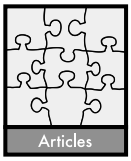

J. Avian Biol. 41: 505–514, 2010
doi: 10.1111/j.1600-048X.2009.04823.x

Received 2 February 2009, accepted 20 July 2009

The evolution of black plumage from blue in Australian fairy-wrens (Maluridae): genetic and structural evidence

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Genetic variation in the melanocortin-1 receptor (MC1R) locus is responsible for color variation, particularly melanism, in many groups of vertebrates. Fairy-wrens, Maluridae, are a family of Australian and New Guinean passerines with several instances of dramatic shifts in plumage coloration, both intra- and inter-specifically. A number of these color changes are from bright blue to black plumage. In this study, we examined sequence variation at the MC1R locus in most genera and species of fairy-wrens. Our primary focus was subspecies of the white-winged fairy-wren *Malurus leucopterus* in which two subspecies, each endemic to islands off the western Australian coast, are black while the mainland subspecies is blue. We found fourteen variable amino acid residues within *M. leucopterus*, but at only one position were alleles perfectly correlated with plumage color. Comparison with other fairy-wren species showed that the blue mainland subspecies, not the black island subspecies, had a unique genotype. Examination of MC1R protein sequence variation across our sample of fairy-wrens revealed no correlation between plumage color and sequence in this group. We thus conclude that amino acid changes in the MC1R locus are not directly responsible for the black plumage of the island subspecies of *M. leucopterus*. Our examination of the nanostructure of feathers from both black and blue subspecies of *M. leucopterus* and other black and blue fairy-wren species clarifies the evolution of black plumage in this family. Our data indicate that the black white-winged fairy-wrens evolved from blue ancestors because vestiges of the nanostructure required for the production of blue coloration exist within their black feathers. Based on our phylogeographic analysis of *M. leucopterus*, in which the two black subspecies do not appear to be each other's closest relatives, we infer that there have been two independent evolutionary transitions from blue to black plumage. A third potential transition from blue to black appears to have occurred in a sister clade.

Identifying the genetic basis underlying adaptation is a central goal for evolutionary biologists. Related to this, coloration patterns in vertebrates have been studied extensively. In some instances, intraspecific variation in color has been shown to be controlled by a simple genetic mechanism. For example, variation at the melanocortin-1 receptor (MC1R) locus is known to affect plumage color in some species of birds (Takeuchi et al. 1996, Theron et al. 2001, Kerje et al. 2003, Ling et al. 2003, Mundy et al. 2003, 2004), coat coloration in some mammals (Majerus and Mundy 2003, Nachman et al. 2003), and hair coloration in humans (Schiöth et al. 1999). The receptor is expressed in the membrane of melanocytes, and is an important regulator of the synthesis of melanin pigments. Activation of the MC1R increases the production of brown and black eumelanin pigments (Mundy 2005). In a number of vertebrate species, non-synonymous substitutions in the MC1R gene are correlated with melanism (Takeuchi et al. 1996, Theron et al. 2001, Kerje et al. 2003, Ling et al. 2003, Majerus and Mundy 2003, Mundy et al. 2003, 2004, Nachman et al. 2003). In many of these cited cases, the mechanism by which melanism is triggered is essentially the same – mutations in the same region of the MC1R gene disrupt the hormone-recognition site and cause constitutive activation of the gene (Majerus and Mundy 2003). This results in the over-production of melanin that eclipses the normal coat or plumage color.

One of the consistent features of avian species for which MC1R has been shown to be important is that the melanistic forms occur within the same population as non-melanistic forms (Theron et al. 2001, Mundy et al. 2004). Additionally, the expression of the melanistic phenotype is seldom sex limited – well-known examples exist in which both males and females are melanistic (Mundy 2005, Hoekstra 2006), although sex-specific effects of MC1R variation have been observed in the chicken (Ling et al. 2003). The extent to which MC1R affects coloration varies, but in general, the entire plumage of an individual is changed. For example, in the bananaquit *Coereba flaveola* all white and yellow colored areas are completely obscured by melanin (Theron et al. 2001); in the arctic skua *Stercorarius parasiticus* the entire plumage of the bird is

darkened; and in the lesser snow goose *Anser caerulescens caerulescens*, although the head of the most melanistic birds remains white, the rest of the plumage is entirely dark (Mundy et al. 2004). In both arctic skuas and snow geese, individuals homozygous for the melanism mutation have the darkest plumage while the plumage of heterozygotes is intermediate (Mundy et al. 2004).

In this paper we examine coloration and MC1R variation in fairy-wrens (Maluridae), an Australo–Papuan family of passerines exhibiting a wide range of plumage patterns. Most species in the family exhibit sexual dichromatism with strikingly colored males and dull colored females, but important exceptions include the brightly colored, but essentially monochromatic broad-billed fairy-wren *Malurus grayi* in Papua New Guinea, and the grass-wrens *Amytornis* and emu-wrens *Stipiturus* of Australia (Rowley and Russell 1997) in which sexual dichromatism is less pronounced than in *Malurus*. Within the genus *Malurus*, geographic variation in plumage can occur in females (the red-shouldered species group; Schodde and Mason 1999) and in males (the blue wren species group; Schodde and Mason 1999).

One of the most unusual patterns of plumage variation in fairy-wrens occurs in the white-winged fairy-wren *Malurus leucopterus*. The mainland subspecies (*M. l. leuconotus*), which is distributed over most of the arid regions of the Australian continent, exhibits no noticeable variation in plumage coloration across this vast range. Males are universally bright cobalt blue with white wings, females are fawn colored, and immature males resemble females. In contrast, in the two island-endemic subspecies in Western Australia, *M. l. leucopterus* on Dirk Hartog Island and *M. l. edouardii* on Barrow Island, males are entirely black with white wings. The plumages of females and immature males of the island subspecies are similar to those of the mainland – largely fawn-colored. Based on mitochondrial DNA evidence, Driskell et al. (2002) demonstrated that: 1) the two black-plumaged island subspecies are not sister taxa; 2) *M. l. edouard* on Barrow Island is the only genetically distinct clade; and 3) *M. l. leucopterus* on Dirk Hartog Island is genetically indistinguishable from the birds on the mainland in Western Australia, in which males are blue and white (Driskell et al. 2002). These islands are each closer to the mainland (Dirk Hartog ~1 km, Barrow ~55 km) than to each other (~600 km) and gene flow between them is unlikely. Thus, either black male plumage evolved independently, and in parallel in the two island populations, or the mainland subspecies has evolved bright blue plumage from a *M. leucopterus* ancestor with black male plumage.

In a study comparing the black Dirk Hartog (*M. l. leucopterus*) and mainland blue subspecies (*M. l. leuconotus*), Doucet et al. (2004) found five amino acid substitutions in the MC1R sequence associated with the plumage difference between these two subspecies. In addition, they concluded from their examination of the feather nanostructure of these two subspecies that the ancestor of the black plumaged island subspecies was blue plumaged (Doucet et al. 2004).

In this paper we examine MC1R variation in all three subspecies of *M. l. leucopterus*, the closely related and black plumaged red-backed fairy-wren (*M. melanocephalus*), other species of *Malurus*, and exemplars of most other fairy-wren genera (Fig. 1, Appendix 1). Additionally, we examine the nanostructure of feather barbs of the subspecies of the white-winged fairy-wren, *M. melanocephalus*, and one blue-plumaged fairy-wren, the splendid fairy-wren (*M. splendens*). As we will demonstrate, patterns of genetic variation at the MC1R locus within all three subspecies of white-winged fairy-wren as well as among multiple other species of fairy-wrens indicate there is no association between MC1R variation and plumage variation in fairy-wrens. However, feather nanostructure does vary in a consistent manner across the subspecies of white-winged fairy-wrens, revealing that the black island subspecies have similar, though not identical, structure and evolved from blue ancestors.

Methods

Taxon sampling

To examine MC1R variation in *Malurus*, tissue or blood samples were obtained from seven of the 11 species in the *Malurus* and three of the remaining four genera in Maluridae. For *M. leucopterus*, 12 individuals from Barrow Island (*M. l. edouardi*), five individuals from Dirk Hartog Island (*M. l. leucopterus*), and 13 individuals from the mainland of Australia (*M. l. leuconotus*) were sampled.

To update our published study of the evolutionary relationships among the subspecies of *M. leucopterus* (Driskell et al. 2002), mitochondrial data for five additional specimens of *M. leucopterus edouardi* from Barrow Island and five additional specimens of *M. l. leuconotus* from Western Australia were added to the published matrix. In addition, two *M. l. leuconotus* specimens had been misidentified as *M. l. leucopterus* in the previous study and this is corrected here. Specimens, collection localities, and lending institutions are listed in Appendix 1.

DNA extraction, amplification and sequencing

DNA was extracted from ethanol-preserved tissue or blood using standard proteinase-*k* digestion followed by a protein precipitation method as implemented in the Puregene or Qiagen DNAeasy kits. Multiple primers were designed for amplification of MC1R in fairy-wrens as previously published primers did not amplify reliably. Two primers were primarily used for amplification: F31 – (5′-GCTGGCCCCCCTGCGYCTGCTGC-3′) and R908 (5′-ACCACCTCCCGCAGCGTCCGCC-3′), which produced a ~870 bp fragment of the MC1R gene. In a few instances, the primers MSHR72 and MSHR73 of Mundy et al. (2003) were also used for either amplification or sequencing. Initially, amplification products were directly sequenced using the amplification and other internal primers. However, allelic variation was apparent in a number of specimens as indicated by the appearance of 'double-peaks' in resultant electropherograms. In order to accurately record all MC1R alleles present in the populations, the majority of MC1R data were produced by sequencing cloned PCR products.

A multi-step procedure was used for sequencing clones: 1) PCR amplification from DNA extracts using the primer pair F31/R908, 2) Gel purification using a 1% low-melt

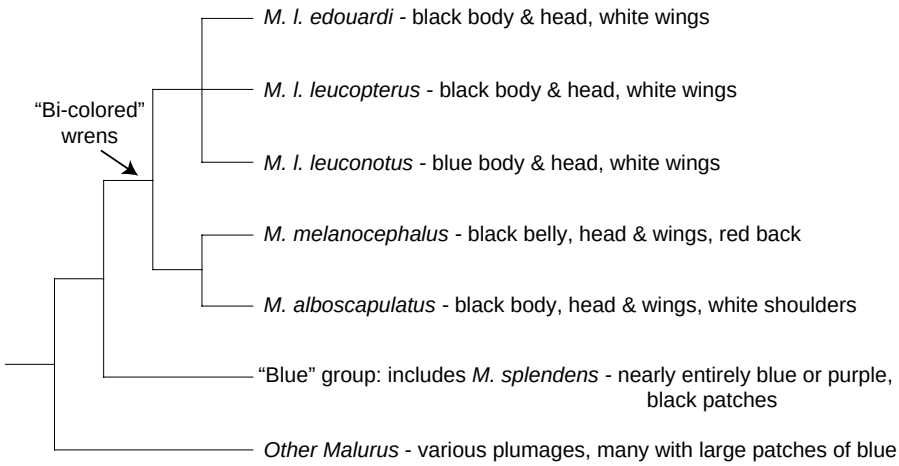

Figure 1. Relationships among the species and subspecies examined in this study and descriptions of their plumages. Diagram based on results published in Driskell et al. (2002) and Norman et al. (unpubl.).

agarose gel and excision of the band of interest, 3) Digestion of excised agarose plug using the Gelase enzyme, 4) Cloning of digested amplification products using the TOPO TA-cloning kit, 5) Checking 1–4 clones (on average, two) from each specimen by boiling picked colonies for 10 min and PCR amplification with the primer pair F31/R908, 6) Clean-up of the amplified product using the ExoSAP-IT protocol (USB), 7) Double-stranded sequencing using standard protocols.

Mitochondrial data were produced for the new *M. leucopterus* specimens following the procedures of Driskell et al. (2002).

Sequence alignment and comparison

MC1R sequences were translated using the Se-AL ver. 2.0 program (Rambaut 2001). All apparent base substitutions, in particular non-synonymous substitutions, were verified by careful examination of the electropherograms. When ambiguity persisted, the specimen was re-amplified and re-sequenced. No insertions or deletions were observed in any of the protein-coding sequences.

Phylogenetic and phylogeographic analyses of mtDNA data

The phylogeographic analyses were conducted using PAUP* ver. 4.0b10 (Swofford 2002). Heuristic searches under the parsimony criterion were unweighted with 50 multiple random addition sequences and TBR branch-swapping limited to 20 min per replicate. An overall maximum of 5000 trees was saved and strict and majority rule consensus trees were then calculated using this tree set. One hundred replicates of bootstrap analysis with TBR branch-swapping and two random addition sequences per replicate were used to evaluate nodal support. Branch-swapping was again limited to 20 min per replicate. Time limits were implemented with this data set as the majority of sequences were identical or nearly so, and almost no well-supported relationships with *M. leucopterus* exist. Additional time spent branch-swapping almost certainly would not result in greater resolution of the final tree.

Feather anatomy

We examined the microstructure of feathers of *M. splendens*, a typical blue plumaged fairy-wren, the three subspecies of *M. leucopterus* and *M. melanocephalus*, a primarily black plumaged sister species of *M. leucopterus* (Christidis and Schodde 1997, this study). Contour feathers were washed and prepared for microscopy following the methods outlined in Dyck (1971a). Transmission electron micrographs of thin ($\sim$100 nm) sections were taken with a Jeol JEM-1200 using a Soft-Imaging Megaview II CCD camera (1024×1200 pixels).

Results

MC1R analysis

Fourteen amino acid residues were variable within *M. leucopterus* (Table 1). Residue 38 was the only position correlated with male plumage color in this species – specimens of both island subspecies had an isoleucine at this position, while mainland birds had asparagine. However, all other species of fairy-wrens, regardless of plumage color, and the non-melanistic form of *Coereba* also had isoleucine at this position (Theron et al. 2001), and the black-plumaged *M. alboscapulatus* individuals had either isoleucine or valine here. Hence, at residue 38 the blue mainland *M. l. leuconotus*, not the black island subspecies, have a genotype (asparagine) unique among the other species examined. For each of the 14 variable amino acid residues, the two island subspecies were either homozygous or heterozygous for the same amino acid as all of the other species, as well as the distantly related bananaquit *Coereba.* Furthermore, at these 14 sites the island variant was not present in all island individuals sampled, whether homozygous or heterozygous – ruling out the possibility that these variants affect plumage color via genetic dominance. The blue mainland subspecies, *M. l. leuconotus*, had the

Table 1. Variable amino acid residues in the MC1R sequence of *Malurus leucopterus* and other fairy-wrens. All residue differences shared by two or more haplotypes of *Malurus leucopterus* are shown. Numbers indicate the ratio of haplotypes with each residue, in the cases where more than one amino acid was present in a taxon. In this study, 16 haplotypes were identified in *M. l. edouardi*, 7 in *M. l. leucopterus* and 19 in *M. l. leuconotus*. Shaded columns are the residues Doucet et al. (2004) identified as correlated with plumage color. *Coereba* sequence (GenBank A362594) from Theron et al. 2001.

	Amino acid residue													
	16	28	31	38	111	122	157	166	182	192	248	269	280	296
Barrow Is *M. l. edouardi*	A/T 14/2	A	G/S 14/2	I	V	I/V 14/2	Q	I	R	G	F	F	F	A
Dirk Hartog Is *M. l. leucopt.*	A	A/T 5/2	G	I	V/D 6/1	I	Q	I	R	G	F	F	F	A
Mainland *M. l. leuconotus*	T	A	G	N	I	I	Q/R 11/8	V/I 16/3	R/H 11/8	G/S 13/6	F/L 16/3	F/L 17/2	F/S 17/2	A/P 17/2
M. alboscapulatus	A	A	G	I/V	I/V	I	Q	V	R/H	G	F	F	F	A
M. melanocephalus	A	A	G	I	V	I	Q	I	R	G	F	F	F	A
M. splendens	A	A	G	I	V	I	Q	I	R	G	F	F	F	A
M. lamberti	A	A	G	I	V	I	Q	I	R	G	F	F	F	A
M. pulcherrimus	A	A	G	I	V	I	Q	I	R	G	F	F	F	A
M. cyaneus	A	A	G	I	V	I	Q	I	R	G	F	F	F	A
Clytomias insignis	A	A	G	I	V	I	Q	I	R	G	F	F	F	A
Stipiturus mallee	A	A	G	I	I	I	Q	I	R	G	F	F	F	A
Amytornis striatus	A	A	G	I	V	I	Q	I	R	G	F	F	F	A
Coereba flaveola y20	A	A	G	I	V	I	Q	V	R	G	F	F	F	A

most divergent amino acid sequence of all those examined – at nine positions it was either fixed or heterozygous for an amino acid not seen in any of the other taxa. There were no fixed synonymous differences among the subspecies and a possible cause for the amino acid divergence of *M. l. leuconotus* from the other two subspecies is unknown.

Phylogeographic analyses within *M. leucopterus*

Analysis of the *M. leucopterus* data set yielded 10 375 equally parsimonious trees. A strict consensus of these trees showed very little structure within *M. leucopterus* – likely due to the reasons of historical demography, which result in very low levels of sequence divergence within the species (97% of the 980 bp of sequence was invariant; Driskell et al. 2002). A strict consensus tree showed no phylogenetic structure within *M. leucopterus* (not shown). However, the majority rules consensus tree (Fig. 2) retained a monophyletic *M. leucopterus*, and three additional clades: A) a monophyletic Barrow Island *M. l. edouardi* clade (present in 77% of the parsimony trees); B) a monophyletic, but not well-supported, clade containing all Dirk Hartog specimens; and C) a clade comprised of all Dirk Hartog *M. l. leucopterus* and all but two of the Western Australian *M. l. leuconotus* specimens (present in 100% of the parsimony trees). Thus, both island subspecies appear as monophyletic groups in the majority rules consensus tree, albeit neither clade obtains overwhelming bootstrap support and they are not necessarily each other's closest relatives. Bootstrap analysis provided strong support for only the node subtending the Barrow Island clade. Sequence divergence among the *M. leucopterus* specimens ranged from 0–0.92% (=9 bp different). The greatest genetic distance observed in the *M. leucopterus* data set was between a specimen from Barrow Island and a specimen from Dirk Hartog Island. Barrow Island specimens had at least 3 and as many as 9 nucleotide differences from all other *M. leucopterus* specimens.

Feather anatomy

The morphology of the barbs of blue feathers of *M. splendens* (not illustrated) and *M. leucopterus leuconotus* (Fig. 3A, 4A–B) of mainland Australia (including those from Western Australia in close proximity to the island populations) was very similar. The spongy medullary layer of the feathers of both species was nanostructured in a system of complex air channels, and is similar to that found in blue feathers of parrots (Psittaciformes), rollers (Coraciidae), leafbirds (Irenidae), and thrushes (Turdidae) (type f of Dyck 1976; Prum et al. 1999, 2003). The barb rami of both *M. splendens* and *M. l. leuconotus* had 1) elliptical cross-sections, 2) a thin and uniform cortex completely devoid of melanosomes, 3) a medullary layer composed of large, boxy-shaped cells with extensive spongy keratin matrices, air-filled vacuoles, and 4) a layer of melanosomes between the central vacuole and the more superficial spongy keratin (Fig. 3A, 4A–B). The position of melanosomes within the spongy medullary cells is typical of almost all structurally colored barb rami (Fig. 4A; Dyck 1971a, 1971b, 1976, Prum et al. 1998, Prum 1999). These medullary melanosomes function as a light-absorbing layer below the structurally colored spongy matrix that prevents the incoherent scattering of white light from the central air vacuoles (Prum 2006, Shawkey and Hill 2005). Thus, the melanosome layer contributes to the saturation of the structural blue color. The barbs of *M. splendens* and *M. l. leuconotus* differ only slightly in shape; *M. splendens* barbs are narrower and higher than those of *M. l. leuconotus*.

In contrast, the black feather barbs from the island forms *M. l. edouardi* and *M. l. leucopterus*, and the black-plumaged red-backed wren (*M. melanocephalus*) were substantially different in morphology from the blue feather barbs of *M. splendens* and *M. l. leuconotus* (Fig. 3B–D, 4C, 4E). Black barbs from all three taxa revealed extensive melanosome deposition in nearly all cells, including the cortex (Fig. 3B–D). Furthermore, all three black taxa showed a substantially thicker cortex with a slight to elongate ridge in

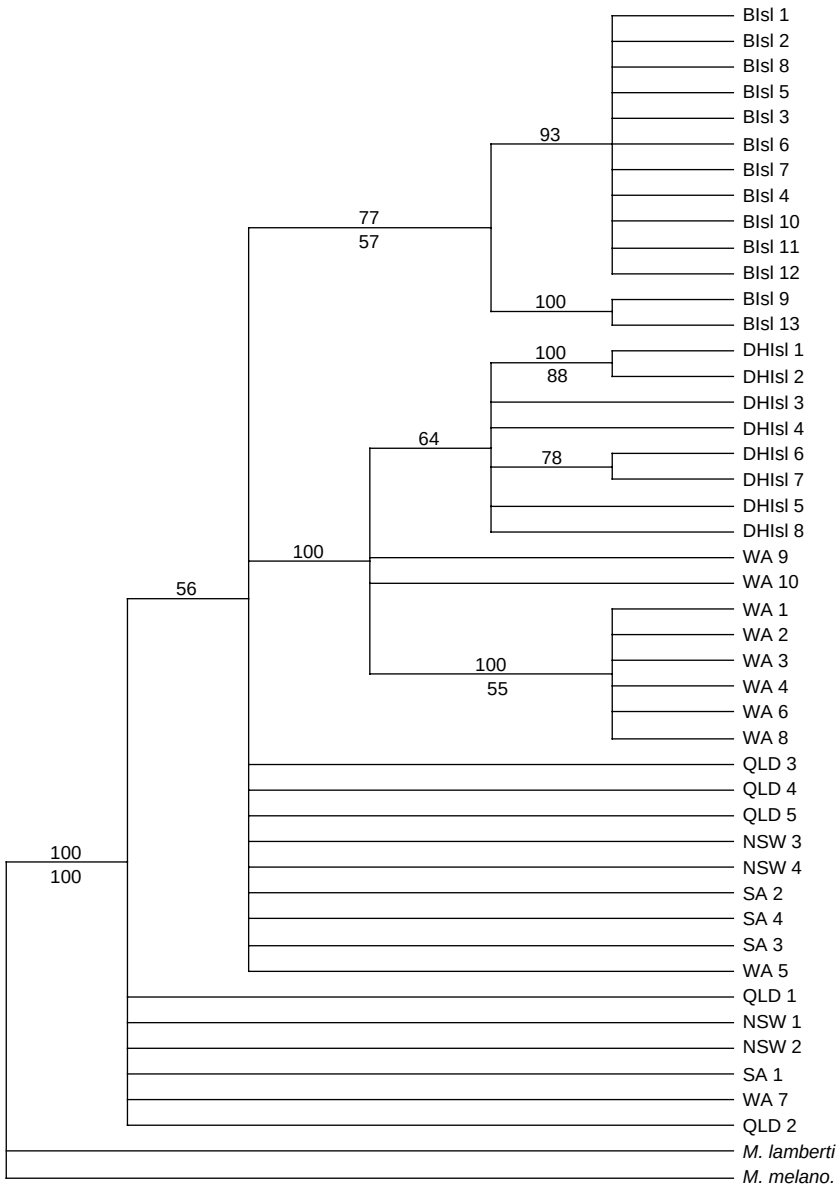

Figure 2. Majority-rule consensus of 5000 equally parsimonious trees. Numbers above the nodes indicate the percentage of trees in which that node is found. Numbers below the nodes are parsimony bootstrap proportions. Where no bootstrap value is indicated, the node received <50% bootstrap support. Tree length = 193.

the cortex on the upper (or obverse) surface (or margin) of the rami (Fig. 3B–D). The barb rami of *M. l. edouardi* have the most modest ridge (Fig. 3B), whereas the barb rami of *M. l. leucopterus* and *M. melanocephalus* have elongate, obverse ridges creating a generally tear drop shaped ramus in cross-section (Fig. 3C–D).

Most interestingly, the barb rami of the two black subspecies of *M. leucopterus* and the black *M. melanocephalus* showed various evidence of plesiomorphic but vestigial (i.e. secondarily non-functional) spongy medullary keratin (Fig. 4D, 4F). *Malurus l. edouardi* showed a substantial spongy keratin matrix that is similar in nanostructure to that found in blue barbs of blue *M. l. leuconotus* and *M. splendens* (Fig. 4D). However, these cells also include a dense concentration of melanosomes, distributed in the cortex and throughout the medullary keratin (Fig. 4C). These melanosomes would function to absorb incoming light before it could interact with the spongy medullary keratin to produce any structural blue color. Black barb rami from *M. l. leucopterus* showed an even more reduced medullary layer composed of fewer cells with less extensive spongy keratin within each cell (Fig. 4E). However, these medullary cells had melanosomes distributed throughout, blocking any direct optical interaction between the rudimentary spongy keratin matrices and incident light. Lastly, *M. melanocephalus* exhibited the most rudimentary medullary layer made of fewer cells with an extensive volume of unstructured ß-keratin (not illustrated).

Discussion

The evolution of color patterns in birds, and vertebrates in general, is complex and involves the interaction of genetics

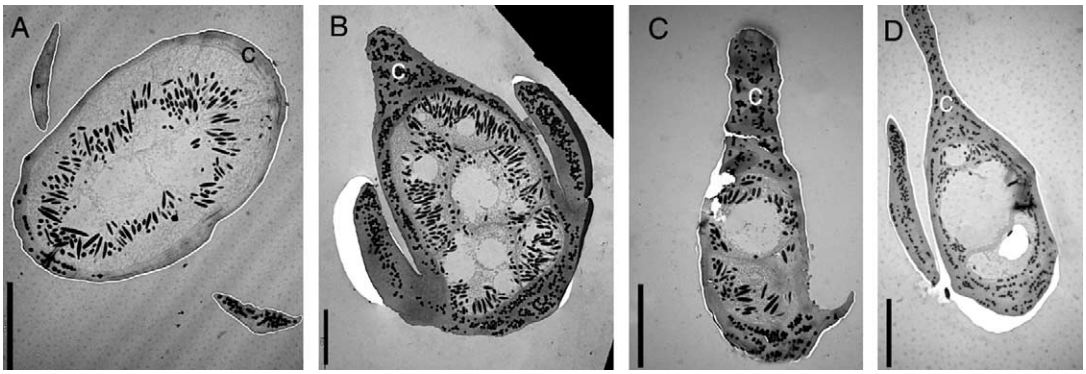

Figure 3. Transmission electron micrographs of the barb rami of white-winged fairy-wrens *Malurus leucopterus* and red-backed fairy-wren *Malurus melanocephalus* (Maluridae). (A) blue, mainland white-winged fairy-wren *M. leucopterus leuconotus*, (B) black, Barrow Island white-winged fairy-wren *M. leucopterus edouardi*, (C) black, Dirk Hartog Island white-winged fairy-wren *M. leucopterus leucopterus*, (D) black feather from red-backed fairy-wren *M. melanocephalus.* The cortex (c) differs greatly in thickness and presence of melanization. Scale bars: (A), (C), (D) = 10 μm, (B) = 5 μm.

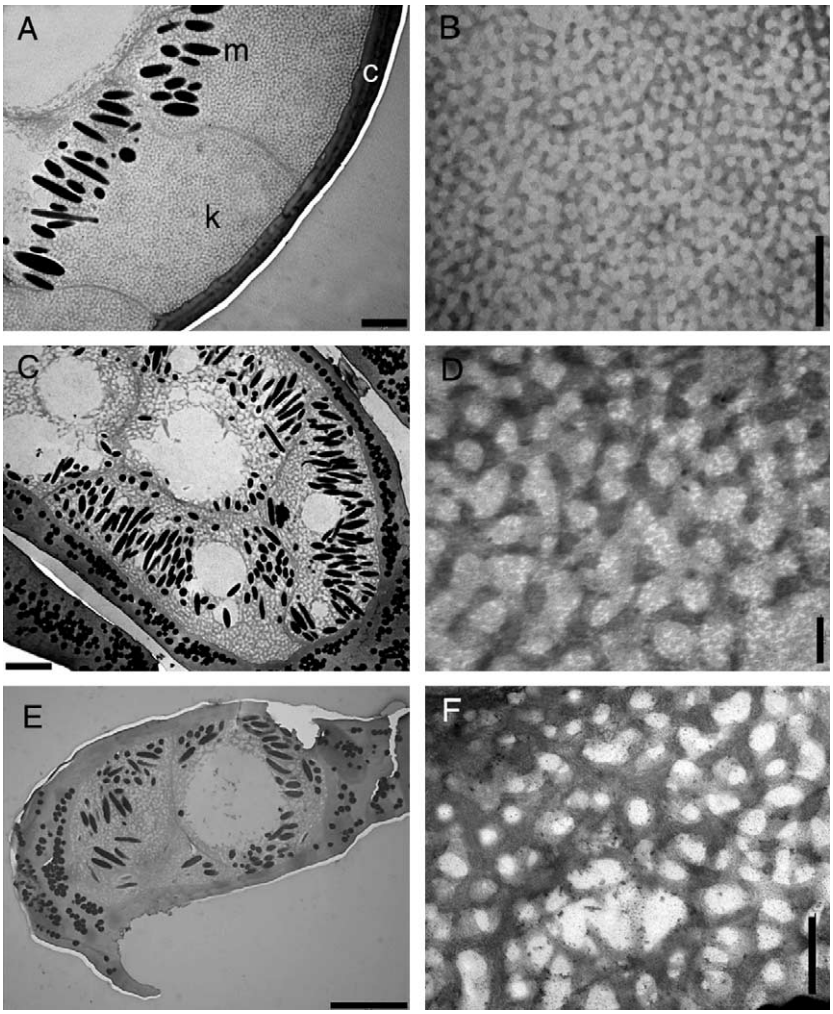

Figure 4. Transmission electron micrographs of spongy medullary cells from subspecies of white-winged fairy-wrens *Malurus leucopterus.* (A)–(B) blue, mainland white-winged fairy-wren *M. leucopterus leuconotus*, (C)–(D) black, Barrow Island white-winged fairy-wren *M. leucopterus edouardi*, (E)–(F) black, Dirk Hartog Island white-winged fairy-wren *M. leucopterus leucopterus.* Both blue and black feathers have spongy medullary keratin. Blue *M. l. leuconotus* feathers have melanosomes distributed below the spongy medullary keratin, whereas black *M. l. edouardi M. l. leucopterus* feathers have melanosome distributed throughout the spongy medullary keratin. c = cortex, k = spongy medullary keratin, m = melanosomes. Scale bars: (A), (C) = 2 μm, (B) = 1 μm, (D), (F) = 500 nm, (E) = 5 μm.

and development with aspects of habitat structure, ecology and breeding biology. The discovery that one or a very few mutations in a single gene, the MC1R locus, can have a major effect on melanism within a number of vertebrate species has provided a unique opportunity to investigate the interaction between genes and habitat structure and its influence on evolution (Majerus and Mundy 2003, Mundy et al. 2003, 2004, Nachman et al. 2003, Hoekstra 2006).

Although there are instances in which mutations at the MC1R locus clearly underlie melanistic plumage in some birds (Takeuchi et al. 1996, Theron et al. 2001, Kerje et al. 2003, Ling et al. 2003), other examples are known in which intra- or interspecific variation in melanistic plumage is unrelated to genetic variation at the MC1R locus (MacDougall-Shackleton et al. 2003). Additionally, other studies clearly reveal the important role that habitat structure and the light environment play in influencing evolution of color patterns in birds (Marchetti 1993, Johnson and Lanyon 2000, Gomez and Théry 2007). In studies of bananaquit, skua and other birds in which MC1R appears to influence plumage coloration, the amino acid substitutions in MC1R affect the overall quantity of produced melanin. Such variation cannot entirely explain the complexity of within-feather pigmentation patterns that contribute greatly to plumage coloration (Prum and Williamson 2002).

Our data show that the melanin color variation in male fairy-wrens generally, and the white-winged fairy-wren specifically, is unrelated to variation at the MC1R locus. We observed no genotypic variation in MC1R that correlated with black plumage in the two island subspecies. At the only residue (38) at which both island subspecies differed from the mainland subspecies, the island subspecies shared this amino acid with other non-melanistic species examined, and it was the mainland *M. l. leuconotus* which possessed the unique character state. The MC1R locus varied at a number of amino acid positions, but the most common allele in the black island populations was the same as that in the majority of other species examined, be they blue or black and in no instance was this 'island' allele present in every specimen examined. Although we acknowledge that the genetic mechanism underlying the plumage variation in white-winged and other fairy-wrens remains unknown, we contend it does not appear to be variation at the MC1R locus.

Doucet et al. (2004) found genetic differences in their comparison of mainland birds and those on Dirk Hartog Island, but their statement that these differences demonstrate a concordance between MC1R variation and plumage evolution is not justified based on their two sample comparison. Any pattern of genetic variation found in a two-taxon sample would lead to the conclusion that the variation was associated with differences between the taxa, but this is not sufficient as a test of the hypothesis of concordance. Like a regression based on two data points, Doucet et al.'s (2004) two population sample was insufficient to make the claim of evolutionary concordance between genetic and morphological evolution, and our broader analysis has falsified their claim.

It is unsurprising that constitutive activation of MC1R, proposed as the mechanism driving melanism in the bananaquit and many other vertebrates (Mundy et al. 2003, Hoekstra 2006), does not underlie the black plumage of the *M. leucopterus* subspecies. Not all of the island adult males' plumage is obscured by melanin; white wing patches persist in all subspecies. This seems inconsistent with continuous activation of MC1R which often completely obscures plumage and coat patterning by overproduction of melanin. In addition, in *M. leucopterus leuconotus* and *M. l. edouardii* only the reproductively mature males have black plumage – females and immature males retain the same fawn-colored plumage as the mainland blue subspecies. Clearly, the MC1R locus cannot be consitutively activated in females and reproductively-immature males, as their plumage is not melanistic. Color production is a complex process and involves a very long list of loci other than MC1R, intricate regulatory mechanisms, and details of developmental biology (Hoekstra 2006). The biological and evolutionary explanation for the appearance of black plumages within fairy-wrens likely lies somewhere 'upstream' of the MC1R locus.

In combination with the phylogeographic results of Driskell et al. (2002) and those presented here, which show the two island populations nested within the mainland subspecies, our data on feather structure imply that the ancestors of both black-plumaged subspecies of *M. leucopterus* were structurally blue-plumaged. Doucet et al. (2004) also reached a similar conclusion based on their examination of the Dirk Hartog subspecies. The barbules of black feathers from *M. l. leucopterus* and *M. l. edouardi*, and the closely related black species *M. melanocephalus* all showed morphological evidence that these feathers were derived from the plesiomorphic blue *Malurus* feather barbs. However, the different black-plumaged taxa show different degrees of alteration from the inferred ancestral form. We believe the differences reflect three separate and convergent acquisitions of black plumage in these taxa. Within *M. leucopterus*, the feathers of *M. l. edouardi* are the most similar to those of its blue mainland relatives, while those of *M. l. leucopterus* are more derived. Moreover, the rami of the feather barbules from the two black island subspecies were different from one another in shape and the two taxa exhibit different quantities of spongy medullary keratin. As the two islands are much closer to the mainland than to each other, convergent evolution of black plumage in *M. leucopterus* seems a reasonable conclusion.

The black feathers of *M. melanocephalus* differ the most from a typical fairy-wren blue feather; they have completely lost the spongy medullary nanostructure required for the production of the structural blue color. If degradation of feather microstructure occurs at a roughly consistent rate, *M. melanocephalus* evolved its black feathers much earlier than either *M. leucopterus* subspecies. In fact, in light of recent phylogenetic information (Fig. 1), it is parsimonious to assume that black plumage evolved in the ancestor of *M. melanocephalus* and *M. alboscapulatus* – placing the event still further back in time, before the divergence of the *M. leucopterus* subspecies. We predict that examination of the nanostructure of black feathers of *M. alboscapulatus* will show a degree of structural degradation comparable to that of *M. melanocephalus*.

We propose the first step in the evolution of black feathers from blue in the fairy-wrens is hypermelanization of the cortex and medullary cells of the feather barbules. The additional melanin obscures the structural blue color

and renders blue feathers black. Once the feathers are black, pressure to maintain a feather structure specific for the production of structural blue color is relaxed. Over time, the ramus of the feather deforms and the spongy medullary cortex is reduced and simplified. We believe this progression is more likely than the alternative hypothesis wherein the ancestor of the entire 'bi-colored' clade (Fig. 1) evolved black plumage and the mainland *M. l. leuconotus* re-evolved blue. This alternative requires the complete re-evolution of the morphology of blue feather barbules. It would necessitate the evolution of non-functioning spongy medullary keratin morphology within a continuous black plumage as well as the subsequent de-melanization of the cortex. It is hard to imagine the selective (or even neutral) process that would cause this to occur. The evolution of black from blue plumage, as we believe occurred, requires only a greater deposition of melanin and subsequent degradation of feather nanostructure.

In summary, we find no correlation between sequence variation at the MC1R locus and variation in blue and black plumage in white-winged and other fairy-wrens. The evolution of black plumage in *M. leucopterus* was not triggered by a mutation causing constitutive activation of the MC1R locus, as is proposed for other avian species. Changes in regulation or developmental biology are more likely causes. Feather nanostructure in the black subspecies of *M. leucopterus* and *M. melanocephalus* is consistent with initial evolution of barb hypermelanization, subsequent derived changes in ramus shape for pigment presentation, and the continuing degradation of spongy medullary keratin organization. The observed pattern could have evolved entirely through initially neutral variation in genes for melanin deposition, followed by a selection on barb morphology for pigment presentation and release of selection on spongy matrix morphology. Each of the three black *Malurus* taxa has a unique barb morphology and because phylogeographic and phylogenetic analyses indicate that they are not each other's closest relatives, we propose at least three instances of evolution from blue to black plumage in the Maluridae. Exactly why the island subspecies of *M. leucopterus* are black remains an open question, but we are certain that it is not the result of sequence variation at the MC1R locus.

Acknowledgements – This research was supported by the National Science Foundation (all authors), the Division of Biological Sciences, University of Chicago (SP-J), the Wettenhall Foundation (SP-J), Western Australian Petroleum (SP-J), Chevron Corporation (SP-J), the Chicago Zoological Society (SP-J) and the Section of Evolution and Ecology, Univ. of California Davis (ACD). We wish to thank the curators at Australian National Museum, National Museum of Victoria, Western Australian Museum, Burke Museum, the Univ. of Kansas Natural History Museum, Academy of Natural Sciences, and Leo Joseph and Melanie Rathburn for access to specimen samples, the many field and laboratory assistants that helped us during this work, Trevor Price, Emily Kay and Leo Joseph for comments on the manuscript, and lastly T. Quinn for electron microscopy.

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Appendix 1. Specimens of fairy-wrens examined in this study. Source = Museum or individual providing specimen; Specimen No. = museum voucher or collection number; Bk. Conserv. Park, SA = Brookfield Conservation Park, SA; Dryandra SF, WA = Dryandra State Forest, WA; Hattah-Kulkyne NP = Hattah-Kulkyne National Park, VIC; WA Museum = Western Australian Museum; ANS, Phil. = Academy of Natural Sciences, Philadelphia; Mus. Victoria = Museum of Victoria; Burke Mus. = University of Washington, Burke Museum; U. Kansas = University of Kansas, Natural History Museum. Genbank accession numbers for newly generated ATP6 sequences: HM156563-HM156572; ND3 sequences: HM156573-HM756582; MC1R sequences: HM156499-156562.

Taxon	Collection Locality	Latitude	Longitude	Source	Specimen No.
Malurus leucopterus edouardi	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW001
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW002
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW003
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW004
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW006
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW007
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW008
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW009
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW011
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW012
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW013
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW015
	Barrow Island, WA	20°47′S	115°28′E	Pruett-Jones	BW038
M. leucopterus leucopterus	Dirk Hartog Island, WA	25°50′S	113°05′E	WA Museum	A26806
	Dirk Hartog Island, WA	25°50′S	113°05′E	M. Rathburn	60
	Dirk Hartog Island, WA	25°50′S	113°05′E	M. Rathburn	81
	Dirk Hartog Island, WA	25°50′S	113°05′E	M. Rathburn	123
	Dirk Hartog Island, WA	25°50′S	113°05′E	M. Rathburn	141
M. leucopterus leuconotus	Bk. Conserv. Park, SA	43°21′S	139°31′E	Pruett-Jones	WW014
	Bk. Conserv. Park, SA	43°21′S	139°31′E	Pruett-Jones	WW020
	Bk. Conserv. Park, SA	43°21′S	139°31′E	Pruett-Jones	WW030
	Bk. Conserv. Park, SA	43°21′S	139°31′E	Pruett-Jones	WW040
	Bulloo Downs, QLD	28°32′S	142°57′E	Mus. Victoria	D075
	Bulloo Downs, QLD	28°32′S	142°57′E	Mus. Victoria	D084
	Ueendoo Creek, WA	25°03′S	113°41′E	ANS, Phil.	ANSP11230
	Ueendoo Creek, WA	25°03′S	113°41′E	ANS, Phil.	ANSP11232
	Ueendoo Creek, WA	25°03′S	113°41′E	ANS, Phil.	ANSP11234
	Ueendoo Creek, WA	25°03′S	113°41′E	ANS, Phil.	ANSP11236
	Ueendoo Creek, WA	25°03′S	113°41′E	ANS, Phil.	ANSP11256
	Cape Range, WA	21°55′S	114°04′E	Mus. Victoria	MV2512
	Lancelin, WA	31°01′S	115°20′E	M. Rathburn	309
Malurus alboscapulatus	Tetebedi, PNG	9°10′S	148°04′E	Mus. Victoria	E121
Malurus melanocephalus	Atherton, QLD	17°16′S	145°28′E	J. Karubian	RBW01
Malurus splendens emmottorum	Longreach, QLD	24°10′S	143°15′E	Burke Mus.	SAR7063
Malurus cyaneus	Bk. Conserv. Park, SA	43°21′S	139°31′E	Pruett-Jones	SFW91-12
Malurus lamberti	Bk. Conserv. Park, SA	43°21′S	139°31′E	Pruett-Jones	VW104
Malurus pulcherrimus	Dryandra SF, WA	32°46′S	117°08′E	Burke Mus.	PLG300
Clytomyias insignis				U. Kansas	4619
Stipiturus mallee	Hattah-Kulkyne NP	34°42′S	142°25′E	Pruett-Jones	MEW1
Amytornis striatus	Hattah-Kulkyne NP	34°42′S	142°25′E	Pruett-Jones	SGW1