresolved haplotype pairs with very little increase in false positives in a number of studies (Harrigan et al. 2008; Garrick et al. 2010). Gel-purified PCR product was cloned using the TOPO TA cloning kit (Invitrogen). After 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 10 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 unexpected indels and stop codons that often flag the sequence.

<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>C. tibicen terraereginae</td>
<td>Black back</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>Hydeaway Bay</td>
<td>C. tibicen terraereginae</td>
<td>Black back</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>Dubbo</td>
<td>C. tibicen terraereginae/ tibicen</td>
<td>Black back</td>
<td>8</td>
<td>5.35</td>
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<tr>
<td>Brisbane</td>
<td>C. tibicen tibicen</td>
<td>Black back</td>
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<td>Graffon</td>
<td>C. tibicen tibicen</td>
<td>Black back</td>
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</tr>
<tr>
<td>Ouyen</td>
<td>C. tibicen tyrannica/terraeregina</td>
<td>White back/ Hybrid</td>
<td>16</td>
<td>5.50</td>
</tr>
<tr>
<td>Seymour</td>
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<td>Black back/ White back/ Hybrid</td>
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<td>5.5</td>
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<tr>
<td>Horsham</td>
<td>C. tibicen tyrannica/terraeregina</td>
<td>Hybrid</td>
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The number of sampled haplotypes according to subspecies, site location, and back color was as follows: Charters Towers 10, Hydeaway Bay 10, Dubbo 8, Brisbane 8, Graffon 8, Ouyen 16, Seymour 6, Horsham 4, Rowsley 14, Phillip Island 10, Tasmania 8, Nullabor 12, Esperance 16, Albury 10, Busselton 10, Mandurah 8, Pilbara 16, Nth. WA 6, Kimberley 6, Northern Territory 10. The table also includes coordinates for each site.

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<td>C. tibicen eylandensis</td>
<td>Black back</td>
<td>6</td>
<td>5.50</td>
</tr>
<tr>
<td>Northern Territory</td>
<td>C. tibicen eylandensis</td>
<td>Black back</td>
<td>10</td>
<td>5.50</td>
</tr>
</tbody>
</table>

The number of sampled haplotypes according to subspecies, site location, and back color was as follows: Charters Towers 10, Hydeaway Bay 10, Dubbo 8, Brisbane 8, Graffon 8, Ouyen 16, Seymour 6, Horsham 4, Rowsley 14, Phillip Island 10, Tasmania 8, Nullabor 12, Esperance 16, Albury 10, Busselton 10, Mandurah 8, Pilbara 16, Nth. WA 6, Kimberley 6, Northern Territory 10. The table also includes coordinates for each site.
unintentional amplification of pseudogenes, using DNASP 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) for comparison (GenBank Accession no. EU348721-630).

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-Onsins 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; Egea 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 MC1R 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. Fst values of these global AMOVAs indicated differences between populations and east/west accounted for 8.11–4.81% of genetic variation in MC1R, respectively (Table 3). These results indicate that the MC1R 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.

**MC1R as a Candidate Gene for Magpie Plumage Differences**

The 861 base pairs sequenced represent the majority of the MC1R gene, which varies slightly in length between species but is generally close to 954bp in vertebrates (Wlasiuk and Nachman, 2007). The magpie MC1R gene is highly similar to MC1R regions characterized in other birds, with 7 trans-membrane domains. The 861 base pairs correspond to amino acids 21–306 of the Gallus gallus MC1R 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.

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. 2006; Nadeau et al. 2006; Baiao 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.
Table 3  Analysis of molecular variance (AMOVA) results and tests of neutrality and selection

<table>
<thead>
<tr>
<th>AMOVA tests</th>
<th>Pairwise Fst</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>by population</td>
<td>0.081</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>by back color (WB vs. BB vs. Varied)</td>
<td>0.026</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>by East–West mtDNA division</td>
<td>0.048</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>by subspecies</td>
<td>0.040</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>by East–West mtDNA division (Sth only)</td>
<td>0.051</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>by population without Tasmania</td>
<td>0.060</td>
<td>P = 0.000</td>
</tr>
</tbody>
</table>

Neutrality and selection tests

Fu’s Fs | Fs = -24.222 | P = 0.000 |
Tajima’s D | D = -1.775 | P = 0.016 |
McDonald-Kreitman Neutrality | P = 0.18 |
(Fisher’s exact 2-tailed) Index = 0.45

A Glu92Lys mutation has been implicated in pigmenta-
cion changes in bananaquits (Theron et al. 2001), Japanese
quail (Nadeau et al. 2006), chickens (Takeuchi et al. 1996;
Kjer et al. 2003) and has been shown to lead to constitutive
activation of MC1R during in vitro experiments of both chick-
ens 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 bananaquit. 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 melanin or nonmelanin 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 numer-
ous other studies of MC1R and color variation in bird spe-
cies. 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; Bai-
o 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 carrion/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 fur-
ther 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 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 mag-
pies. 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 (Kjer 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 ben-
efit from further investigation of such genes.

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
1995Moore, 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
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 Fs 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 negative Tajima’s D value, highly significant Fu’s Fs 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 (Toon, 2007) found evidence to support this contemporary gene flow in north–western Australia with microsatellites, which are rapidly evolving nuclear markers (Ellegren, 2007).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 Nullabor-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. E. Dobson 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 SF003903, Queensland Parks and Wildlife permit W4/002670/01/SAA, 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