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Mutations in different pigmentation genes are associated with parallel melanism in island flycatchers

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The independent evolution of similar traits across multiple taxa provides some of the most compelling evidence of natural selection. Little is known, however, about the genetic basis of these convergent or parallel traits: are they mediated by identical or different mutations in the same genes, or unique mutations in different genes? Using a combination of candidate gene and reduced representation genomic sequencing approaches, we explore the genetic basis of and the evolutionary processes that mediate similar plumage colour shared by isolated populations of the *Monarcha castaneiventris* flycatcher of the Solomon Islands. A genome-wide association study (GWAS) that explicitly controlled for population structure revealed that mutations in known pigmentation genes are the best predictors of parallel plumage colour. That is, entirely black or melanic birds from one small island share an amino acid substitution in the melanocortin-1 receptor (MC1R), whereas similarly melanic birds from another small island over 100 km away share an amino acid substitution in a predicted binding site of agouti signalling protein (ASIP). A third larger island, which separates the two melanic populations, is inhabited by birds with chestnut bellies that lack the melanic *MC1R* and *ASIP* allelic variants. Formal F_{ST} outlier tests corroborated the results of the GWAS and suggested that strong, directional selection drives the near fixation of the *MC1R* and *ASIP* variants across islands. Our results, therefore, suggest that selection acting on different mutations with large phenotypic effects can drive the evolution of parallel melanism, despite the relatively small population size on islands.

1. Introduction

Convergent evolution of phenotypic traits across taxa, such as limb length in *Anolis* lizards [1] and pelage colour in *Peromyscus* mice [2], provides some of the strongest evidence of natural selection in the wild [3]. Although there is clear evidence that these convergent traits evolve in response to similar ecological pressures, surprisingly little is known about their underlying genetic basis [4,5]. Uncovering the genetic basis of convergent or parallel traits is essential, as it provides key insights into the genetic and developmental constraints that underlie diverse phenotypes [5–10], and the operation of selection as it acts on existing or novel mutations to drive convergence [11–13]. For instance, in threespine sticklebacks *Gasterosteus aculeatus*, multiple populations evolve similar plate armour morphology as they colonize fresh water lakes from marine habitats [14]. This

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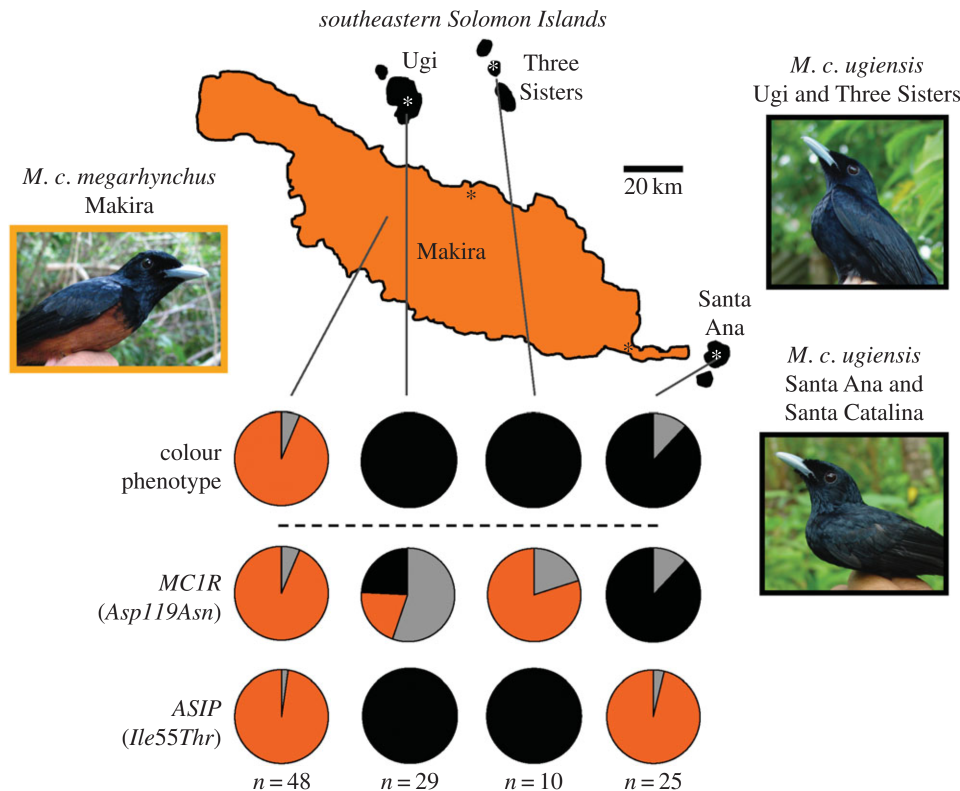

Figure 1. Plumage colour phenotype, and *MC1R* and *ASIP* genotype frequencies for melanic and chestnut-bellied subspecies of *Monarcha castaneiventris*, which consists of the chestnut-bellied *Monarcha castaneiventris megarhynchus* on Makira (orange island) and melanic *Monarcha castaneiventris ugiensis* subspecies on Ugi and Three Sisters to the north, and Santa Ana to the southeast (black islands). The top row of pie diagrams shows proportion of individuals, by locality, that were chestnut-bellied (orange), melanic (black) and partially melanic (grey) in plumage colour. The middle and bottom rows of pie diagrams show proportions of individuals that were homozygous for the ancestral alleles (orange), homozygous for the derived alleles (black) and heterozygous for the derived and ancestral alleles (grey) for *MC1R Asp119Asn* and *ASIP Iln55Thr*, respectively. Asterisks indicate study sites where samples were collected. See figure 3 for the phenotypes of the *MC1R* and *ASIP* heterozygotes (grey).

parallel adaptation is mediated by repeated selection on allelic variants of the *Ectodysplasin* (*EDA*) gene [7]. These *EDA* variants are present in low frequency in marine populations, suggesting that standing genetic variation facilitates the repeated and rapid colonization of freshwater lakes by sticklebacks [7]. Knowledge of the genetic basis of parallel traits, combined with understanding the evolutionary processes that favour convergence, can therefore provide a comprehensive understanding of how natural selection operates [5].

In island birds, one of the most variable traits among closely related populations is plumage colour, with geographically disjunct populations sometimes converging on similar colour patterns [15,16]. Unlike, for example, bill variation, which has been linked to feeding ecology, the genetic basis of convergent plumage colour and the evolutionary processes that mediate its occurrence across islands remain little understood [15,17]. Because the efficacy of selection should be reduced in islands with small effective populations (N_e), the evolution of plumage traits on islands has been attributed to alternative mechanisms, such as relaxed selection or pleiotropy (reviewed by Grant [15]). This is because reduced N_e should affect the efficacy of selection either directly (i.e. $N_e \times s$) or indirectly through the Hill–Robertson effect, which enhances interference by background, deleterious mutations through reduced recombination rates in smaller populations [18]. However, observations of multiple taxa converging on similar colour patterns implicate selection in driving the predictable evolution of plumage colour [19]. Information on the genetic basis of and the evolutionary processes that mediate parallel plumage colour could, therefore, provide unique insights into the evolutionary processes that operate in small populations.

In his seminal work on speciation, Ernst Mayr [20] presented the variable chestnut-bellied flycatcher *Monarcha castaneiventris* complex as a prime example of incipient speciation and parallel evolution of plumage colour. The *M. castaneiventris* clade comprises at least six named taxa that differ in plumage colour across narrow geographical gradients throughout the Solomon Islands [16,20]. A notable pattern is that subspecies in the southeastern part of the archipelago are similar in body size and bill morphology, but strikingly different in plumage colour [20]. A population on the large island of Makira has a chestnut belly and iridescent blue-black upper-parts (*Monarcha castaneiventris megarhynchus*; 'chestnut-bellied form'), whereas nearby populations, approximately 8 km away, on the small satellite islands to the north (Ugi & Three Sisters) and southeast (Santa Ana and Santa Catalina) are entirely iridescent blue-black (*Monarcha castaneiventris ugiensis*, 'melanic'; figure 1). The northern and southeastern melanic populations are over 100 km apart, yet are considered a single subspecies because of their similarity in plumage colour [20]. Phylogenetic analyses of the *M. castaneiventris* complex indicate that *M. c. megarhynchus* and *M. c. ugiensis* form a separate, well-supported clade that is sister to the other *M. castaneiventris* subspecies [21]. Neither taxon, however, is reciprocally monophyletic owing to gene flow between islands and the retention of ancestral polymorphisms, reflecting their recent divergence [22,23]. More recent phylogenomic analyses, however, suggest that the melanic population of Santa Ana is sister to the chestnut-bellied

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population of Makira, with the melanic population of Ugi as the outgroup to the two taxa [23]. Given that chestnut belly is the most likely ancestral state for the *M. castaneiventris* clade [21] and the entire *Monarcha* genus, in general [24], the phylogenomic pattern suggests that melanism evolved convergently between the southeastern Santa Ana and northern Ugi populations of *M. c. ugiensis* [23]. Alternatively, hybridization could explain similar plumage colour between melanic populations, given evidence of gene flow among islands. Information on the genetic basis of plumage colour could, therefore, help resolve the origins of melanism in this clade.

In the past century, over 100 genes have been identified that influence colour in vertebrates [25–27]. Most notably, mutations in the melanocortin-1 receptor (MC1R) and its antagonist, agouti signalling protein (ASIP), have been shown experimentally to have large effects on the expression of melanin-based coloration [28–31]. MC1R is a seven-transmembrane receptor that regulates melanogenesis in vertebrates. When bound to alpha melanocyte stimulating hormone (α-MSH), MC1R signals the production of eumelanin in melanosomes, which creates black to grey coloration. By contrast, when bound to ASIP, MC1R signals the production of phaeomelanin in melanosomes, which creates chestnut to brown coloration [27]. Association studies, complemented by direct mutagenesis, biochemical and pharmacological experiments, show that simple, point mutations in either of these genes can result in large changes in colour [25,26]. Taking advantage of this well-known genetic pathway and complemented by reduced representation genomic sequencing data [32], we explore the genetic basis of and the evolutionary processes that mediate parallel melanism between the two geographically isolated populations of *M. c. ugiensis* inhabiting small islands to the north and to the southeast of Makira Island (figure 1). We discuss our results in light of the expectation that a smaller effective population size should reduce the efficacy of natural selection on small islands.

2. Material and methods

(a) Collection

From 2006 through to 2012, tissue or blood samples were collected from 45 chestnut-bellied birds in Makira, 22 melanic birds in Santa Ana, 29 melanic birds in Ugi and 10 melanic birds in Three Sisters (electronic supplementary material, table S1). In addition, blood samples were taken from six birds with intermediate belly colours (e.g. partial melanic): three individuals from Makira and three individuals from Santa Ana. Birds were caught with mist nets then measured, tagged, blood-sampled and released as part of a long-term study [21,22]. Genomic DNA was extracted from blood samples using standard protocols (DNEasy DNA Extraction Kit, Qiagen, Valencia, CA, USA).

(b) Candidate gene and next generation sequencing approaches

For our candidate gene study, we sequenced the following genes/loci: *MC1R* (813 bp of the first and only exon) and *ASIP* (411 bp of all three exons, 704 bp of intron 1, 532 bp of intron 2 548 bp of the upstream region (approx. 400 bp from start of exon 1)). Ancestral state for the *MC1R* and *ASIP* allelic variants was inferred by sequencing the sister species *Monarcha cinerascens* (electronic supplementary material, table S1). Primers for sequencing *MC1R* and *ASIP* were developed using the zebra finch genome [33] and are detailed in the electronic supplementary material, table S2. Unincorporated primers and dNTPs from PCR products were removed using Exo-SAP-IT following the manufacturer's protocol (USB, Cleveland, OH, USA). Sequencing reactions using both forward and reverse primers were carried out at Cornell University's Life Sciences Core Laboratories Center and at the University of Miami's Molecular Core Facilities in the Department of Biology using an ABI 3730xl capillary DNA sequencer. Sequences were aligned and annotated using SEQUENCHER 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). Population genetic parameters, such as F_{ST} (Wright's estimator) and diversity (π), were calculated using the program DNASP v. 5.10 [34].

We complemented the candidate gene approach with genome-wide single nucleotide polymorphism (SNP) data recovered from restriction site associated DNA sequencing (RAD-seq) [32] of 22 chestnut-bellied and 2 partially melanic birds from Makira, 1 partially melanic and 19 melanic birds from Santa Ana, and 24 melanic birds from Ugi (a subset of birds used in the candidate gene approach). Multiplexed RAD tag libraries were constructed for each population following a modified version of the protocol described by Parchman *et al.* [35]. Briefly, the genomic DNA was double digested with *Eco*RI and *Mse*I, and then ligated to barcoded Illumina sequencing adapters, which had been customized to recognize the restriction sites. Adapter-ligated DNA was then purified using Beckman-Coulter's AMPure purification kit (cat. no A63880) to remove adapter dimers, amplified via PCR and size-selected using gel electrophoresis. Samples were organized into libraries according to population, resulting in three libraries with a maximum of 24 barcoded individuals each. Libraries were sequenced at the Hussman Institute of Human Genetics, University of Miami in one-half of a lane on an Illumina HiSeq 2000, which produced 100 bp paired-end reads. Raw Illumina reads were sorted by barcode and filtered for quality (any read with a phred quality $<$20 was removed) using process_radtags from the Stacks package [36]. Filtered RAD tags were de novo assembled using the Stacks package, and individual reads were re-aligned to the consensus using BWA [37], with up to 10 mismatches allowed (note, however, that 99% of the aligned reads had five or fewer mismatches).

SNPs were called using vcfutils in the SAMtools package [37], with a minimum quality threshold of 20, and a minimum and maximum individual read depth threshold of 10 and 50, respectively. The read depth cut-offs were determined by examining the empirical distribution of coverage in our aligned data, which indicated that a depth above 50 represented an outlier. Using custom Perl scripts, we removed any sites with more than two alleles or fewer than 10 individuals per population, and then verified the heterozygous and homozygous variants called by SAMtools. We removed polymorphisms with less than 5% minor allele frequency in order to eliminate potential sequencing errors. We also required at least 20% of reads within an individual to contain the minor allele in order for that individual to be called heterozygous; individuals with between 0 and 20% minor alleles at a particular site were treated as missing data at that locus. These procedures were similar to the methods and cut-offs used by Rheindt *et al.* [38], although we were more conservative because we had lower overall coverage in our data. More details about our pipeline and the scripts can be found online at: https://github.com/eacooper400/RAD_Pipeline_Info.

Our study system comprises geographically isolated populations with nearly fixed phenotypes but limited population genetic structure [22] (see the electronic supplementary material, figure S1). We, therefore, used two complementary approaches to recover potential candidate genes for plumage colour and to infer the evolutionary processes that mediate the differences in plumage colour across islands: (i) a genome-wide association study (GWAS), which is more appropriate for studies of groups with little background, genetic structuring; and (ii) an F_{ST} outlier test, which is more appropriate for studies of distinct populations.

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Combining the genome-wide SNP data from RAD-seq, and the *MC1R* and *ASIP* SNPs from the candidate gene approaches, we conducted a GWAS to scan for genetic variation associated with plumage colour using the mixed linear model implemented in the software package GEMMA [39]. Because plumage colour is correlated with locality (i.e. islands are fixed or nearly fixed for divergent plumage colour), we controlled for intrinsic, population genetic structure by running a principal component analysis (PCA) to calculate the major axis of genetic variation between populations (implemented in PLINK v. 1.7; [40]), then used the major PC axis as a covariate in the GWAS. Given that our previous results from a candidate gene approach suggested that the underlying genetic basis of melanism between the Santa Ana and Ugi populations are different [22], we ran separate GWAS for the Makira and Ugi (8907 SNPs), the Makira and Santa Ana (14 305 SNPs), and Santa Ana and Ugi (13 030 SNPs) comparisons. Chestnut-bellied birds were assigned a phenotypic value of 0, melanic birds were assigned a phenotypic value of 1, and partially melanic birds were assigned an intermediate phenotypic value of 0.5. For each SNP, significant association with plumage colour was determined using Wald's statistic, which was Bonferroni corrected for multiple comparisons (i.e. cut-offs for calling candidate SNPs were $p = 5.6 \times 10^{-6}$, 3.5×10^{-6} and 3.8×10^{-6} for the Makira and Ugi (8907 SNPs), Makira and Santa Ana (14 305 SNPs) and Santa Ana and Ugi (13 030 SNPs) comparisons, respectively).

Given that our comparisons involve distinct islands, we complemented the GWA approach by implementing F_{ST} outlier tests for the Makira and Ugi (8907 SNPs), Makira and Santa Ana (14 305 SNPs) and Santa Ana and Ugi (13 030 SNPs) comparisons to uncover SNPs that may be subject to directional selection and thus likely linked to candidate genes for divergent plumage colour [41]. We executed F_{ST} outlier tests in BAYESCAN v. 2.1 [42], which implements a Bayesian method to estimate the posterior probability that a particular locus or SNP is subject to selection. That is, BAYESCAN calculates a posterior probability (α) for a model in which selection better explains the data than the null model of neutrality, with positive values suggesting directional selection and negative values suggesting stabilizing selection. We used the default parameters for our runs; however, the false discovery rate (FDR) was set at 0.05 and the prior odds for the neutral model was set at 100 (i.e. the neutral model was set at 100 times more likely than a model with selection) to reduce the likelihood of false positives [41]. We checked for adequate mixing and convergence of our Markov chain Monte Carlo runs by evaluating trace plots of F_{ST} and Geweke's diagnostic tests [43], executed in CODA [44].

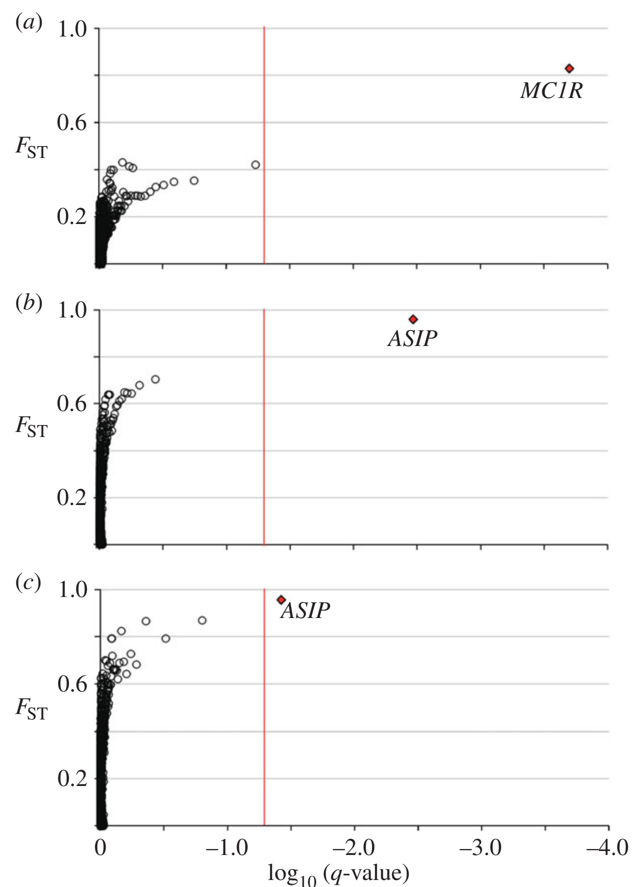

Figure 2. Results of the BAYESCAN F_{ST} outlier analyses for the comparisons between (*a*) Makira (chestnut-bellied) and Santa Ana (southeast melanic; 14 303 SNPs), (*b*) Makira and Ugi (north melanic; 8905 SNPs) and (*c*) Santa Ana and Ugi (13 208 SNPs) populations. The horizontal axis shows $\log_{10}$ of the *q*-value, which is the false discovery rate (FDR) analogue of the *p*-value. The vertical axis shows the F_{ST} for each SNP (open circles), with the diamonds indicating outliers detected by BAYESCAN. The vertical line indicates the $\log_{10}$ of FDR = 0.05. (Online version in colour.)

3. Results and discussion

(a) A point mutation in *MC1R* predicts melanism in the southeastern Santa Ana population

Consistent with our previous work [22], a comparison of most of the coding region of *MC1R*, here with a substantially larger sample size, indicates that a derived amino acid substitution at position 119 of MC1R (aspartic acid → asparagine; *Asp119Asn*) is strongly associated with melanism in the southeast Santa Ana population (figure 1; electronic supplementary material, table S3). All melanic birds from Santa Ana ($n = 22$) were homozygous for *Asn119*, while all chestnut-bellied birds from Makira ($n = 45$) were homozygous for *Asp119*. A mixed linear model GWAS using 14 305 SNPs distributed throughout the genome of 20 birds from Santa Ana and 24 birds from Makira recovered *MC1R Asn119* as the best predictor of melanism, with only two other SNPs approaching *MC1R*'s strong association (electronic supplementary material, table S3). That is, when the *MC1R* SNP is combined with the RAD-seq dataset, the GWAS that incorporated intrinsic population structure as a covariate recovered *MC1R* as the best predictor of plumage colour between melanic Santa Ana and chestnut-bellied Makira birds, with *Asp119Asn* having a β-value of 0.50 ± 0.00 ($p = 0.0$). Only two other SNPs were significant, with β-values of 0.31 ± 0.06 ($p = 2.6 \times 10^{-6}$) and 0.29 ± 0.06 ($p = 3.3 \times 10^{-6}$). The two additional, significant SNPs did not map onto or near known genes in the zebra finch genome. Likewise, the BAYESCAN F_{ST} outlier test using the same dataset recovered *MC1R Asp119Asn* as the only significant outlier (figure 2*a*).

Additional lines of evidence strongly suggest a causal role for *Asn119* in melanism for Santa Ana birds. First, we sequenced *MC1R* for six unusual birds with intermediate belly colour from Makira ($n = 3$ of 48 birds) and Santa Ana ($n = 3$ of 25 birds). All six were heterozygous for *Asp119Asn*, suggesting that this *MC1R* allele is partially dominant. Note also that the genome-wide level of heterozygosity based on approximately 14 000 genome-wide SNPs did not differ among the three partially melanic, 22 chestnut-bellied and 19 melanic birds included in the RAD-seq study (electronic supplementary material, figure S2), suggesting that heterozygosity in *Asp119Asn* for partially melanic birds is not simply due to them being F_1 hybrids of matings between Makira and Santa Ana birds (e.g. overall high heterozygosity

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in the genomic background). Second, experimental and association studies in other taxa indicate that point mutations in this region of *MC1R* can lead to melanism [25], and, in fact, *Asn119* is the same mutation in the homologous position of *MC1R* responsible for melanism in some breeds of sheep and pigs [45,46]. Finally, *in vitro* functional experiments indicate that the position corresponding to *Asp119Asn* is necessary for high affinity ligand binding [45], and that the *Asn119* mutation may activate MC1R by mimicking ligand binding [47]. In summary, the co-segregation of genotype and phenotype, including six birds with intermediate belly colour, and the results from previous functional experiments and comparative studies strongly implicate *Asn119* as the cause of melanism in the southeastern Santa Ana birds.

(b) A point mutation in *ASIP* is the best predictor of melanism in the northern Ugi and Three Sisters populations

Because birds from the southeastern and northern satellite islands are both melanic in plumage colour (figure 1), we tested if *MC1R Asn119* likewise predicts melanism in the northern satellite islands of Ugi and Three Sisters. We found a much weaker association between *MC1R Asn119* and melanism (figure 1), suggesting that northern melanic birds may have a different or additional genetic basis for melanism. That is, the derived *MC1R Asn119* allele is present in high frequency in the melanic populations of Ugi and Three Sisters, but the association between colour and genotype is weak (figure 1). In fact, several melanic birds were homozygous for the ancestral, chestnut *Asp119* allele (figure 1). This pattern suggests the possibility that a different or additional mutation mediates melanism in the northern *M. c. ugiensis* populations of Ugi and Three Sisters.

We, therefore, sequenced the entire coding region of *ASIP* (three exons), the antagonist of *MC1R*, and found a polymorphic site in exon 1 that results in an amino acid substitution at position 55 of ASIP (isoleucine → threonine; *Ile55Thr*). Exons 2 and 3 were not variable. All melanic birds from the northern satellite islands ($n = 29$ on Ugi Island; $n = 10$ on Three Sisters) are homozygous for threonine in this position (*Thr55*; figure 1). The *Thr55* allele, however, is nearly absent in the chestnut-bellied Makira ($n = 48$) and melanic Santa Ana ($n = 25$) populations, with both melanic and chestnut-bellied birds carrying copies of isoleucine instead of threonine (*Ile55*; figure 1). In the chestnut-bellied population of Makira, the *Thr55* allele is present as a single copy in a heterozygote, and this bird was intermediate in belly colour. In the melanic Santa Ana population, we likewise caught a single bird heterozygous for *ASIP* position 55 but this individual was entirely melanic. A GWAS that explicitly controlled for population structure, and used 8907 SNPs from the combined RAD-seq and candidate gene approaches recovered *ASIP Ile55Thr* as the best predictor of plumage colour for the melanic birds of Ugi and chestnut-bellied birds of Makira. *ASIP Ile55Thr* had the highest β-value of 0.49 ± 0.02 ($p = 1.18 \times 10^{-25}$), and only one other candidate marker was uncovered by the GWAS, which had a β-value of 0.29 ± 0.05 ($p = 1.8 \times 10^{-7}$; see the electronic supplementary material, table S4). This SNP did not map onto a known gene in the zebra finch genome. *MC1R Asp119Asn* was not recovered by the GWAS as a predictor of plumage colour. Similar patterns were found in the Ugi and Santa Ana comparison, with *ASIP Ile55Thr* having the highest β-value of 0.46 ± 0.03 ($p = 3.7 \times 10^{-21}$). Seven other candidate SNPs were uncovered by the GWAS, but none mapped onto or near annotated genes in the zebra finch genome (see the electronic supplementary material, table S5). The BAYESCAN F_{ST} outlier analyses corroborated the results of the GWAS, recovering *ASIP Ile55Thr* as the only significant outlier for the Makira versus Ugi, and Ugi versus Santa Ana comparisons (figure 2*b*,*c*).

Based on sequences from an outgroup species, *M. cinerascens*, and other more distantly related avian species, isoleucine is the ancestral state for ASIP's 55th amino acid residue [48]. Alignment with other vertebrate ASIP protein sequences indicates that *Ile55Thr* is in the N-terminal chain of ASIP [48]. The N-terminal chain is predicted to bind to attractin, an accessory receptor adjacent to MC1R thought to mediate ASIP's antagonistic activity [48]. In fact, because of its important role in binding, the N-terminal chain is highly conserved across distantly related mammalian and avian taxa [48]. Further, the *Ile55Thr* mutation results in a change from a non-polar to a polar residue (hydrophobic to hydrophilic, respectively), which can influence the affinity of the N-terminal chain to attractin [49] and could thus limit ASIP's antagonistic activity on MC1R. In the Japanese quail *Coturnix japonica*, dark plumage (*recessive black*) results from a frameshift mutation in the N-terminal chain that causes the dysfunction of ASIP as an antagonist of α-MSH [50], while light plumage (*yellow*) results from a large > 90 kb deletion in *RALY*, which regulates *ASIP* [51]. Similarly, in two *Peromyscus* mice populations, a premature stop codon eliminating the N-terminus of ASIP is associated with melanism [29], while a deletion in the N-terminal chain is associated with light pelage colour [12]. Although the point mutation in the final *Peromyscus* example results in light and not melanic coloration, the result from this study suggests that relatively simple mutations in the N-terminal domain can have large effects on ASIP's interaction with α-MSH and MC1R. Overall, therefore, a causal role for *ASIP Thr55* in mediating melanism in the northern satellite islands of Ugi and Three Sisters is suggested by the clear genotype-phenotype association from the candidate gene and GWAS approaches, coupled with the experimental and comparative data indicating the N-terminal chain's critical function in receptor binding and highly conserved nature across vertebrates [48,49].

Without functional experiments to directly quantify the effects of *ASIP Thr55*, we cannot definitively exclude the possibility that this point mutation is simply in linkage disequilibrium with other mutation(s) in *ASIP*. We explored this hypothesis by surveying the scale of linkage disequilibrium in *ASIP*, sequencing upstream and downstream of *ASIP*'s first exon where *Thr55* resides.

Between the northern melanic *M. c. ugiensis* and chestnut-bellied Makira *M. c. megarhynchus* populations, the F_{ST} for *ASIP Ile55Thr* is 0.99 when partially melanic birds are included or 1.0 when only chestnut-bellied and melanic birds are included (electronic supplementary material, figure S3). By contrast, the F_{ST} values downstream of the first exon (i.e. portions of introns 1 and 2) are much lower, with a sharp decline within 1500 bp downstream (electronic supplementary material, figure S3). The significant reduction in population structure for the intronic sequences downstream of exon 1 (and lack of variation in exons 2 and 3) suggests that the causal mutation is not downstream of *ASIP*'s first exon.

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In the region adjacent and upstream of exon 1, we found six segregating sites in 516 bp sequenced. Five of the six SNPs showed only minor variation across islands. However, a single point mutation is fixed in the northern melanic populations (428 bp upstream of exon 1; *g.428A>T*), resulting in relatively high F_{ST} values between islands for this SNP (electronic supplementary material, figure S3). The strong linkage disequilibrium between *g.428T* and *Thr55* makes it difficult to definitively exclude *g.428T* as the causal mutation for melanism in the northern satellite islands. Genotypes of individuals outside the northern satellite islands, however, provide insights on its possible role in melanism.

The derived *g.428T* allele is present in three heterozygotes in the chestnut-bellied Makira population. All three birds, which were homozygous for ancestral *ASIP Ile55* and *MC1R Asp119* alleles, had entirely chestnut bellies despite carrying a copy of the derived *g.428T* allele. Further, the single bird that was heterozygous for *ASIP Ile55Thr* was homozygous for the ancestral *g.428A* allele, yet it was partially melanic in plumage colour (electronic supplementary material, figure S3). Finally, in *Monarcha castaneiventris obscurior*, a subspecies that is polymorphic in plumage colour (i.e. melanic and chestnut-bellied birds) and found in the Russell Islands over 300 km from Makira, four birds were homozygous for the derived *g.428T* allele but homozygous for the ancestral *ASIP Ile55* and the ancestral *MC1R Asp119* allelic variants. If *g.428T* mediates melanism, these four birds should be melanic. Only two of the four birds, however, were melanic, with the remaining two having pure chestnut bellies (electronic supplementary material, figure S4). The imperfect association between the *g.428A > T* allelic variants and plumage colour, especially the cases where chestnut-bellied birds were homozygous for the derived *g.428T* allele and a partially melanic bird was homozygous for the ancestral *g.428A* allele, suggests that *g.428T* does not cause melanism and is, instead, probably in strong linkage disequilibrium with *Thr55* in the northern *M. c. ugiensis* birds. Overall, however, the strong linkage disequilibrium between *g.428A > T* and *Ile55Thr* prevents us from completely ruling out additional mutations upstream of *g.428A > T* as the causal mutation(s) for melanism in the northern populations. Regardless of which of the two point mutations is causal or if there are additional mutations upstream of *Ile55Thr*, the clear association between these *ASIP* variants and melanism suggests that mutation(s) in *ASIP* mediate(s) melanism in the northern populations of *M. c. ugiensis*.

Breeding and functional experiments will provide more direct tests of the causal role of *ASIP* and *MC1R* mutations in melanism. However, our results showing the clear association between mutations in genes known to mediate pigmentation, after controlling for population structure, implicate mutations in *MC1R* and *ASIP* in causing parallel melanism in the two, geographically separated populations of *M. c. ugiensis*.

(c) Interaction between *ASIP* and *MC1R* allelic variants

With each *M. c. ugiensis* and *M. c. megarhynchus* individual genotyped for the coding sequences of *ASIP* and *MC1R* and having sampled six birds of intermediate belly colour in Makira and Santa Ana, we can infer how alleles from the two loci interact to create specific plumage colours. Figure 3 summarizes the genotype combinations and corresponding belly colour in a 3×3 genotype interaction table. Individuals homozygous for the ancestral alleles for both *MC1R* and *ASIP* had chestnut bellies (top left in figure 3). Individuals homozygous for the derived *ASIP* allele were melanic regardless of their *MC1R* genotype (right column in figure 3). Similarly, individuals homozygous for the derived *MC1R* allele were melanic regardless of their *ASIP* genotype (bottom row in figure 3). Individuals heterozygous for *MC1R* or *ASIP* were intermediate in belly colour as long as they were not homozygous for either the derived *MC1R* or *ASIP* alleles. These patterns suggest that: (i) the derived *MC1R* and *ASIP* alleles are partially dominant; (ii) being homozygous for either the derived *ASIP* or *MC1R* alleles is, by itself, sufficient to cause melanism; and (iii) *ASIP* and *MC1R* interact epistatically. Overall, these patterns suggest a simple genetic mechanism for melanism, similar to other avian and mammalian species (reviewed in [25,26]).

(d) Directional selection drives the differentiation of *MC1R* and *ASIP* between islands

Results from our F_{ST} outlier analyses are consistent with the hypothesis that *MC1R* and *ASIP* are subject to strong directional selection (figure 2). In the comparison between the chestnut-bellied Makira and the melanic Santa Ana populations, *MC1R Asp119Asn* was the only significant outlier detected, with an α score of 2.02 (figure 2*a*). Similarly, in the comparison between the chestnut-bellied Makira and the melanic Ugi population to the north, *ASIP Ile55Thr* was the only outlier and had an α score of 1.76 (figure 2*b*). Finally, in the comparison between the two melanic populations of Santa Ana and Ugi, *ASIP Ile55Thr* was the only outlier detected, with an α score of 1.59 (figure 2*c*). Overall, the significant and positive alpha scores, as estimated by BAYESCAN, indicate that *MC1R* and *ASIP* are under strong directional selection, which drives the near fixation of alternative *MC1R* or *ASIP* allelic variants between the chestnut-bellied Makira population and the melanic populations of Santa Ana and Ugi, respectively.

Because islands typically have a smaller effective population size (N_e) than their mainland counterparts (or larger islands in a chain), the efficacy of natural selection should be reduced on small islands. Based on field observations of territory size in this species [22] and the size of each island, we estimate the effective population size at 2342 and 6746 for the Santa Ana and Ugi populations, respectively (see the electronic supplementary material for our methods of estimating N_e). By contrast, assuming that birds only breed in comparable habitats (lowland, secondary growth rainforest, which is approx. 1/3 of Makira) in the large island of Makira, a very conservative estimate of N_e for this population is 149 942, which is about 20 to 60 times greater than the melanic populations of the satellite islands. As such, despite Ugi and Santa Ana's relatively small effective population size, our results suggest that strong directional selection can drive the evolution of parallel melanism on very small islands.

4. Conclusion

In mice (*Peromyscus* spp.; *Chaetodipus intermedius*) and lizards (*Sceloporus undulates*, *Aspidoscelis inornata* and *Holbrookia maculata*), natural selection for crypsis across a variable visual background maintains a stable colour polymorphism that

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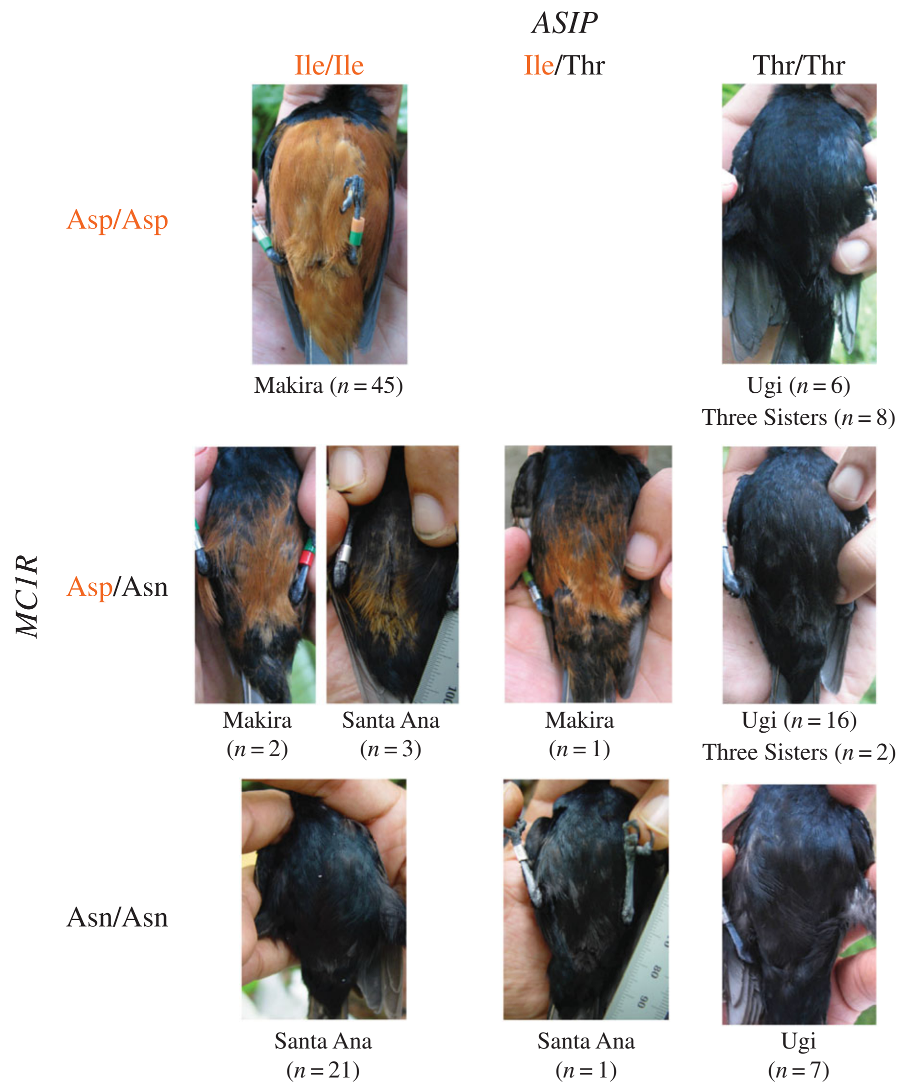

Figure 3. Association between *MC1R* and *ASIP* genotypes, and belly colour. The islands where each genotype combination was sampled (and corresponding sample size) are listed below each representative photograph of an individual's chest and belly.

is similarly mediated by mutations in *MC1R* or *ASIP* [10–12,52]. Conversely, loss of pigmentation across independent populations of the cave-dwelling fish *Astyanax mexicanus* is mediated by unique mutations in *MC1R* [9]. These studies suggest that parallel ecological selection can favour the evolution and maintenance of convergent phenotypes between independent populations, and that these convergent traits can be mediated by the same [53,54] or different [9,10,29,52] genetic mechanisms. Likewise, our results suggest that parallel, diversifying selection may favour similar phenotypes that are associated with different pigmentation genes across two geographically separated populations of a single subspecies. One possible mechanism of selection between islands is assortative mating or conspecific recognition based on plumage colour. Our previous field experiments indicate that divergent plumage colour is used by melanic birds from Santa Ana and chestnut-bellied birds from Makira in recognizing conspecifics, suggesting divergent plumage colour as the basis for premating reproductive isolation or discrimination against hybrid phenotypes [22,55]. Mate choice experiments should provide more direct insights into the role of divergent plumage colour in assortative mating as a mechanism for disruptive selection. Regardless of the specific mechanism, however, our results highlight the diversity of simple molecular pathways that can give rise to similar morphological traits between populations of a single species, as well as the effectiveness of natural selection even in small island populations.

Ethics. This research adhered to the Institutional Animal Care and Use Committee guidelines for the use of vertebrate animals in research (protocol no. 11–116) and the legal requirements of the country in which the work was carried out. Permission to work in the Solomon Islands was granted by the Ministry of Education, and the Ministry of Environment, Conservation and Meteorology of the Solomon Islands.

Data accessibility. RAD-seq SNP data for GWAS: Dryad ID 877n3 (http://dx.doi.org/10.5061/dryad.877n3). DNA sequence: GenBank submission numbers: 1842234, 1842238–40, 1842246–47, 1842249, 1842252, 1842255, 1842259, 1842266.

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Competing interests. The authors declare no conflict of interest.

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