GENETIC BASIS OF MELANIN PIGMENTATION AND SEXUAL DICHROMATISM IN DOMESTIC PIGEONS

by

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Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2016

In loving memory of Penny

To my parents

Acknowledgements

It is with great pleasure that I express thanks to Trey Fondon for giving me this rare opportunity and great insights and training, although equally great is the misfortune that our vision was not shared by the university. None of our work would have been possible without help from several batches of interns: Andrew, Trevor, Raj, Nhu thinh, Johnny, Steven, Michael, Heinz, Todd, Lucio, Treyce, Russell, Julie, Abraham, Spencer, Amanda, Arielle, Clifford, Curtis, Edward, Laura, Bimal, Courtney, Himani. Thanks also to Eldon Prince and Kristen Correll for help and support in the lab. Richard Cryberg, and Kerri Hendricks among several fancier/geneticists played an important role in fast-forwarding our learning curve of pigeon genetics.

I am thankful to my committee members for their time, patience, advice and support, especially Andre. Special thanks to the office staff: Linda, Gloria, Sherri, Paulette, and GCF staff Melissa and Kim. Linda and Gloria have come to my aid too many times to keep count of.

Special thanks to my several friends who have tolerated my long periods of silence, but have always been available to keep me afloat. Vikas and Todd especially have been a great help when I required taking a break. Sean (my roommate) has been a bedrock in the last year and especially these last few weeks. My brothers who are always a phone call away have been my connections to the world beyond science. Finally, none of this would be possible without the unwavering support of my parents who have been waiting patiently all these years since I first set sail to the Nicobar Islands adamant to work in the life sciences.

Everyone of you has had an important role in my success. During the six-plus years of my PhD, my two little best friends Penny and Daisy made every day seem effortless.

November 2, 2016

Post script: Penny endured this PhD program with me. At the time of this defense she was being treated for respiratory issues, little did I know that after two failed treatments we would get a diagnosis of metastasized cancer in her lungs.

Penny took her last breath on 25 Nov ’16 at 4:58 am

Abstract

GENETIC BASIS OF MELANIN PIGMENTATION AND SEXUAL DICHROMATISM IN DOMESTIC PIGEONS

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Deciphering the genetic basis of pigmentation in domestic pigeons (*Columba livia*) affords a unique opportunity to connect microevolution to macroevolution. Domestic pigeons have far greater phenotypic diversity than other domesticates that often recapitulate traits that occur in nature. By using classical genetic approaches and by leveraging the innumerable meiotic events since the advent of pigeon domestication, and population structure, I have mapped traits to genes and begun to deconstruct more complex traits. In this process of investigation one expects to discover mechanisms that may be generalizable to some extent to other birds; however, their generality may be limited in their extent as they are products of intense artificial selection.

In this volume I have mapped well characterized monogenic or Mendelian pigeon pigmentation traits to their genetic underpinnings. As pioneers in this system, since the last workers (up until the 1940s), my approach is akin to using pigeon breeds as Petri dishes and intercrosses as inoculation experiments. In this process, by moving traits into different genetic contexts I am deconstructing genetic pathways and validating and extending our knowledge of melanin synthesis. Some of my results initially appear contradictory to expectation, as in the case of the phenotype associated with the gene

Mc1r and the characteristics of the *smoky* trait. In other cases including some not presented in this volume we have concordance with the predictions from pigmentation studies in other systems.

Birds undoubtedly have among the most flamboyant displays and colors used in courtship and combat rituals, and possess some dramatic sexual dimorphisms, yet the sexes of wild rock pigeons (*Columba livia*) are uniform in appearance, a slate blue-gray with black markings. Sexual dichromatism can evolve in several ways involving antagonistic and/or sexual selection. While sex-linkage is not necessary for the evolution of sexual dimorphisms, theory predicts that sex linkage should favor the evolution of such traits. Early pigeon fanciers characterized sexually dichromatic sex-linked pigmentation traits and created breeds in which sexes can be readily distinguished as early as hatchlings. Mapping this locus helps us understand the possible roles of as yet poorly known players in the melanogenesis pathway and mechanisms by which they may contribute toward pigmentation diversity,

The repeated evolution of traits due to the same genetic players and pathways is support for the conserved nature of the genetic toolkit. The genetic associations in this volume can be leveraged in future studies to dissect more complex pigmentation traits and our knowledge from domestic pigeon pigmentation genetics may be extended to birds in general.

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Chapter 1 Introduction

Avian color diversity is elaborate and diverse, exemplified by ornate sexually dimorphic traits that are used in courtship displays. Understanding the genetic basis of such diversity requires teasing out causative mechanisms that underlie traits from the surrounding genetic and genomic context. The essential difficulty is that traits of interest are typically fixed within species, precluding the rigorous genetic dissection necessary to discern causative events from the obscuring background of genetic noise that accumulates within lineages. To the extent that evolutionary changes in different taxa are due to changes in a common genetic tool kit, the evolution of similar traits in related lineages may have similar genetic origins. By discovering the genetic basis of plumage traits in bird species harboring polymorphisms amenable to study, we may better comprehend the processes by which similar traits arise through focused studies in less tractable species. Domestic pigeons harbor tremendous color variation that parallels diversity seen among numerous avian taxa, and are amenable to rigorous genetic analysis. My goal has been to determine the genetic basis of color traits in domestic pigeons in order to better understand the mechanisms by which color traits evolve in nature.

The genetic origins of naturally arising traits have been mapped in only a few wild vertebrates, while the majority of examples are from domesticates. Moreover, of the handful of successful efforts to map pigmentation traits in wild birds, most were directly guided to their targets by prior knowledge of the genetic basis of related traits in domesticates. Several factors contribute to this bias towards domesticates, not the least of which is that domestic animals are almost by definition relatively easy to breed (albeit

some more so than others), but the most important factor is that inherited variation is relatively abundant in domestic animals. In this regard, there is perhaps no species better endowed than the domestic pigeon, which exceeds all other domesticates in the number of breeds and numbers of traits in which they vary (Price 2002). The domestic pigeon possesses key attributes that can be exploited for genetic mapping studies, including the ability to perform experimental crosses between any of hundreds of varieties harboring an array of diverse traits. In making the case for the principle of natural selection, Darwin argued that the same process of selection has been affected by man on domestic species, giving rise to diverse varieties, races, and breeds of plants and animals (Darwin 1859; Darwin 1868). Confidence in the descent of all domestic pigeon breeds from a single ancestral species, and the greatest availability of material (i.e. diversity) led Darwin to choose pigeons over other domesticates as his primary demonstration and research model. Although presenting scant variation in nature, the wild rock dove (*Columba livia*) has under domestication evolved a tremendous spectrum of diverse variations in color, structure, voice, and behavior representing a microcosm of evolution (Figure 1-1), offering an excellent and largely untapped resource to understand the origins of novelty and diversity. The strength of domesticates and specifically pigeons is enhanced by a population structure that facilitates distilling causal associations from nonfunctional background variation. The availability of pedigreed samples from fanciers, and ability to conduct controlled crosses and other breeding experiments makes pigeons a powerful, but underutilized, genetic resource.

Figure 1-1 Previous page – sample of domestic pigeon variation (photographs – Stephen Green-Armytage 2003).

Columba livia, the wild-rock pigeon

The pigeon has a historical association with man, having been used in different cultures as a source of meat and also as symbols of worship, wealth, and peace (Levi 1963; Blasco et al. 2014). For centuries it has been used in relaying messages using its homing ability (Tegetmeier 1863; Levi 1963). In the early 1900s, at the time when *Drosophila* was emerging as a genetic model, several early geneticists, including Thomas Hunt Morgan, were using domestic pigeons as a model system to study principles of inheritance and inherited variation. These genetic pioneers were able to show how Mendel's laws applied to vertebrates, including genetic dissection of polygenic quantitative traits, while working out the modes of inheritance of several Mendelian pigmentation characters (Morgan 1918; Christie and Wriedt 1931; Steele 1931; Hollander 1937; Hollander 1982; Mowrer 1940; Lange 1952; Levi 1963).

The diversity of domestic pigeons exceeds all other domesticates in number of breeds and numbers of traits in which they vary (Price 2002). The wild pigeon is blue-gray with black bar patterns on its wing shield and tail, and remnant wild populations harbor little color variation. But the domestic varieties have been selected for diverse traits including size, mass (breast size), posture, colors, plumage, and flight variations. Performance breeds, used in contests of speed, homing, rolling or tumbling, have been selected primarily for performance with little or no focus on other traits and utility breeds have been developed for meat production. Many pigeon hobbyists, however, specializing in what are termed fancy breeds, have selected for conformation to breed standards or

standards of beauty (Figure 1-1). The diversity of color and pattern among domestic pigeons is often used like a painter's palette by fanciers of certain breeds, for which “color projects” to introduce new colors into the breed are common. This has often resulted in colors and patterns that are reminiscent of those observed among species in nature, indeed, some breeds are developed specifically to resemble other species of birds in color and/or structure (e.g. Egyptian swifts, Figure 1-1 - top left corner). To a geneticist, deconstructing and mapping the complex color phenotypes can provide rare insight into the numerous ways that similar colors and patterns can be produced in pigeons and perhaps in other species.

Pigmentation in birds

Birds are heavily dependent on color for both inter and intra-specific signaling, and this has manifested in immense diversity of colors, structures, and displays. Several functions are attributed to color and pattern evolution in birds including camouflage, concealment of the eye, deflective markings, advertisement against predation, perception advertisement, allurement to conspecifics, group cohesion and coordination, startle, flash and confusion markings, disguise and mimicry, thermoregulation, and protection from ultra-violet radiation (Bortolotti 2006). As with other vertebrates, avian coloration is primarily the result of a combination of dietary pigments (primarily carotenoids) or melanin based pigments and structural color (Gower 1936; Shawkey et al. 2011; Stoddard and Prum 2011). While many types of dietary pigmentation exist, most are restricted to relatively highly narrow set of taxa, not including the wild rock dove and its derivatives. Melanin based pigmentation, however, is both ancient and conserved, and relatively well understood (Borovansky and Riley 2011). To efficiently map pigmentation traits using candidate gene based mapping approaches it is essential to both understand

the development and biochemistry of color. and to borrow from the numerous mapping efforts in other domestic animals and model organisms (see Chapter 2 Melanogenesis review).

Genetic mapping approaches

Most genetic mapping experiments can broadly be classified as either genetic association or linkage mapping (Song, Feingold, and Weeks 2002; Hanson et al. 2006; Al-Chalabi 2009; Smith 2012). Genome-wide association, candidate-based association, extended haplotype homozygosity mapping, and selective sweep mapping are different approaches to exploit statistical correlations between alleles and traits within/between populations or samples that result from linkage disequilibrium (LD) and population structure. An important factor critical for the success of the genetic association is the existence of LD between the genetic markers tested and the causal mutation(s). This LD can be undermined by several factors, with genetic heterogeneity (multiple genetic bases for same or similar traits) being of greatest concern here. Linkage mapping studies work within families segregating for a trait, with the variable of interest being recombination frequency between trait and marker (Sham et al. 2000; Stephens and Bamshad 2011). Linkage-based approaches minimize or eliminate the problems caused by genetic heterogeneity or weak LD, but are constrained by the availability and size of families, and offer much more limited mapping resolution.

With the availability of high-throughput genotyping technology, genome-wide association studies (GWAS) have become commonplace in human genetics. In GWAS, thousands of markers from across the genome are screened with the expectation of LD between markers and causal mutations for the traits of interest. While advances in

genotyping technologies have driven increased reliance on whole genome scans, these advances come at a statistical cost that requires larger sample sizes to compensate for the large number of comparisons being performed (So and Sham 2011). The multiple comparison statistical penalty is incurred by both genetic association and genetic linkage based approaches that test many markers distributed across the genome. This penalty may be avoided with *a priori* knowledge (or good guesswork) of likely locations of causative loci, so that by limiting testing to just one or a few loci power can be increased dramatically, permitting traits to be mapped efficiently with much smaller samples. The key is to be able to make accurate guesses about the likely locations of causative mutations, something that is rarely possible for most types of traits, but is possible for pigmentation traits. Modern genetic mapping in domestic animals is often performed using a hybrid approach, in two stages, with the first stage like in linkage mapping identifying large chromosomal regions associated with the trait within which to fine map by association (Smith 2012).

Inaccurate trait diagnoses and genetic heterogeneity can cause either type of approach to fail, but association-based approaches are far more susceptible to these issues. Trait diagnosis errors can adversely affect any type of experiment, decreasing power and inflating required sample size. With accurate diagnosis and no genetic heterogeneity, candidate-based genetic association can be successful with very small samples. For example, the Fisher's exact test probabilities for complete association in 10 cases and 10 controls without assignment errors results in a significance of $p = 5 \times 10^{-6}$, but the significance collapses to $p = 1 \times 10^{-3}$ with only three incorrect assignments. So, in choosing an appropriate approach for any trait, careful consideration must be made to assess the potential for errors and heterogeneity, and taking steps to avoid, minimize, or

mitigate such errors. These considerations factor heavily into the approaches chosen for mapping the *Stipper* locus, where the concerns differ for different alleles and populations (Stephens and Bamshad 2011).

Chapter 2 Melanogenesis - Review

Studies across several vertebrates have identified more than 150 genes that affect color directly or indirectly and another 150 unmapped color loci have been characterized (Yamaguchi and Hearing 2009; Cieslak et al. 2011). In pigeons as in several other vertebrates the majority of color diversity is the result of the interplay between incident light and layers of keratin, underlying which are melanin pigment granules. Melanin polymers fall in two classes, eumelanin that is black or brown, and pheomelanin that is yellow or red. Synthesis of melanins occurs within specialized neural crest-derived melanocytes, and chromatic variation in pigeons is primarily the result of different quantities and proportions of each pigment (Haase et al. 1992; Mollaaghababa and Pavan 2003; Goding 2007).

Melanogenesis occurs in melanocytes that are descended from cranial neural crest-derived melanoblasts. Several upstream genes in the melanogenesis pathway are responsible for melanoblast migration, in the early embryo, toward their final destinations where they differentiate and commit to melanocyte lineages. Classical melanocytes located in the epidermis and dermis directly or indirectly affect the skin and integumentary color. Other types of melanocytes occur that develop color in the retina and irides, cochlea, cardiac cells, and neural cells. Iris color results from uveal melanocytes; loss of melanocytes in the uvea results in colorless irides giving the eye the color of the retina. The retinal color pigments are synthesized within the melanocytes of the retinal pigment epithelium (RPE) only in early development. Loss of melanocytes in the RPE results in colorless retina giving the eye a red color appearance as observed in albinos. Cardiac and neuronal melanocytes provide some color to respective tissues, but

coloration is thought to be incidental to the function of these cells (Colombo et al. 2011). Mutations in many of these upstream genes often have similar color phenotypes including complete or partial lack of pigmentation, and candidate gene lists can be narrowed down by examining which genes can account for the correlated traits (pleiotropic effects) such as ocular or auditory defects.

The synthesis of the melanin pigments within melanocytes occurs in the melanosome, a lysosome-derived organelle (Figure 2-1). As melanosomes mature with their melanin payload they are transported toward the cell membrane from the periphery of the nucleus, where they exocytose from melanocyte dendrites into keratinocytes (Park et al. 2009). Developing feather barbules then uptake melanosomes giving them their color and patterns. The biochemistry of melanogenesis requires the key melanosomal enzymes Tyrosinase (TYR), Tyrosinase-related protein 1 (TYRP1), and Dopachrome tautomerase (DCT) to be present within the melanosome under very specific conditions. TYR is the rate-limiting enzyme in the absence of which melanins are not synthesized. Pheomelanin synthesis is a rapid cascade of reactions that requires only the enzyme TYR and the pheomelanin precursor, cysteine. Eumelanin synthesis, however, is a slower reaction that is catalyzed only after cysteine has been depleted, and in the presence of the three additional melanosomal enzymes. The concentrations of melanosomal enzymes and maintenance of the melanosomal environment are regulated by proteins and transcription factors external to the melanosome. For proper pigment production a complex suite of genes governing the migration of melanoblasts, transcription factors that initiate production of melanosomal enzymes, ion transporters, melanosomal matrix proteins, melanosome transporters, and melanosome uptake by keratinocytes from melanocyte dendrites, must all be choreographed together. Mutations

at any stage of the complex pathways can affect the ultimate phenotypes, and certain steps in the pathway allow us to predict the class of resulting phenotypes. For instance, elimination of *Tyr* by a loss of function mutation should result in no melanin production and a resulting white plumage, and unpigmented iris and retina. In chicken, the *C* locus has three alleles causing red-eye white, recessive white, and autosomal albino; the last of which was mapped to a six base pair deletion in the gene *Tyr* (Tobita-Teramoto et al. 2000). We can also predict that a regulatory mutation at *Tyr* could be responsible for producing mottled phenotypes of eumelanism, pheomelanism, and amelanism. The mouse *Tyr* allele c^{m1OR} is caused by a transposable element insertion that in heterozygosity with the albino allele produces a black and white mottled mouse and in homozygosity produces a reduced level of mottling (Wu et al. 1997). Loss of function mutations in *Tyrp1* that catalyzes later steps of eumelanin synthesis predictably results in brown-like traits because of a reduction in eumelanin to pheomelanin ratio, as demonstrated in the mouse *A* locus and in Tibetan brown pigs (Zdarsky, Favor, and Jackson 1990; Ren et al. 2011b).

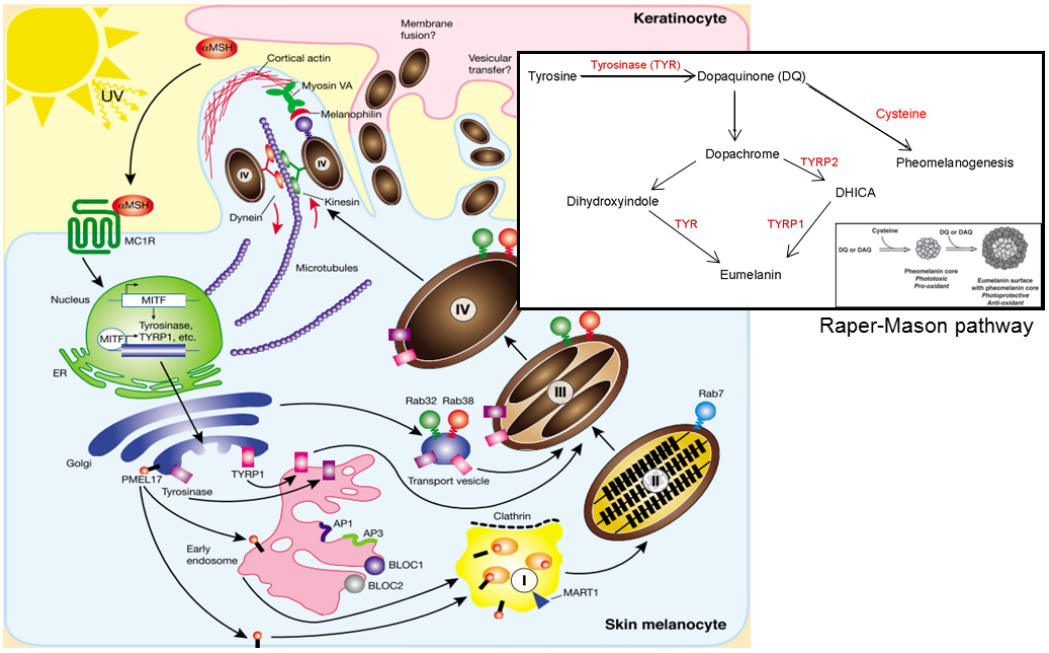


Figure 2-1 Melanogenesis is the result of complex interactions between melanocytes and their physiological environment. Melanin is synthesized within organelles called melanosomes. Critical enzymes and proteins essential for these reactions are regulated by their proper transcription, processing of precursors, and trafficking of proteins. Inset- The Raper-Mason pathway within the melanosomes results in the synthesis of eumelanin (blue/black). Pheomelanin (yellow/red melanin) is a consequence of the rapid degradation of dopaquinone (also a precursor to dopamine) and polymerization of the pheomelanin precursors in the presence of cysteine. (Figure reproduced from Ito and Wakamatsu 2008 and Wasmeier et al. 2008)

The regulation of melanosomal fate toward eumelanism or pheomelanism begins with the melanocyte membrane receptor encoded by the single exon gene, *melanocortin receptor 1* (*Mc1r*). MC1R, a seven transmembrane G protein-coupled receptor that is expressed in melanocytes and leukocytes, functions at a pivotal juncture in the pathway

as a switch regulating the type of pigment, eumelanin or pheomelanin to be produced (Barsh et al. 2000; Yang 2011). The receptor's natural agonist, alpha-melanocortin stimulating hormone (α-MSH), encoded by the gene *propiomelanocortin* (*Pomc*) that also encodes adrenocorticotropic hormone and beta endorphin, is produced by both the melanocyte and the adjacent keratinocytes so that it stimulates MC1R by both autocrine and paracrine signaling. Stimulation of MC1R by α-MSH activates the cyclic AMP secondary messenger pathway that in turn activates transcription of transcription factors that are required for production of melanogenesis enzymes. Basal levels of transcription factors like SOX10 and MITF are responsible for constitutive production of melanosomal enzymes that are required for eumelanin production and also for pheomelanin production (Kushimoto et al. 2003). Elevated cyclic AMP levels by α-MSH stimulation of MC1R results in increased eumelanin production due to increased availability of melanosomal enzymes and a consequent darkening in several species (Baião, Schreiber, and Parker 2007; J. A. Johnson, Ambers, and Burnham 2012). MC1R also interacts with a second ligand Agouti (ASIP), an inverse agonist, resulting in depressed activity of cyclic AMP and a consequent pheomelanic trait due to lowered transcription of the rate limiting and eumelanogenesis enzymes (Barsh et al. 1999). In dogs a mutation in the beta defensin protein was found to antagonize MC1R resulting in constitutive synthesis of eumelanin, the underlying basis of black allelomorph at the K locus (Candille et al. 2007).

Solute carrier proteins are involved in maintaining cellular and organellar homeostasis, through transport of proteins and their precursors, metal ions, amino acids, etc., consequently mutations in these genes can result in hypomorphs and varied levels of albinism in different vertebrates: occulocutaneous albinism 4 in humans, mouse underwhite, white tigers, sex-linked imperfect albinism in chicken and quail (Fredriksson

et al. 2008). Mature melanosomes, bearing melanins laid on a matrix derived from fragments of the PMEL protein, are transported to the periphery of the melanocyte by transporter proteins, like melanophilin (MLPH), RAB27A, and MYOVA, where the melanosomes are taken up by the developing feathers. Disruption of this transport mechanism can lead to different kinds of plumage/coat color dilution as observed in melanophilin mutants in chicken, quail, dog, mink etc. (Philipp et al. 2005; Vaez et al. 2008; Bed'hom et al. 2012; Cirera et al. 2013) and Myosin Va mutant in horse (Brooks et al. 2010)

Chapter 3 An expanded pigment-type switching regulatory network may explain deficiency of *Mc1r*-associated pheomelanic traits in birds

Abstract

Variation at the *Melanocortin 1 receptor* (*Mc1r*) has been shown in several mammalian and avian species including humans to be associated with darkening and reddening traits. Due to lack of known or limited pleiotropic effects this locus has been repeatedly mutated, sometimes altering the same amino-acid residues or transmembrane domains, resulting in similar phenotypes in different species. Recently two independent groups attempted unsuccessfully to map pigeon *Mc1r* coding variants responsible for eumelanic traits (darkening color variation) in other bird species to eumelanizing traits in domestic fancy pigeons (*Columba livia*). I undertook a linkage mapping approach to map variation at this locus to pigmentation traits and discovered a large frameshift deletion that occurs frequently among domestic and feral populations to be associated with the *smoky* (*sy*) phenotype. Contrary to expectation that inactivating mutations and hypomorphs of *Mc1r* result in pheomelanic (reddish) traits in mammals, *smoky* pigeons have a mild phenotype that affects dorso-ventral and wing pigmentation pattern, while lightening the beak, claws, and skin. *Smoky* pigeons do not have any red plumage except in some genetic contexts. Failure to detect this mutation may explain the lack of association and misleading results by previous workers. Based on these findings I conclude that avian *Mc1r* is not the sole pigment switch regulator, and deficiency of null and hypomorphic alleles of this gene among birds in general suggests that such variants have subtle pigmentation phenotypes and some fitness costs.

Background

Variation at the *Melanocortin 1 receptor* (*Mc1r*) has been shown in several mammalian and avian species to be associated with darkening (eumelanic) and reddening (pheomelanic) traits, for example red hair in humans (Beaumont et al. 2008). Among birds color polymorphism has been associated with *Mc1r* in several species, however, most characterized variants are melanizing with the exception of a null allele reported in the turkey, and a putative hypomorph in chicken (Kerje et al. 2003; Vidal, Viñas, and Pla 2010; Mundy 2005). There appears to be a deficiency of null and hypomorphic *Mc1r* alleles among birds, and both alleles in turkey and chicken are only marginally pheomelanic in contrast to similar class of alleles in mammals (Kerje et al. 2003; Vidal, Viñas, and Pla 2010; Beaumont et al. 2008). Further, several authors have concluded that *Mc1r* is not associated with color polymorphism in several bird species (MacDougall-Shackleton et al. 2003; Haas et al. 2009; Dobson, Schmidt, and Hughes 2012; Corso et al. 2013; Derelle et al. 2013). While the high degree of polymorphism at this locus in domesticates in general can be explained as either tolerance or selection for color polymorphism, recent work in domestic pigeons was unable to find traits associated with variants in this gene (Derelle et al. 2013). Quite perplexingly an independent study by Guernsey, et al. (2013) of *Mc1r* polymorphism in domestic pigeons concluded that a shared eumelanizing variant (Val85Met) is associated with pheomelanization (Guernsey et al. 2013; Baião, Schreiber, and Parker 2007; Mundy et al. 2004).

Melanocortin 1 receptor (*Mc1r*), a seven trans-membrane G-protein coupled receptor, is reported to function like a switch at a key position in melanogenesis from where it directs pigment synthesis toward eumelanin or pheomelanin (Mundy 2005; Barsh 2006; Walker and Gunn 2010). The receptor is expressed on melanocytes and

leukocytes and stimulated on melanocytes by ligands alpha-Msh and Asip (Yang 2011). Lack of any significant pleiotropic effects may explain the abundance of *Mc1r* variants affecting pigmentation that fall along a continuum of blue-black to red-yellow in several breeds of domestic mammals and birds and some polymorphic species (Figure 3-1, Hubbard, Uy, Hauber, Hoekstra, & Safran, 2010; Mundy, 2005).

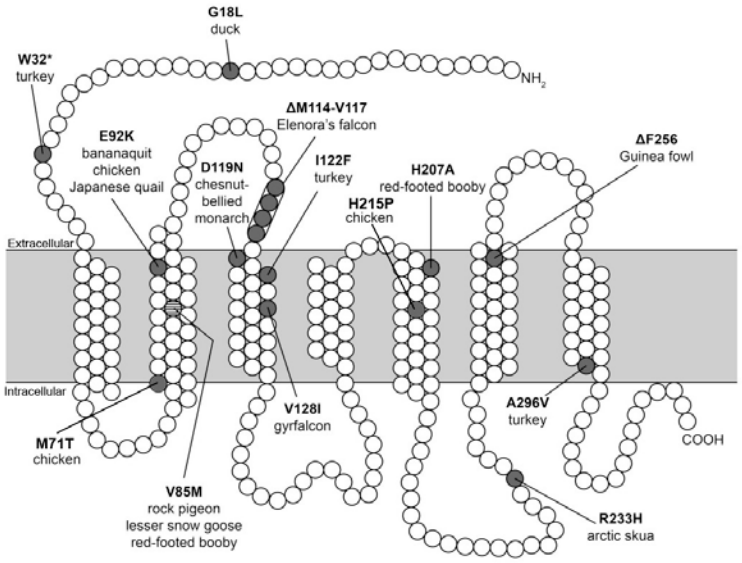


Figure 3-1 Most avian *Mc1r* variants have been mapped to eumelanizing traits with the exception of the chicken *buttercup* allele (haplotype: Thr71, Lys92, Pro215; Kerje et al., 2003) and the turkey *bronze* allele (haplotype: Ile122, Ala296; Vidal et al., 2010). [figure adapted from Mundy, 2005]

We used domestic pigeons for their magnitude of color diversity that has resulted from unconstrained selection by man, to map the genetic bases of melanin based pigmentation traits (Levi 1963; Sell 2012). Classical genetic work by pioneers in the early part of the 1900s and continued efforts by amateur geneticists has resulted in characterization of several complex pigmentation traits, their mode of inheritance, allelism

and interactions, which have enabled forward genetics approaches in domestic fancy pigeons (Domyan et al. 2014). Pigmentation traits in pigeons can be broadly classified as darkening, reddening, lightening, and pie-balding. In domestic pigeons we have a red and black trait labelled by fanciers as *recessive red* and *Spread* respectively. Although the similarity of the *recessive red* and *Spread* to phenotypes of hypomorphic and hypermorphic mutations, respectively of *Mc1r* in other species has prompted other researchers to test this association, so far any trait association to *Mc1r* has remained elusive [Derelle et al., 2013; Miller & Shapiro, 2011; & Dan Smith (unpublished)]. *Recessive red* (symbol *e*; Horlacher, 1930), is an autosomal recessive trait, reportedly epistatic to the *Spread* locus. The *Spread* locus (*S*), an autosomal dominant trait results in black coloration (Sell 2012). Following the similarities between *recessive red* and *Spread* with the *extension* loci of several mammals (all mapped to *Mc1r*), the pigeon *recessive red* (*e*) has been dubbed the *extension* locus and the *Spread* locus has been qualified as an extension or spreading/smearing of black pigment (Horlacher 1930). During the course of this study we mapped *recessive red* (*e*) to another gene (*Sox10*) that encodes a key melanogenesis transcription factor and is a primary target of *Mc1r* regulation (Domyan et al. 2014). However, Domyan, et al. (2014) did not formally rule out other genetic players like *Mc1r* from association with *recessive red*.

To map *Mc1r* variants to phenotypes I designed further experiments using a linkage mapping approach.

Methods

DNA samples:

Genomic DNA from EDTA preserved whole blood was extracted by phenol-chloroform extraction (Sambrook, Fritsch, and Maniatis 1989).

Genotyping and statistical analysis:

To characterize the *Mc1r* locus, a panel comprising *Spread* (SS^{-} E^{+}-; n = 3) and *recessive red* ($S^{-}S^{-}ee$; n = 4) and wild-type ($s^{+}s^{+}E^{+}$-; n = 1) was assembled. The chicken *Mc1r* mRNA sequence was BLASTed against the pigeon pre-release genome to identify the genomic scaffold and gene coordinates. Three amplicons, over-lapping end-to-end, were staggered across the 942 bp single exon gene and flanking non-coding sequence of *Mc1r* with the objective of Sanger sequencing (Figure 3-2). A redesigned amplicon spanning the 5' and middle amplicons was used for further genotyping, to screen parentals of several crosses, some of which segregate for one, both or neither *Spread* nor *recessive red*. All F_1 and F_2 were genotyped to test for departure from Mendelian segregation. Two families (referred to as Family1 and Family2) were selected for characterization of the *Mc1r* phenotype as these families segregate for the deletion allele. The *Mc1r* locus was Sanger sequenced in the sire and dam of both families. To understand the population genetics of *smoky* under natural conditions, two feral populations were surveyed.

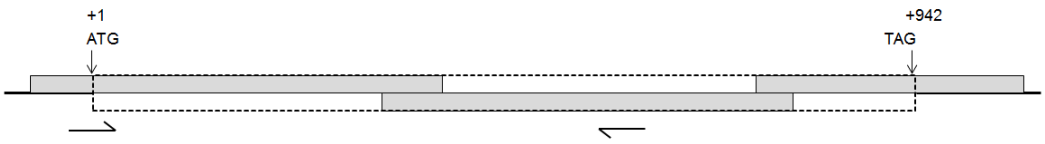


Figure 3-2 The *Mc1r* exon is shown with three amplicons spanning its length. Arrows indicate primers of the redesigned assay.

Genotyping was performed using polymerase chain reaction (PCR) standardized for 10 µL volume reactions, 0.1 µL *Taq* DNA polymerase, 0.5 µL left and right primers (40 µM) respectively, 5 µL *Failsafe* premix (EpiCentre), 2.9 µL H_2O, and 1 µL genomic DNA template. PCR cycling conditions were 96 °C – 3 min, (96 °C – 0.30 min, 61.7 °C – 0.30 min, 72 °C – 0.30 min) x 29, 72 °C – 6 min, hold at 4 °C.

Phenotyping:

All individuals were phenotyped and photo-documented under standardized light conditions. Photography was targeted to document bilateral profiles and dorsal and ventral views of both wings and tail of each adult bird. Final phenotype calls were made independently by two evaluators, blind to genotypes. *Spread*: Only genetically blue birds were used; these pigeons appear black when possessing at least one copy of the *Spread* allele. *Recessive red*: Pigeons that are red over the entire body except in piebald backgrounds (piebald: lack of pigmentation in patches); phenotype at *Spread* and *B-locus* (refer chapter 4) are masked by *recessive red*. *Smoky*: In phenotypically blue individuals, the wing pattern appears diffuse and smudgy, bill is depigmented, and under-wing and lateral tail feathers are pigmented. *Dirty*: Neonates have blackened bill, skin and scutes; adults bear similarities to *smoky* individuals, but distinguished by presence of pigmented bill and albescent tail strip (depigmented lateral tail feather). Pigeon eggs hatch in about 20 days. All neonates were photographed upon hatching to phenotype *smoky* and *Dirty*. Only late stage embryos that failed to hatch were sampled after day 20. Bill pigmentation was reliably diagnosed in late stage embryos.

Results

Early efforts with the objective of Sanger sequencing to investigate occurrence and association of variants at *Mc1r* with various color traits employed three amplicons staggered to span the single exon gene and flanking sequences. The PCR amplification of the 5' and middle amplicon of *Mc1r* failed in all *recessive red* birds (n = 4), but not individuals of other colors. The terminal 3' amplicon of *Mc1r* amplified successfully in all samples. Redesigning the PCR assay with primers distal to the external primers of the 5' and middle amplicons gave amplification in the *recessive red* birds, however the product was several hundred base-pairs shorter than expected, suggestive of an intragenic deletion (Figure 3-3A). Sanger sequencing identified a 500 bp deletion, corresponding to positions 5700250-5700749 of scaffold123 (Cliv1.0), beginning in the fourth codon of *Mc1r* that removes the next 167 codons and disrupts the reading frame, resulting in truncation of the first 3 ½ trans-membrane domains toward the N terminus of the predicted wild-type Mc1r protein (*Mc1r*$^{\Delta 500fs}$; Figure 3-3B). The deletion allele was found to segregate in a Mendelian fashion in several families (χ^2:= 9.441, p = 0.225, df = 7).

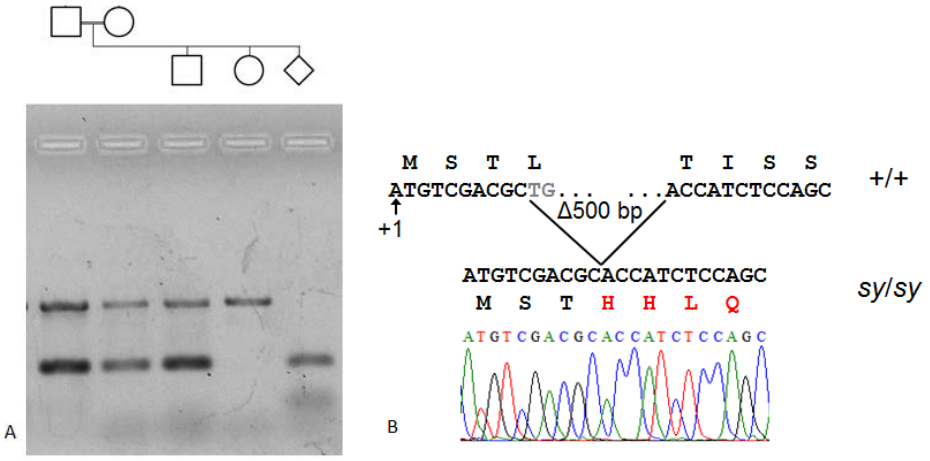


Figure 3-3 (A) A 500 bp (approximately) intragenic deletion was discovered in *Mc1r* in homozygosity, which segregates in a Mendelian fashion. (B) Sanger sequencing revealed the deletion to be a frameshifter of exactly 500 bp length.

Screening a larger panel (*recessive red* n = 8, wild-type n = 10, *Spread* n = 5) showed that this deletion was highly enriched in *recessive red* birds: it occurred in homozygosity in some wild-type individuals, and one *recessive red* individual was homozygous for the full length allele. Although statistically associated, the deletion was clearly neither necessary nor sufficient for *recessive red*. The color of *recessive red* birds is enriched when combined with other modifying factors, and so these birds are expected to be loaded with modifying genetic factors selected by fanciers for aesthetic reasons. Although the modifying factors are generally poorly characterized, one exception is *smoky* (symbol *sy*, autosomal recessive), which increases the color uniformity prized in *recessive red* pigeons. On an otherwise wild-type genetic background, *smoky* produces a complex constellation of phenotypic effects, including a darker hue to the light blue and bleached regions of the plumage, a lighter or washed-out appearance to the black wing pattern (described to appear as if viewed through a veil of smoke), a shift in shape/position of the black sub-terminal tail band to effectively extend the blue termini of the rectrices (tail feathers), and the appearance of albescent areas in the base of the primaries (Hollander 1937). In addition to affecting the plumage pigmentation, *smoky* also lightens the base of the bill, eye cere, and skin, features also desired by most breeders of exhibition *recessive red* pigeons (Hollander 1937; Hollander 1938). Although *smoky* affects the color of most parts of the bird, the effects are typically subtle, and easily overlooked by the casual observer. In addition, the combination of considerable variation in the expression of the trait, and the existence of other genetic factors with overlapping

phenotypic effects and/or uncharacterized epistatic interactions means that there is no single reliable diagnostic feature. However, with experience, *smoky* can be diagnosed reliably, particularly in cohorts with a common genetic background.

Screening the founders of crosses in our colony for the *Mc1r*$^{\Delta 500fs}$ deletion identified two families segregating for the deletion with potential to be highly informative for identifying phenotypic effects of the deletion via co-segregation. Both families were also segregating for *smoky*: in Family1 a 14-member sibship including some *smoky* was derived from unaffected parents, identifying the parents as carriers; in Family2, a large cohort of F_2 including several *smoky* was derived from a *smoky* male and wild-type female P_0 (Figure 3-4). As the genotypes of the founders were consistent with the *Mc1r*$^{\Delta 500fs}$ deletion causing *smoky*, we tested for co-segregation in both families under an autosomal recessive model. Phenotype calls made blind to genotypes by two independent observers (SK and JWF) were fully concordant, and genotyping revealed complete co-segregation between *Mc1r*$^{\Delta 500fs}$ and *smoky* in both families: All *smoky* birds were homozygous for the deletion, while all non-smoky individuals were either homozygous or heterozygous for the full length allele, and obligate carriers were heterozygous (Figure 3-4). The binomial probabilities of obtaining the observed complete co-segregation by chance are $p = 7.33 \times 10^{-5}$ for Family1 and $p = 7.8 \times 10^{-9}$ for Family2, with a combined significance of $p = 5.68 \times 10^{-13}$ (LOD = 12.24). The pigeon *smoky* genotype was, however, enriched of among eggs that failed to hatch; the binomial probability of exactly 7 homozygous *Mc1r*$^{\Delta 500fs/\Delta 500fs}$ out of 13 embryos is significant ($p = 0.018$). Survey of two feral domestic pigeon populations showed a significant departure of *smoky* from Hardy-Weinberg expectations (χ^2 18.166, $p = 0.0001$, $df = 2$, $n = 39$).

The alternate full length allele segregating in Family1 and Family2 are Val85 and Met85 respectively (Table 3-1). The Met85 variant was shown to underlie eumelanic plumage in red footed boobies and lesser snow geese (Baião, Schreiber, and Parker 2007; Mundy et al. 2004). In Family2, every non-*smoky* individual is an autosomal, dominant, eumelanic trait *Dirty* (*V*). .

Table 3-1 *Mc1r* coding haplotypes identified in Family1 and Family2.

	G253A	A279G	G343A	T354C	A520G
	Val85Met		Asp115Asn		Ser174Gly
C.livia (Val85)	G	A	G	T	G
C.livia (Met85)	A	G	G	T	A
C.livia (∆500fs)	-	-	-	-	A

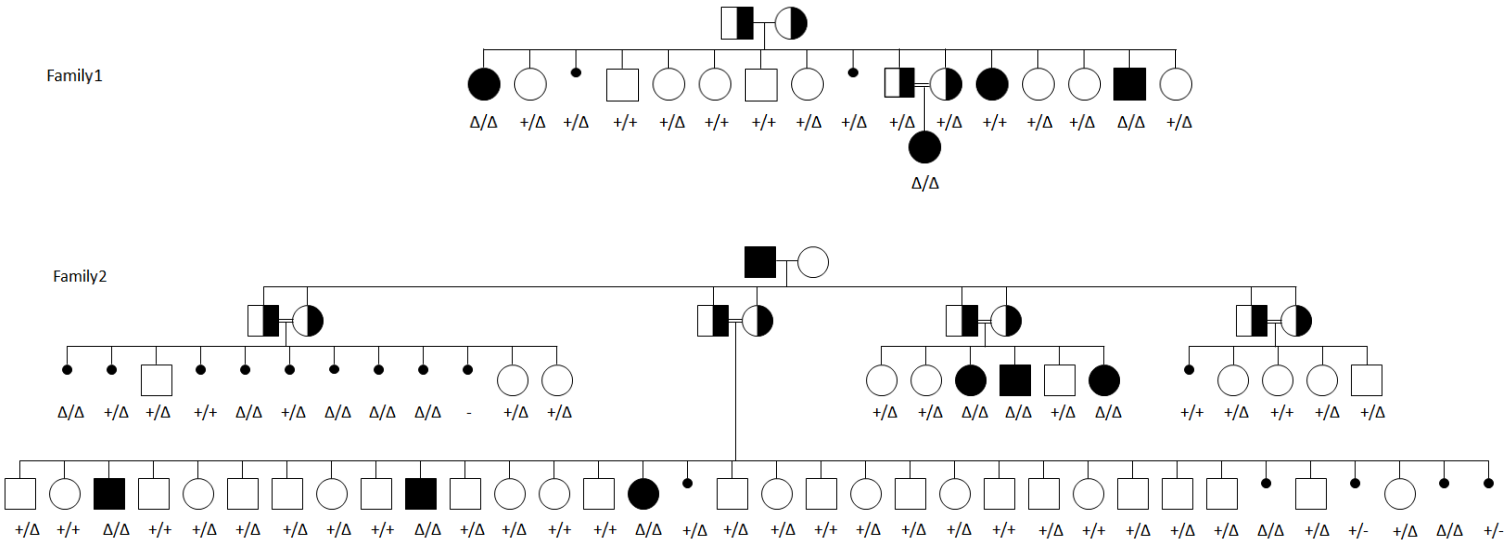


Figure 3-4 Two sibships were used in a linkage mapping approach to map the *Mc1r*$^{\Delta 500fs}$ to a trait. Family1 and Family2 segregate for the deletion allele and *smoky* (*sy*), a Mendelian, autosomal recessive trait. Family1 was founded by unaffected individuals and Family2 was founded by an affected sire and an unaffected dam. Only individuals that hatched were included in calculating the significance of cosegregation. Out of six *smoky* F_2 in Family2, one individual died prior to fledging, but was diagnosed based on neonatal *smoky* phenotype (distinct bill ring).

Trait descriptions:

Smoky (*sy*) (Bol 1920; Hollander 1937; Sell 2012): autosomal, recessive (in some genetic contexts expresses in heterozygosity); neonatal skin lightened, bill rostrally pigmented with distinct band; adult bill pigmented rostrally only, eye cere and skin lightened; overall plumage darkened, primaries and secondaries pigmented distally and on lateral vane only (medial portion of vane albescent); underwing coverts pigmented, not albescent; loss of definition and intensity of wing pattern; lateral rectrix albescent strip absent (pigmented), tail bar broader; epistatic to some piebald traits. *Smoky* intensifies or enriches black (*Spread, Blue*) and red (*recessive red*); non-*smoky*, *recessive red* ($Sy^{+}/-$; *e/e*) individuals have albescent strip; permits red-bronze (*bronze atlas*) in neck and shield plumage.

Dirty (*V*) (Mangile 1973; Sell 2012): autosomal, dominant, possible genetic heterogeneity; neonatal beak, skin, and feet darkened- diffuse black to intense black, feet lighten to wild-type in adult molts; adult cere and plumage darkened, beak and claws intense black; lower back darkened not albescent (continuous with tail color); underwing coverts pigmented, not albescent; well defined wing pattern; lateral rectrix albescent strip present or half tone (absent/pigmented in *Smoky*); white feathers in lower back/rump, or vent, or head (post-ciliary eye-tick).

Discussion

The canonical melanogenesis pathway describes *Mc1r* as a switch that discretely alternates melanin synthesis between eumelanin or pheomelanin. The majority of avian *Mc1r* variants have been shown to be eumelanizers with the exception of couple alleles in chicken and turkey that are only mildly pheomelanic (Kerje et al. 2003; Mundy 2005;

Vidal, Viñas, and Pla 2010). We have exploited the power of classical genetics to dissect pigmentations traits in domestic pigeons within homogenized genetic backgrounds, demonstrating 1) the first proof that *Mc1r* is conserved in function as a melanin switch among birds and mammals, 2) that the avian *Mc1r* is not the sole regulator of melanin type, and also 3) an explanation for the apparent deficiency of hypomorphs and loss of function mutations reported among birds for this gene. We predict that our findings may lead to reexamination of avian *Mc1r* variants that have been dismissed from phenotype associations and discovery of subtle phenotypes associated with this gene.

In domestic pigeons, *Mc1r* is not required for the eumelanic or pheomelanic extremes, *Spread* and *recessive red* respectively, and loss of *Mc1r* (*Mc1r*$^{\Delta 500fs}$) results in a surprisingly mild phenotype, scarcely distinguishable from wild-type in most genetic contexts (Figure 3-5 A & B). In Family2, however, sired by an Egyptian swift, a breed in which *smoky* is known to be fixed, the *smoky* phenotype perfectly cosegregates with red-bronze neck plumage (Figure 3-4, Figure 3-5 C & D). Sell (2013) describes *bronze atlas* (*ab*), a recessive modifier trait that he found to segregate in a *smoky* genetic background, which expresses pheomelanin in the neck (i.e. *Mc1r* loss of function permits pheomelanin synthesis in some contexts). Our approach and findings are significant in contrast to Vidal et al., (2010), because we had the *smoky* allele (*Mc1r*$^{\Delta 500fs}$) segregating in two independent crosses that are large enough for us to ascertain the complexity of the trait and the conserved pigment type switching function played by *Mc1r*.

A wild-type pigeon (*Sy*$^{+}$*Sy*$^{+}$) is light blue presumably due to synthesis of only eumelanin like the *Black* (*B*) turkey (Vidal et al. 2010). [A wild-type (blue) pigeon has eumelanin deposited in clumps along the length of the barbule, so that the optical effect

of the incident light results in the blue-gray hue and not black (Hollander 1938).] The darker intensity of the *smoky* plumage may be a consequence of increased pheomelanin deposition, distributed uniformly along barbules resulting in a net decrease in reflectance. The *smoky* (*sy*) phenotype has overlapping characteristics with the autosomal dominant *Dirty* (*V*) phenotype including darkening the bird and loss of counter-shading (loss of ventral wing albescence) (Hollander 1937). *Dirty* is reported to be a collection of autosomal dominant darkeners with few distinguishing characteristics. Loss of ventral wing albescence similar to loss of dorso-ventral dermal patterning in *Asip* mouse mutants suggests that the two traits (*smoky* and *Dirty*) may be close in the melanogenesis pathway or even allelic (*Asip* is a ligand of *Mc1r*). Both families used in our linkage mapping approach segregate for *Dirty* traits and for the Val85 and Met85 full length *Mc1r* alleles respectively (Table 1). Met85 has been associated with eumelanic traits in other species, and every non-*smoky* individual in Family2 is *Dirty* (Mundy et al. 2004; Baião, Schreiber, and Parker 2007). Recent workers on the pigeon *Mc1r* locus failed to find any eumelanic association with the Met85 variant, presumably because their approaches were unable to detect the 500 bp deletion allele (*smoky*), thus leading to conflation of deletion heterozygotes with homozygotes of the full length *Mc1r* alleles (Derelle et al. 2013; Guernsey et al. 2013). In addition, the Val85 and Met85 haplotypes in each family (this study) share a non-synonymous variant at residue 115 of the polypeptide, which is deleted (in-frame) in the Eleonora's falcon resulting in a eumelanic morph (Gangoso et al. 2011). The same transmembrane domain (Asp119Asn) is affected in island flycatchers resulting in melanic plumage (Uy et al. 2016). It is, therefore plausible that *Dirty* in Family1 is an allele of *smoky* or it is encoded by an upstream factor, and similarly *Dirty* in Family2 is a second melanizing allele (*Dirty*) caused by Met85 or Asp115 or an upstream factor.

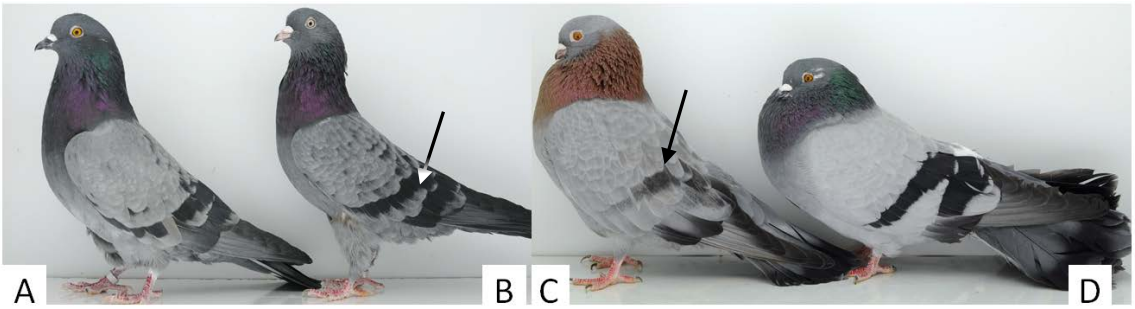


Figure 3-5 Non-*smoky* (A & D) and *smoky* (B & C) from Family1 and Family2. The *smoky* (B & C) individuals are distinguished in profile by the unpigmented bill (except at the tip) and smudgy or smoky appearance of wing bars (see arrow in B and C). In the non-*smoky* (A & D) the black bill, distinct wing bar boundary and white tail feather (albescent strip of lateral rectrix) are visible. Loss of *Mc1r* in some backgrounds as in Family2 (C) permits pheomelanin expression in the neck and wing shield plumage (*bronze atlas*).

Deficiency of null *Mc1r* alleles among birds and enrichment of the *smoky* genotype among unhatched embryos suggests lowered fitness effects from loss of this gene (Figure 3-4). However, our survey of feral pigeons suggests that *smoky* may be segregating in natural feral populations, albeit at a low frequency.

Chapter 4 Molecular characterization of sexually dichromatic traits in the domestic pigeon

Abstract

Birds have classic examples of exaggerated sexually dimorphic traits, including colors. The wild rock dove and its derived domestic breeds, however, are quite indistinguishable and sex can only be ascertained through genotyping or egg laying and successful hatching of eggs. Yet, the pigeon fancy has discovered sexually dimorphic traits and harnessed these traits in some auto-sexing breeds. Early genetics pioneers characterized and mapped four pigmentation traits to the Z chromosome. I used this positional information together with a candidate gene approach to map these traits to genes on the Z chromosome. I have shown that the pigeon *dilution* locus is mapped to the solute carrier gene *Slc45a2*, the same gene responsible for sex-linked albinism in chicken and quail. I have mapped the *B-locus* to *Tyrosinase related protein 1*, which is associated with brown-like traits in chicken, quail, and several mammals. The dominant *Ash-red* allele of the *B-locus* exhibits mild sexual dimorphism in the degree of plumage pigment variegation. Extreme sexual dimorphism is associated with alleles of the tightly linked *Stipper* locus (3 cM from the *B-locus*) and its alleles. These findings were published in Current Biology (Domyan et al., 2014). I have leveraged my mapping results for *dilution* and *B-locus* to map the *Stipper* locus to a large tandem duplication (77 kb) centered on the gene *Melanoma antigen recognized by T cells* (*Mlana*). Sexual dimorphism at this locus is a consequence of dosage and severity of the phenotype. The alleles of the *Stipper* locus are incompletely dominant, with heterozygotes of less severe alleles having similarities to wild-type, and those of more severe alleles approaching the homozygous mutant state.

Background

Although sexual dimorphism is prevalent throughout vertebrate and invertebrate animal taxa, it is perhaps best known among birds, which boast some of nature's most spectacular and iconic examples of sexually dimorphic ornamentation. Sexual dimorphism may evolve as a consequence of natural or sexual selection, or by neutral processes (Darwin 1874; Lande 1980; Székely, Reynolds, and Figuerola 2000; Allen, Zwaan, and Brakefield 2011). When dimorphism is sexually selected, the traits may serve as signals of availability or readiness to mate, health, genetic quality, etc. (Darwin 1874; Hamilton and Zuk 1982). Sexual dimorphism may be the result of sexually antagonistic natural selection on traits with contrasting fitness effects in males and females (Ellegren and Parsch 2007). Finally, sexual dimorphism may arise as a by-product of other processes, such as genetic sex determination and dosage compensation. These selective distinctions are dynamic and non-exclusive; sexual dimorphisms likely pass through multiple selective regimes; their origins are nearly always unknowable. To understand how sexually dimorphic traits arise and evolve, we need to study them at their earliest stages, something that is rarely possible. However, pigeons, which are sexually monomorphic in nature, have acquired de novo sexually dimorphic pigmentation traits under domestication, providing a unique opportunity to understand the genetic basis of their origins.

Classical genetic work in pigeons has identified four sex-linked color loci: *Stipper*, *B-locus*, *Dilution* and *Reduced*, two of which exhibit sexual dimorphic effects (Cole and Kelley 1919; Hawkins 1931; Hollander and Cole 1940). *Stipper* and *B-locus* are closely linked (~3 cM), and *Dilution* and *Reduced* are also closely linked (~5 cM), but the order of the members of these two tightly linked pairs with respect to the other pair are unknown

(Figure 4-1; Hollander, unpublished). Of these four loci, *Stipper* and *B-locus* alone are recognized for sexually dimorphic plumage pigmentation, and breeder selection has specifically targeted alleles of the former locus for their remarkable variegation and for auto-sexing purposes.

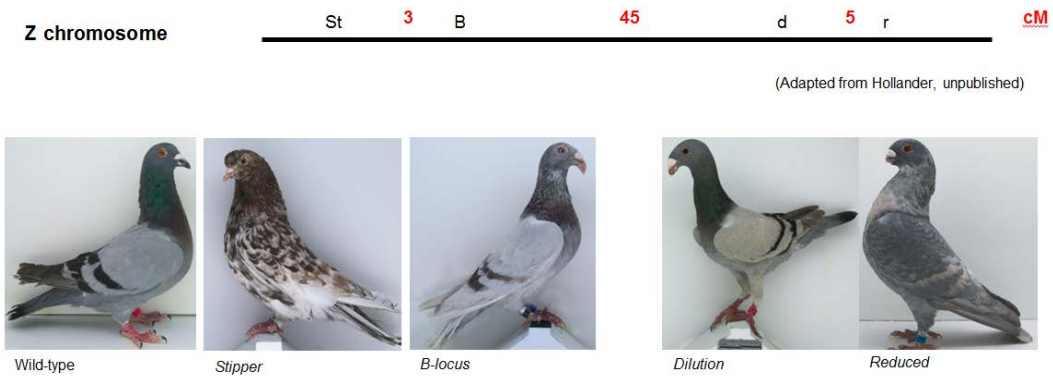


Figure 4-1 The pigeon Z chromosome has four color loci (top panel): *Stipper* (*St*), *B-locus* (*b*), *Dilution* (*d*), *Reduced* (*r*), depicted above the line and separated by the linkage distance in red. While these data are not published, pigeon fanciers report similar observations. The order of *St/B* and *d/r* is arbitrary, i.e. it is not known whether *B* or *St* is closer to *d/r* and similarly, whether *d* or *r* is closer to *St/B*. The panel below shows a representative allelomorph at each locus.

B-locus (*b*): The *B-locus*, also known as the *brown*, or informally as the base color locus or major color locus, harbors two phenotypically distinct traits, *Ash-red* and *brown* (Figure 4-2). Of the three alleles at the locus only *Ash-red* is sexually dimorphic. *Ash-red* is dominant to wild-type (commonly referred to as blue or blue/black), which is in turn dominant to *brown*. In *brown*, the black melanin is replaced with a brown form, so the black bars on the wing shield and tail become a chocolate brown and the light blue areas

become light brown. The irides of *brown* birds are nearly white instead of the orange eyes of wild-type, but differ from stark white irides of the autosomal *pearl eye* trait in having faint traces of yellow pigment. *Ash-red* is described as a reduction or modification of the black pigment (Hawkins, 1931). The color of Ash-red differs from wild-type in being ash-gray to lavender in the wing shield, brick red in the wing bar pattern, and albinistic in the tail bar (the boundaries of the tail bar are, however, discernable). Heterozygous *Ash-red* males develop flecks or variegation on their plumage that bear the color of the recessive alternate allele they harbor (Hollander, 1982). That is, males that are heterozygous *Ash-red* and wild-type develop wild-type (blue/black) flecks, while heterozygous *Ash-red/brown* males develop brown flecks (Figure 4-3). *Ash-red* hens on the other hand develop few subdued black flecks; this observation of eumelanic flecks in the hemigametic hens led early workers to conclude that *Ash-red* and *brown* were linked loci (Figure 4-3; Cole and Kelley, 1919; Hawkins, 1931). This sexual dimorphism in the hue of the flecks of males and females was noticed even in the time of Darwin, including the fact that flecks increase in size in males with every molt (Darwin, 1868).

Figure 4-2 *B-locus* phenotypes: left to right: *Ash-red*, wild-type (blue), and *brown* roller pigeons.

Figure 4-3 Variegation, characteristic of only the *Ash-red* allele at the *B-locus,* is usually discernible as flecks of darker hues on a light ash background. *Ash-red* hens develop few and small flecks in contrast to heterozygous cocks. In *Ash-red* hens ($B^A/\bullet$, hemizygotes) the flecks range in color from black (A) to dun (B) to brown (C). In heterozygous *Ash-red* males the flecks bear the color of the recessive allele at the locus, recur in the same part of the feather in subsequent molts, and expand in size. Here (D), an *Ash-red,* wild-type heterozygote (B^A/B^+), a tail feather has black flecks on one half and the opposite half is entirely flecked, including a tail bar.

Stipper (*St*): Mutations of the *Stipper* locus share some characteristics of *Ash-red*, including an overall lightening effect on the plumage, and variegation/flecking that increases with successive molts, and distinct phenotypic manifestations in the two sexes. However, variation at the *Stipper* locus is characterized by more extreme sexual dimorphism than seen for *Ash-red*, including male-specific lethality for some of the more severe alleles. The locus has seven recognized alleles of increasing severity and dominance that correspondingly exhibit a near-continuum of lightening and variegation. The namesake *Stipper* or *Almond* (*St*) allele sits at the top of the allelic series and in heterozygosity is prized among breeders for the rich colors produced in the flecks, particularly in combination with heterozygosity for a number of autosomal traits (Figure 4-4 A). Homozygotes lack normal natal down at hatching, and have eye defects, and are completely white if they survive to fledge. They typically die before reaching sexual maturity. Consequently, all *Almond* cocks used in breeding are heterozygotes (St/st^{+}). While anecdotal reports suggest that the eye deformities of homozygotes are due to deformed irides, Hollander (1982) reports that the lens is absent in such birds.

Heterozygotes and to a lesser degree the hemizygotes develop flecks or variegation, which is influenced by the genotype and gametic phase at the neighboring *B-locus*, as well as other loci, with distinct *cis*- vs. *trans*- allelic interactions in variegation phenotypes in males doubly heterozygous for *St* and *b* (described below), reminiscent of position-effect variegation seen in other systems (Hollander, 1982). In *Almond, B-locus* double heterozygotes, the flecks bear the color encoded by the dominant *B-locus* allele if the recessive *B-locus* allele is in phase with the *Almond* (*St*) allele. When this phase relationship is reversed, the flecks may bear colors of either *B-locus* allele. As with *Ash-red*, flecks reoccur generally in the same region on the feather and tend to increase in

size with successive molts. However, the abundance, size, and expansion of flecks is much greater in *Stipper*, for which flecks frequently encompass entire feathers. The variegation, large number of alleles and continuum of phenotypes together with the distinct *cis*- vs. *trans*- interaction with the *B-locus* are suggestive of some form of instability at the locus. The recognized alleles of *Stipper* in decreasing order of severity and dominance are *Almond* (*St*), *Sandy* (S^{Sa}), *Qualmond* (St^{Q}), *Hickory* (St^{H}), *Faded* (St^{F}), *Chalky* (St^{Ch}), and *Frosty* (St^{Fy}), with wild-type (st^{+}) at the bottom of the dominance hierarchy (Peter 2015). The degree of blanching in homozygotes is correlated with dominance, and while most alleles exhibit some variegation, only more severe alleles are associated with eye defects. Several of the intermediate alleles are reported to have arisen multiple times as independent and recurrent spontaneous mutations, and additional alleles intermediate to the recognized ones have been reported, but remain unconfirmed (Sell, 2013). Reports and data from breeders suggest frequent *de novo* origins of *Faded* and *Qualmond* alleles arising in stocks segregating for *Almond*.

A less severe allele, *Faded* (St^{F}), with no obvious health effects in homozygosity, was observed as a spontaneous mutation in 1933, by Dr. H. W. Feldman in Jan Metzelaar's laboratory colony at the University of Michigan. An independently originated *Faded* allele was identified by Wendell M. Levi in 1938 that was shown to be identical to Feldman's *Faded* by Hollander (Hollander, 1982). Dose-dependent sexual plumage pigmentation dimorphism of the *Faded* allele was demonstrated in 1940 and harnessed by breeders in the commercial squab production industry, resulting in fixation of this trait in a small number of utility breeds, in particular the Texan pioneer, as well as some strains of giant runts, show kings, and other utility breeds (Figure 4-4 C & D). The Texan pioneer is unique in that its date and origin are known (new breed admission 1962 NPA).

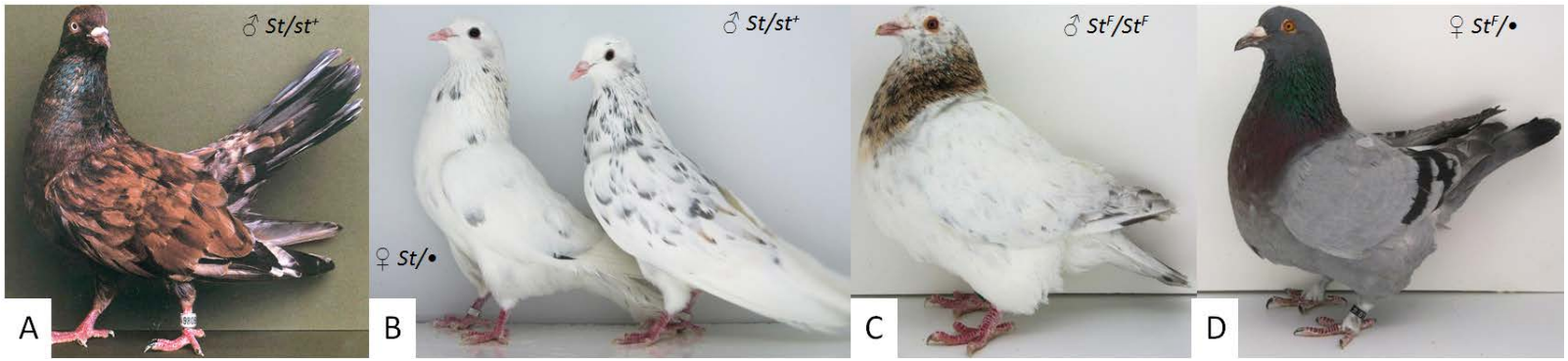


Figure 4-4 Effects of different alleles of the *Stipper* locus. **A**: an adult classic *Almond* male, fledges white, but pigmentation increases with age (Extraordinary Pigeons); **B**: *Almond* females (left, 5 month old) like *Ash-red* hens, develop less variegation than heterozygous males (right, 3 month old). The classic *Almond* phenotype (Figure 7A) in contrast to unrefined *Almond*s (Figure 7B) combines several loci, often in heterozygosity that enhance the richness of the variegation, for example heterozygous *Kite bronze* and *recessive red*. **C** & **D**: Texan pioneer is a squabbing breed (produced for meat), in which the *Faded* allele from a single source has been fixed. **C**: a male blue Texan pioneer appears nearly white (St^F/St^F); **D**: a female blue Texan pioneer appears nearly wild-type ($St^F/•$).

Dilution (*d*): This locus results in a uniform lightening of the color, including the color of the pattern areas (Figure 4-5). Alleles at this locus in decreasing order of dominance are wild-type (D^+), *pale* (d^p), *dilute* (*d*), and *extreme dilute* (d^{ex}). *Dilute* interacts additively with most other pigmentation traits. Alleles at this locus do not affect iris color, but do reduce the amount of natal down (Darwin, 1868; Cole and Kelley, 1919). Using inter-specific hybrids, *dilute* has been demonstrated to be allelic to the *blond* locus of ring-neck doves (*Streptopelia risoria,* Cole and Hollander, 1950). The existence of a more extreme allele at this locus in ring-neck doves prompted Hollander (1982) to predict the discovery of a homologous extreme mutant form of *dilute* in domestic pigeons. This prediction was subsequently confirmed, and at least three independent origins of *extreme dilute* have now been reported in domestic pigeons.

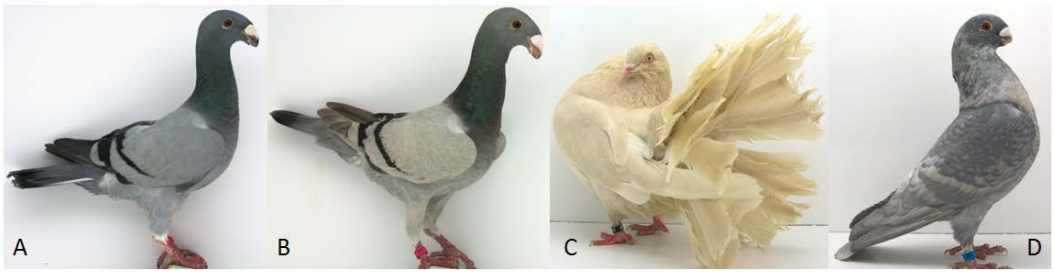


Figure 4-5 A: *Blue* German beauty homer (D^+/D^+ R^+/R^+); **B**: *Dilute, blue,* German beauty homer (*d/d* R^+/R^+); **C**: *Extreme dilute,* presumed *blue,* American fantail (d^{ex}/d^{ex} R^+/R^+)*;* **D**: *Reduced, blue*, Antwerp (D^+/D^+ *r/r*).

Reduced (*r*): Mutations of the *reduced* locus also result in lighter plumage, although unlike *dilute*, the effect is not uniform. Mutations of this locus lighten the neck and the mid-vane of the shield feathers, producing a lacing effect, the visual effect of which is accentuated when combined with other traits (Figure 4-5 D). In decreasing order

of dominance the alleles of this locus are wild-type (R^+), *reduced* (*r*) and *rubella* (r^{ru}). The *reduced* allele was first recognized in 1945 in a pair of pied Birmingham rollers in Ohio, and has subsequently been introgressed into many other breeds (Hollander, 1982). Reports from some fanciers, supported by observations in our lab, indicate that *reduced* hens produce brittle eggs, but no other pleiotropic effects have been reported.

I have used this positional information (Figure 4-1) and leveraged the population structure that has resulted during breed formation and through breeder's color introgression projects to map these sex-linked color loci to sex-linked genes and specific mutations.

Methods

Foundation stock was obtained from hobbyists to screen for marker-phenotype association; in addition *Almond* crosses were established to test co-segregation of markers with phenotype. Blood was drawn from the brachial or axillary vein and stored in ice in EDTA vacutainers, or blood was drawn into heparinized capillary tubes and plunged into 50% ethanol in 1.5 mL centrifuge tubes. All animals were photographed in a customized light box standardized for dimensions, illumination, focal length and field of view using a Nikon D5000 DSLR camera. Detailed interviews were conducted with breeders for each sample to ascertain the phenotypes, genotypes, and to assess confidence. Pedigree information including phenotypes/genotypes of first degree relatives, and information on size and diversity of loft, and breeding goals of the breeder were used to vet and inform decisions on sample ascertainment and confidence. Knowledge of the Z linkage group was used to target samples across breeds such that phase relationships could be deduced with the objective that mapping one locus will help

lead to nearby loci. All experiments involving animals were conducted in accordance with Institutional Animal and Use Committee guidelines (protocol #s A09.009 & A14.009; PI: JWF).

Genotyping was performed using polymerase chain reaction (PCR) standardized for 10 µL volume reactions, 0.1 µL *Taq* DNA polymerase, 0.5 µL left and right primers (40 µM) respectively, 5 µL *Failsafe* premix (EpiCentre), 2.9 µL H_2O, and 1 µL genomic DNA template. PCR cycling conditions were 96 °C – 3 min, (96 °C – 0.30 min, (65 – 55 °C) 0.30 min, 72 °C – 0.30 min) x 29, 72 °C – 6 min, hold at 4 °C, or touchdown protocol [96 °C – 3 min, {(96 °C – 0.30 min, 65-55 °C (-0.5 °C) – 0.30 min, 72 °C – 0.30 min) x 11}, {(96 °C – 0.30 min, 55 °C– 0.30 min, 72 °C – 0.30 min) x 30}] 72 °C – 6 min, hold at 4 °C].

The chicken genome has the most complete annotation (Ggal 3.0), and while the order of genes along the chromosome is variable, the karyotype is highly conserved in birds, and chromosome painting experiments show synteny between chicken Z and pigeon Z to be conserved (Derjusheva et al. 2004; Ellegren 2013). To map the *Stipper* locus I have employed a candidate gene association approach, selecting melanogenesis-related genes that are Z-linked in chicken (Gunnarsson et al. 2007; Nadeau et al. 2007; Ellegren 2013). *Tyrosinase related protein 1* (*Tyrp1*) at 30.6 Mb on the p arm of the chicken Z chromosome (82.3 Mb Ggal 4.0) stood out as the strongest candidate for the pigeon *B-locus* because inactivating mutations of this gene result in phenotypes similar to the pigeon *brown* in several vertebrates, including quail (Figure 4-6; Nadeau et al. 2007; Ren et al. 2011; Hirobe et al. 2014). Similarly, the solute carrier protein *Membrane associated transported protein* (*Slc45a2*), near the telomere on the p arm at 10.1 Mb

(82.3 Mb Ggal 4.0) stood out as a strong candidate for the *dilution* locus because it underlies sex-linked albinism in chicken and quail and in the chicken it is tightly linked to a chicken late feathering trait similar to the short-down phenotype of *dilute* pigeon neonates (Figure 4-7; Gunnarsson et al. 2007). Melanoma antigen recognized by T cells (*Mlana*) at 27.6 Mb, a melanoma antigen gene near *Tyrp1* was chosen for initial screening for coding variants in a discovery panel. Genes in the vicinity of *Tyrp1* that might plausibly account for one or more attributes of *Stipper* include *Tyrp1*, *Mlana*, *Nuclear factor I B* (*Nflb,* melanocyte stem cell homeostasis), *Lens epithelium derived growth factor* (*Ledgf*, maintenance and survival of lens epithelial cells), *Adapter protein complex 3B subunit 1* (*Ap3b1,* adaptor protein complex-*Tyrp1* transporter), *Cyclin dependent kinase inhibitor locus 2B and 2A* (*Cdkn2b, Cdkn2a*) (Nakamura et al. 2000; Pietro et al. 2006; Aydin and Beermann 2009; Dorshorst and Ashwell 2009; Hellström et al. 2010; Aydin et al. 2012; Chang et al. 2013).

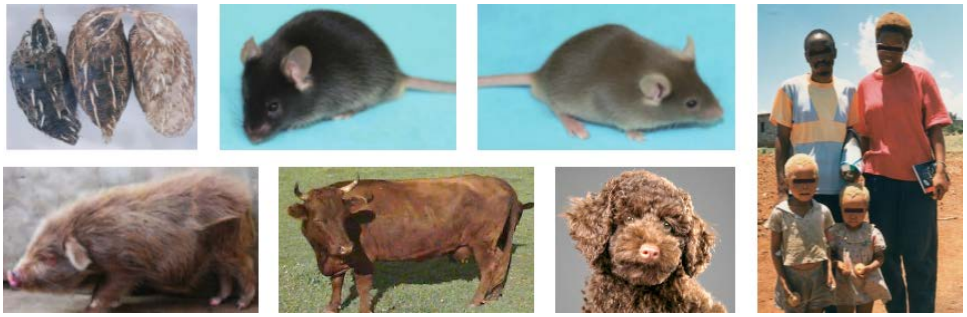


Figure 4-6 *Tyrp1* mutants in diverse vertebrates**.** clockwise from top left: Japanese quail wild-type, *brown*, and *roux* (Minvielle et al. 2000); mouse wild-type *black,* and *brown* (Hirobe et al. 2014); rufous brown albinism in humans (Manga et al. 1997); *brown* dog; dun Dexter cow; rufous brown Tibetan pig (Ren et al. 2011a).

Figure 4-7 Mutations in *Slc45a2* (left to right) result in sex-linked albinism in quail, sex-linked imperfect albinism in chicken (Gunnarsson et al. 2007); white Doberman pinscher (Winkler et al. 2014); albino Lhasa Apso (Wijesena and Schmutz 2014); white tigers (Xu et al. 2013); oculocutaneous albinism type 4 in humans (Newton et al. 2001).

Bioinformatics

Chicken candidate genes were BLASTed against the pigeon genomes to identify pigeon orthologs (Altschul et al. 1990). In the chicken Z chromosome (Ggal 4.0) *Tyrp1* lies at about 10 Mb from the metacentric centromere. To determine the location of the pigeon Z centromere, chicken centromeric PR1 repeat sequences from Genbank (Solovei et al. 1996; Shang et al. 2010) were BLASTed against the pigeon genome. Gene order and orientation on pigeon scaffold6 were manually screened and gene order at the termini of scaffold6 and flanking scaffolds were used to determine scaffold ordering by contrasting with the chicken and hoatzin genomes. Whole genome shotgun sequences from ~40 pigeons, primarily domestic pigeons, but also including two feral pigeons and one wild *C. rupestris* individual, became available in early 2013, and were used to help order scaffolds (Shapiro et al. 2013). Scaffold ordering was later verified using the chromosome bowser (Figure 4-8, Evolution Highway v1.0.6002), and WGS reads were extracted using Samtools and manually aligned to bridge across scaffold breakpoints. The WGS read depth data for the domestic pigeon reference and the resequenced genomes of several breeds of pigeons (Shapiro et al. 2013) was screened

for signatures of structural variation, example: lowered to zero reads compared to flanking regions revealed deletions, and elevated read depth in regions of duplications, and lack of alignment or improper mate pairing in regions of translocations and inversions. For all putative variants, variation between individuals was used to filter out mapping errors and other anomalies resulting from reference assembly errors and repetitive sequences. Read depth per base position 50 kb on either flank of each candidate gene was plotted and examined to identify structural variation in the resequenced genome panel. A second approach scanned read depth in 100 bp windows normalized to genome wide averages to detect large-scale copy number variation that could be missed in the preceding approach. Putative structural variation was verified by manually aligning reads (including unmapped reads) from the WGS data, and empirically confirmed by PCR and/or sequencing. Allele-specific tailed primers targeting breakpoints were designed for use in a three primer PCR assays for routine genotyping.

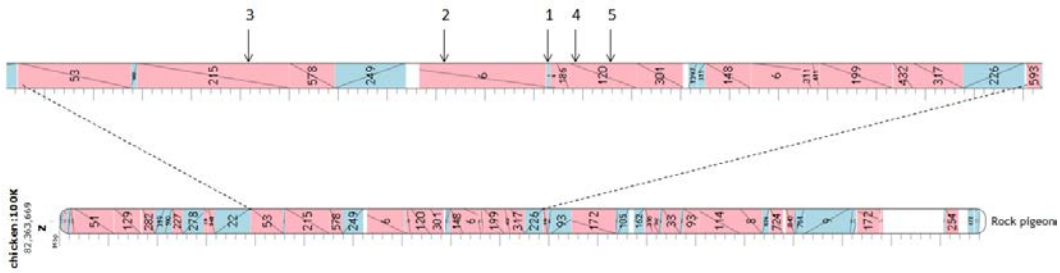


Figure 4-8 Pigeon scaffolds aligned to Chicken Z chromosome (Ggal 4.0), indicating scaffold orientation and relative scale using the interactive Evolution highway chromosome browser (v1.0.6002, http://evolutionhighway.ncsa.uiuc.edu/). Numbered arrows in the top panel represent relative positions of candidate genes for the *Stipper* locus: 1) *Tyrp1*, 2) *Mlana*, 3) *Ap3b1*, 4) *Nfib*, 5) *Ledgf*.

Association mapping

Once scaffolds were identified and genes annotated, primers were designed flanking the pigeon coding exons for PCR amplification and Sanger sequencing. All coding exons of *Tyrp1, Slc45a2,* and *Mlana* were Sanger sequenced in a cross-breed discovery panel of 11 pigeons of both sexes including alleles of *Stipper, B-locus,* and *dilution* in different phase combinations: wild-type, *Ash-red* (B^A), *brown* (*b*), *Almond* (*St*), *Qualmond* (St^Q), *reduced* (*r*), and *dilute* (*d*). *Tyrp1* was tested against the *B-locus* and *Slc45a2* was tested against the *dilution* locus. Due to its proximity to *Tyrp1, Mlana* was screened for polymorphisms that may be associated with *Stipper.* Electropherograms were examined manually to verify calls and zygosity. Single nucleotide polymorphisms (SNPs) were genotyped using Sanger sequencing or restriction fragment length polymorphism assays where applicable. To test genetic association and to rule out flanking genes and variants, SNP and indel data from the WGS resequenced genomes were leveraged, with the objective of breaking up and bounding associated haplotypes.

Mapping Stipper

The characteristics of *Stipper* are consistent with a model in which a chromosomal interval involving one or more genes, perhaps including *Tyrp1*, is genetically eliminated or epigenetically silenced to produce reversionary flecks. The same or similar mechanisms in the germ-line may be responsible for the large number of alleles and high apparent mutation rate. Genetic causes may involve transposable elements, centromeric repeats, structural rearrangements, and/or somatic recombination, among others, that may in turn be modulated by epigenetic processes. To mitigate the concern of genetic heterogeneity, features of pigeon population structure were leveraged to select samples for which genetic heterogeneity can be excluded (Figure 4-9). Each

approach seeks to tune to appropriate strata of population genetic hierarchy, to first bound the interval, and then to rapidly dial in the resolution, by working in families and defined populations.

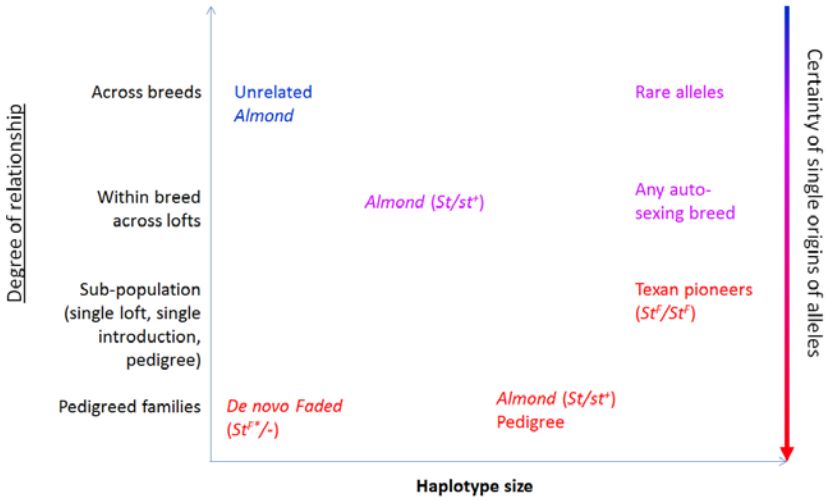


Figure 4-9 Effect of population structure and origins of *Stipper* alleles on haplotype size. Sampling blind to population structure is expected to yield smaller haplotypes in general, specifically for *Almond*, which can be maintained only in heterozygosity. However, due to the elevated potential of multiple independent origins of alleles, this approach is expected fail in unrelated *Almonds* and rare alleles. However, rare alleles and *Almond* within certain breeds (across lofts), and auto-sexing breeds offer greater confidence of single origins of the allele, but haplotype sizes will range from moderate to very large.

To bound the chromosomal interval of the *Stipper* locus relative to *B-locus*, markers (SNP and indel) were genotyped to exclude *Tyrp1* from association, and then walk out in either direction from *Tyrp1* by genotyping markers in samples that would allow

homozygosity mapping or cross-over detection. For example, an informative marker, i.e. heterozygous, at *B-locus* may cross-over proximate to the *Almond* allele at some low frequency, and this cross-over can be detected visually in pedigreed populations due to distinct *cis-*/*trans*- phenotypic effects. Alternatively, in breeds like the Texan pioneer, polymorphism around the *B-locus* is expected to yield to fixation for a single *Faded* haplotype at the *Stipper* locus. The Texan pioneer breed was used for homozygosity mapping among individuals from a single loft (Figure 4-9). Since there is a single origin of *Faded* in Texan pioneers, yet all three *B-locus* alleles occur in the breed, Texan pioneers will share the same, albeit probably large haplotype around *Faded*, but harbor variation at the *B-locus*. A nuclear family, sired by an *Almond* cock was used to test for cosegregation of markers and candidate mutations. Finally, to bound the *Stipper* associated haplotype markers were genotyped in *Stipper* individuals with the expectation that a recombinant haplotype will exclude flanking regions.

Copy-number assessment

A parsimonious model for the phenotypic continuum of the *Stipper* alleles leads one to predict continuous variation in the underlying mechanism, i.e. copy-number correlation with allele severity. To evaluate copy-number correlation with allele severity, the Applied Biosystems ® SYBR green I chemistry (Power SYBR and Fast SYBR) were employed on an ABI7300 instrument. Relative quantification of copy number was assessed using the $2^{-\Delta\Delta C_T}$ method, which contrasts PCR signal at the target or candidate region with an untreated control locus of known copy-number (Livak and Schmittgen 2001). This method controls for variation in template abundance and provides fold change vs exact copy numbers. In the context of amplicons selected for these assays, fold change represents copy number and can be treated as such. ΔC_T values for each

sample were normalized against those of the wild-type hen to estimate fold change in amplification efficiency. Two amplicons of 90 bp length were designed, placing one amplicon in the heart of the candidate region (*Mlana* exon 4) and the second amplicon in a region on the Z chromosome that was ascertained bioinformatically to be invariant. To ensure wild-type genotype (single copy of *Mlana* per chromosome), phenotypically wild-type hens were genotyped for absence of the 25 kb and 77 kb duplication. An ascertained wild-type hen was used for further experiments. To ascertain the reliability of this method a panel comprising a wild-type hen, *Almond* hen (*St*/•) and two unrelated Texan pioneer hens (St^{F}/•), was used. Follow-up experiments were designed to evaluate relative copy numbers of various alleles of the *Stipper* locus in males (homozygotes and heterozygotes) and females. Finally, to independently ascertain the relationship of copy-number and *Stipper* phenotype severity I assayed copy-number in an *Almond* (*St*/•) and de novo *Faded* (St^{F*}/•) racing homer hen derived from an *Almond* (*St*/st^{+}) by wild-type cross (st^{+}/•).

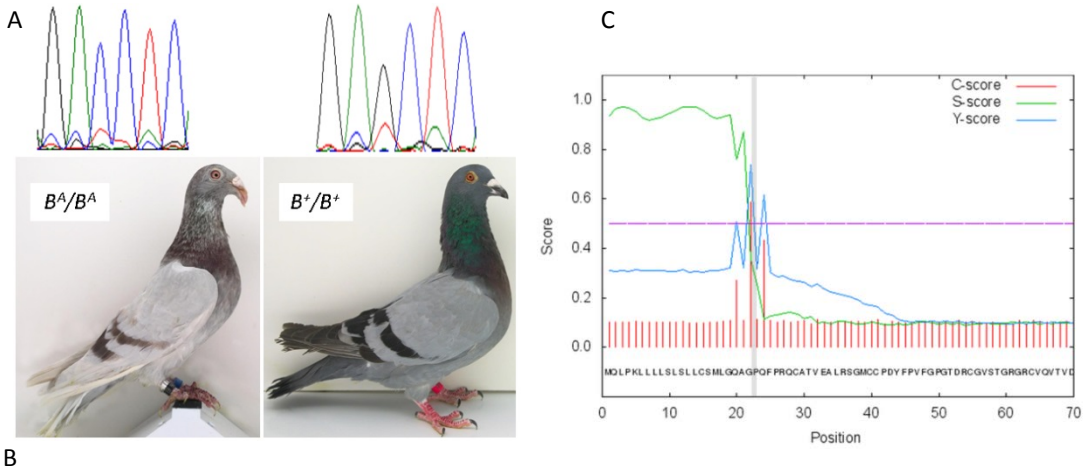
Quantitative PCR was performed using manufacturer's protocol for 20 µL volume reactions, 10 µL SYBR, 0.2 µL left and right primers (20 µM) respectively, 7.6 µL H_2O, and 2 µL genomic DNA template. Cycling conditions for Power SYBR and Fast SYBR on the ABI 7300 were 95 °C – 10 min, [(95 °C – 0.10 min, 60 °C – 0.30 min) x 40] and 95 °C – 0.23 min, [(95 °C – 0.10 min, 60 °C – 0.30 min) x 40] respectively.

Results

Mapping the B-locus – Tyrp1

Sanger sequencing of the candidate gene *Tyrp1* identified a transversion in exon1 resulting in a substitution of alanine at residue 23 to proline (c.G67C; p.Ala23Pro) only in *Ash-red* birds (Figure 4-10 A). An eight-base-pair frame-shifting deletion at position 411 of the cDNA in exon 2 was seen in the *brown* individual only (Figure 4-11).

Multiple species alignment with avian and mammalian orthologs showed alanine at position 23 to be completely conserved among mammals and birds (Figure 4-10 B). The Ala23Pro substitution is predicted to disrupt the signal peptide sequence that is essential for cleavage and proper entry of the mature protein into the secretory pathway (Nielsen et al. 1997; Petersen et al. 2011; Figure 4-10 C). Since a restriction endonuclease SacI cut site GAG'CTC is disrupted by the G67C substitution, a restriction fragment length polymorphism assay was designed to screen for the Ala23Pro variant. Primers were designed to amplify a 186-bp segment spanning the variant of interest and the amplicon was digested with SacI. Experiments were performed to optimize the restriction endonuclease assay and to ensure that heterozygotes could be distinguished from incomplete digestion, and repeated when ambiguous. To test for association of the Ala23Pro variant with *Ash-red*, a panel including 7 unrelated *Ash-red* and 49 non-*Ash-red* birds was screened, yielding a perfect association under a Z-linked dominant model (Fisher's exact test: $p = 4.3 \times 10^{-9}$). SNPs flanking *Tyrp1* were similarly genotyped walking outward from the Ala23Pro variant, and these data were combined with variant calls from sequenced genomes to define the minimum haplotype. All *Ash-Red* chromosomes tested possess the same haplotype over a ~1.7 Mb interval (Cliv0:scaffold6:157101-1.87 Mb) that spans *Tyrp1*, and does not include any other genes (Figure 4-12).



B

```
Domestic pigeon BA      MQLPKLLLL-SLSLLCSMLGQAGPQFPRQCATVEALRSGMCCPDYFP
Domestic pigeon B+      MQLPKLLLL-SLSLLCSMLGQAGAQFPRQCATVEALRSGMCCPDYFP
Hoatzin                 MQLPMLLLL-SLLLFLSMLGQAGAQFPRQCATVESLRSGMCCPDYFP
Tibetan ground-tit      MRIATLLYL-SLLMLLTMSGQAGAQFPRQCATVEALRSGMCCPDYFP
Medium ground finch     MRIPTLLHL-SLLLLLTMLGQVGAQFPRQCATVESLRSGMCCPDYFP
White-throated sparrow  MRIPTLLHL-SLPLLLTMLGRAGAQFPRQCATVESLRSGMCCPDYFP
Budgerigar              MQLPGLLLL-SLPPLLSLLSQAGAQFPRQCATVESLQSGMCCPDYFP
Collared flycatcher     MRMPALPCL-SLLLLLTAPGRAGAQFPRQCATVEALRSGVCCPDYFP
Chicken                 MQLPMLLLV-SLPLLLNMFKPAEAQFPRQCATIESLRSGMCCPDYFP
Turkey                  MQLPVLLFL-SLPLLLNIFEPAGAQFPRQCATIESLRSGKCCPDYFP
Dog                     MKAHKLLSLGSIFLFLLFFHQTWAQFPRECATVEALRNGVCCPDLSP
Human                   MSAPKLLSLGCIFFPLLLFQQARAQFPRQCATVEALRSGMCCPDLSP
```

Figure 4-10 A. Electropherograms (above) from exon1 *Tyrp1* showing the c.G67C (Ala23Pro) variant in *Ash-red* (B^A/B^A) and non-*Ash-red* (B^+/B^+) pigeons (below). **B**. TYRP1 multiple species amino-acid alignment. **C.** Signal peptide prediction shows that Ala23Pro residue coincides with the end of the predicted signal peptide.

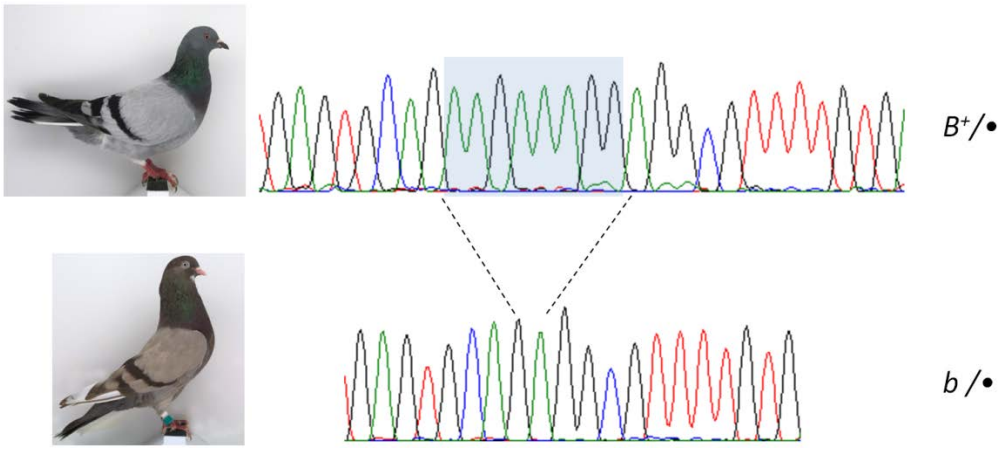


Figure 4-11 An eight base pair deletion in exon two of *Tyrp1* in a *brown* roller results in a frameshift.

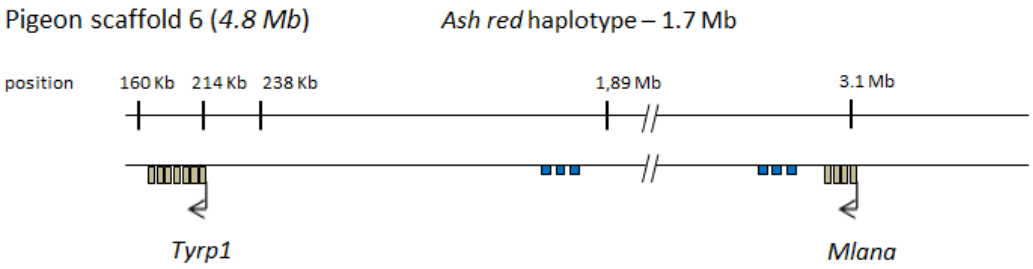


Figure 4-12 SNPs from the sequenced genomes were identified for testing by restriction enzyme assays to identify the minimum *Ash-red* haplotype and to exclude flanking genes. The *Ash-red* haplotype spans a distal regulatory element and centromeric repeats on the 5' end (Murisier, Guichard, and Beermann 2006; Shang et al. 2010). Centromeric repeats indicated by triplets of blue boxes are scattered 5’ of the gene up to the end of the scaffold.

Mapping the dilution *locus* – Slc45a2

The solute carrier protein encoded by *Slc45a2* also a seven exon gene was screened for coding variants in a discovery panel including three *dilute* birds (two cocks *d/d* R^+/R^+ and one hen *d/•* $R^+/•$), an obligate *dilute* heterozygote D^+/d R^+/R^+, a *reduced* hen (non-*dilute,* $D^+/•$ *r/•*), and two wild-type hens ($D^+/•$ $R^+/•$). Sanger sequencing all exons revealed a non-synonymous substitution in exon 4 (c.A1022G; p.His341Arg) that was in perfect association with *dilute* in the small sample of unrelated individuals examined (Figure 4-13; Fisher's exact test: p = 0.029).

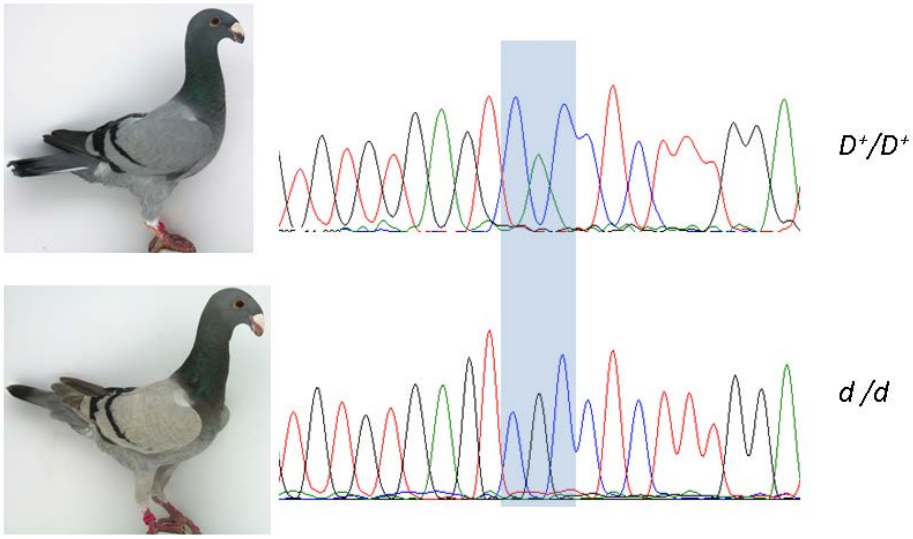


Figure 4-13 Wild-type non-*dilute* German beauty homer (above) and *dilute* German beauty homer (below) aligned against the electropherograms of *Slc45a2* showing the c.A1022G (His341Arg) in exon 4 that is associated with the *dilute* allele.

Mapping the Stipper *locus* – Mlana

Mlana, 2.9 Mb upstream from the transcription start site of *Tyrp1* on scaffold6 stood out as a strong candidate for *Stipper* that is reported to be tightly linked (~3 cM) to *B-locus*. Sanger sequencing results of *Mlana* showed no coding polymorphism within the discovery panel comprising several *Stipper* alleles.

The pigeon *Ash-red* haplotype spans the proximal 1.7 Mb of scaffold6 and includes centromeric repeats at its distal 5' end (Solovei, et al., 1996; Murisier, et al., 2014). The Z centromere sequences were scattered over pigeon scaffold6 approximately 3 Mb upstream from *Tyrp1* and in particular were found to be interrupted by CR-1 type non-LTR transposable element. Chromosome walking along scaffolds in Texan pioneer pigeons (St^F/St^F) excluded *Tyrp1,* and did not identify polymorphism 3' of *Tyrp1* for long stretches (3.2 Mb spanning scaffolds 120, and 301) suggestive of the predicted long runs of homozygosity expected around the *Faded* (St^F) haplotype. This result prompted investigation of the scaffold fragmentation in the region. WGS read alignment 3' of *Tyrp1* to bridge scaffolds 6 and 186 identified a CR1 element within an intron of the gene *Mpdz* that fragments the scaffolding. On the 5' of *Tyrp1,* the tested markers were not polymorphic at the end of Scaffold 6.

Examination of the mapped read depth of WGS genomes revealed two putative large copy number variants (CNVs) 5' of *Tyrp1* centered on *Mlana* (scaffold 6). A 25 kb tandem duplication, neatly encompassing *Mlana* and no other genes, occurred in ten individuals. This duplication was nested within a larger, 77 kb tandem duplication occurring in five of the ten libraries that possessed the 25 kb duplication. The 77 kb duplication appears to have occurred on the 25 kb duplication haplotype, with no

recombinant haplotypes possessing the 77 kb duplication without also possessing the 25 kb duplication among the WGS genomes. It is thus predicted that haplotypes with the 25 kb duplication possess at least two copies of *Mlana*, and those with the 77 kb duplication are expected to possess at least four copies of *Mlana* (per chromosome). Color phenotypes for the WGS sequenced individuals are not available, only breed names. Of the five breeds possessing both duplications, four (Birmingham roller, African owl, Egyptian swift, and Lahore) are known to occur in *Almond* varieties, while the fifth (ice pigeon) has a frosty pale appearance which could be in part the result of one the weaker *Stipper* alleles. Given its breed distribution, lower frequency, and greater apparent potential for various forms of instability, the 77 kb variant was chosen for testing for association with *Almond* and other alleles of *Stipper*. The distinguishing feature among these haplotypes and wild-type are the breakpoint spanning sequences. Reads for the region flanking the boundaries of the putative CNVs were identified by improper mapping orientation and large inferred insert sizes, and manually aligned to identify the breakpoints (see Appendix B for additional details). Breakpoint-specific amplicons were designed to empirically verify the CNV observed in the WGS data. A large CT-rich repeat $(TTTCCCTTTTCCTTCCTTTTCCCCC)_{\sim 25}$ at the right breakpoint of the 77 kb CNV allowed for only a presence/absence assay, and significantly limited the efficiency of genotyping . Nevertheless, in an association panel comprising 19 unrelated individuals from diverse breeds with several *Stipper* alleles (*Almond, Qualmond, Faded, Sandy, Chalky,* and *Frosty*) and 35 wild-type individuals, the 77 kb breakpoint spanning primers amplified in all *Stipper* mutants and failed to amplify in any of the wild-type individuals (Fisher's exact test: $p = 3.56 \times 10^{-15}$). Testing this breakpoint spanning amplicon in a nuclear family sired by an *Almond* cock, showed complete segregation with the *Almond*

phenotype (p = 0.015); however, a priori knowledge of tight linkage (3 cM) of *Stipper* to the *B-locus* makes complete cosegregation unsurprising.

The 77 kb duplication spans only *Mlana* and *Monocarboxylate transporter 2* (*Mct2*) in their entirety, as well as portions of two additional genes (see Appendix B for additional details). To bound the critical interval around the 77 kb duplication for association with *Stipper*, indel markers were genotyped at intervals walking away from the duplication breakpoints in *Stipper* mutant and wild-type individuals. Recombinant haploltypes were identified 3 kb from the left flank (Fisher's exact test: p = 0.011) and 44 kb from the right flank (p = 0.43) of the 77 kb duplication (Figure 4-15).

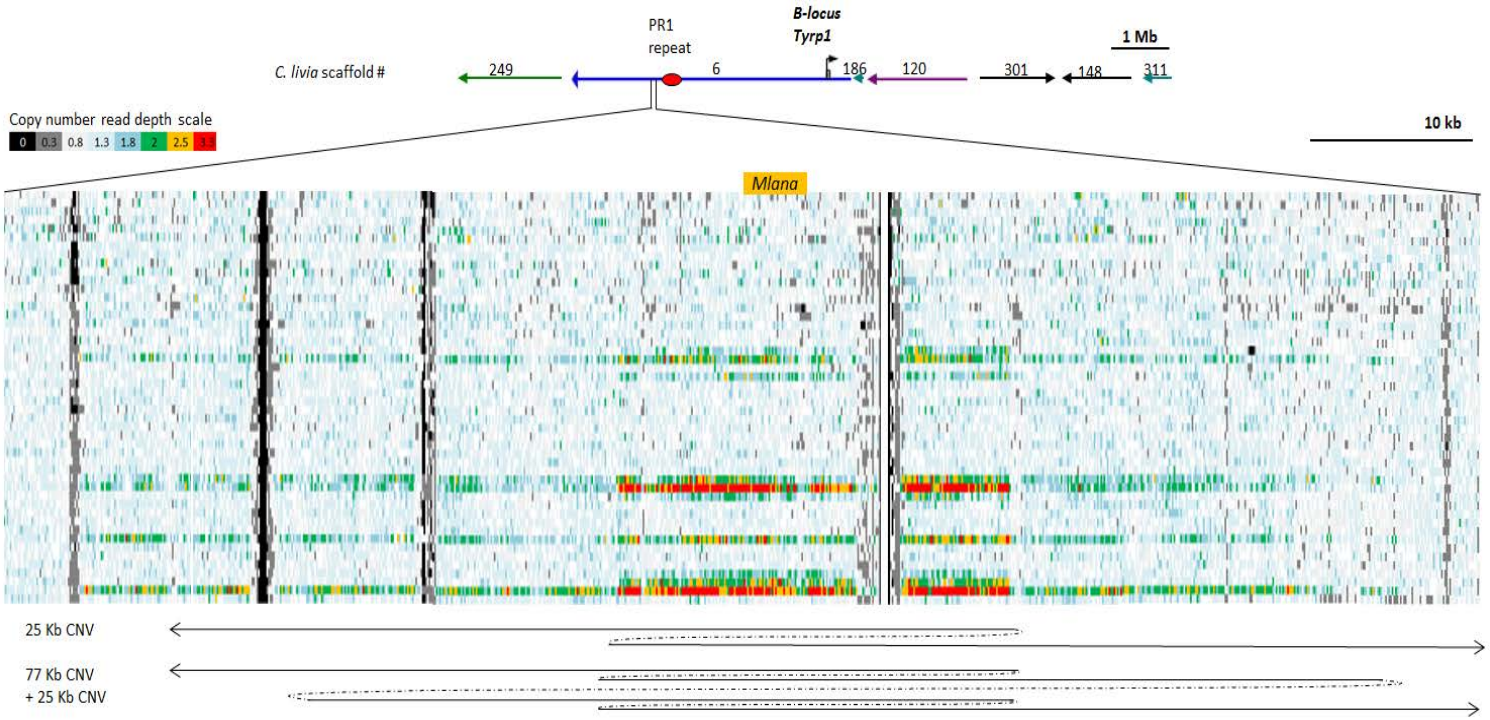


Figure 4-14 Elevated read depth reveals nested serial amplifications of Mlana. Scaffolds in the vicinity of *Tyrp1* were examined for variation in read depth in 100 bp sliding windows, normalized to library-specific genome-wide averages. Elevated read depth spanning 25 kb centered over *Mlana* was observed for ten out of 40 WGS libraries. Five of these ten libraries had further increase in read depth also centered on *Mlana,* but spanning 77 kb. Scaffold 6 – 4.8 Mb, *Mlana* coordinates – 3,173,417-3,178,870, centromeric repeats on scaffold6 are labelled PR1 repeat.

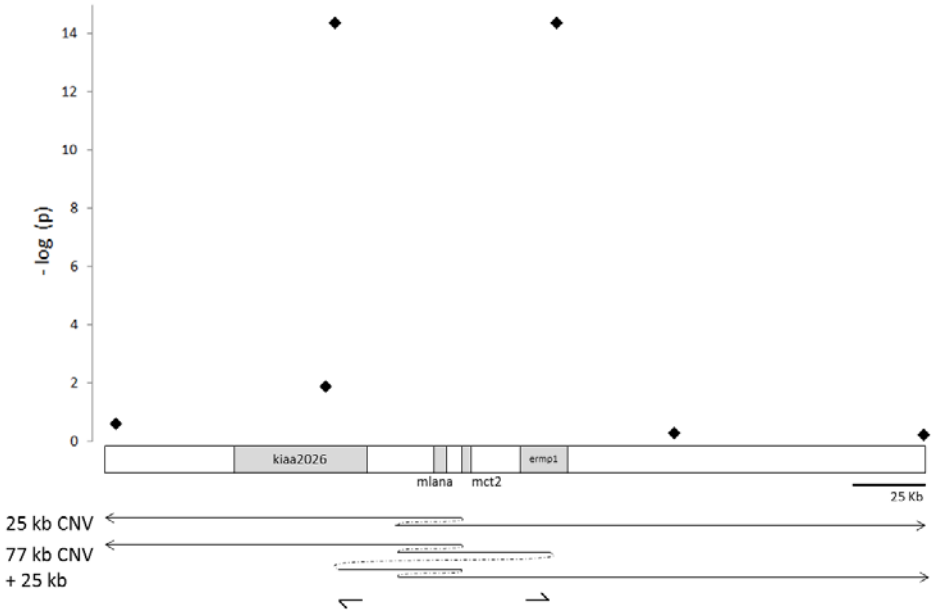


Figure 4-15 Bounding the associated critical interval for *Stipper.*

The significance of the association of markers in the vicinity of *Mlana* with *Stipper* is plotted as –log (p). The two high-significance points represent the breakpoint-spanning amplicon marker (primers indicated by arrows). Schematic below illustrates the structure of the 25 kb and nested 77 kb + 25 kb CNVs.

Copy-number evaluation

Validation of the copy-number assay was based on the premise that the *Faded* allele of Texan pioneer pigeons will have a narrow range of variation due to their known single origin. All Texan pioneer hens (n = 2) tested had seven-fold amplification of *Mlana* in contrast to 13-fold and 15-fold amplification in two unrelated *Almond* hens, relative to an ascertained wild-type hen (Figure 4-16). Copy-number of *Mlana* per chromosome varies from three to 15 in *Almonds* and three to seven in *Faded* (assuming their wild-type chromosome bears a single copy *Mlana* in heterozygotes, and both chromosomes bear

equal numbers of *Mlana* in homozygotes). Copy-number of *Mlana* among unrelated cocks homozygous for *Frosty, Chalky, Faded,* and *Sandy* increases in an apparent linear fashion, however only a single specimen was available for these alleles. The de novo *Faded* hen had a single copy of *Mlana* in contrast to 15 copies in its *Almond* sister (Figure 4-17).

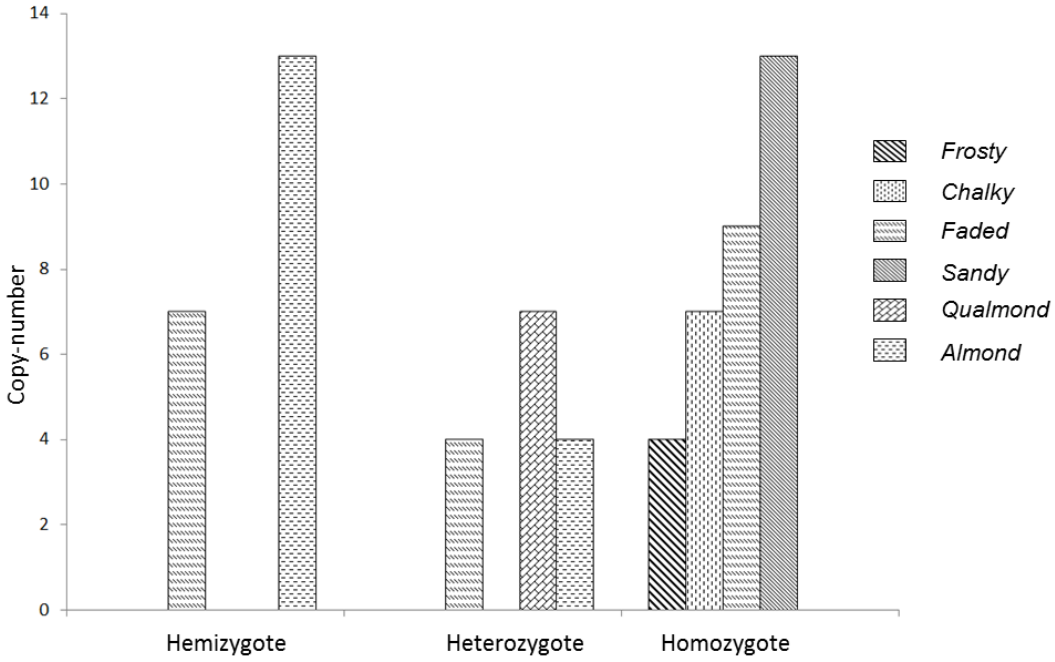


Figure 4-16 Copy-number assay: fold-change in amplification efficiency using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

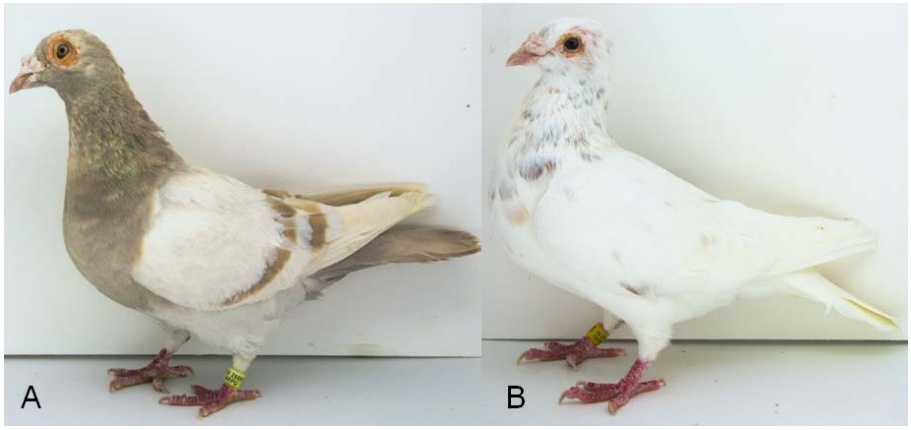


Figure 4-17 Pigeon fanciers report that *Almond* alleles give rise to alleles of lesser severity like *Qualmond* and *Faded* at a frequency of one in hundred meiosis. **A**: de novo *Faded* hen (St^{F*}/•), **B**: Almond sister (*St*/•).

Discussion

Pigeons, which are sexually monomorphic in nature, have acquired de novo sexually dimorphic pigmentation under domestication, providing a unique opportunity to understand its genetic basis. These dimorphic traits were observed by early pioneers including Darwin, and are reported to have been harnessed even in ancient breeds like the Reehani dewlap to discriminate sexes (Darwin 1868; Levi 1963). Four sex-linked pigmentation traits were recognized by early geneticists and the sexually dimorphic expression was recognized for two of the four loci.

The positional information of the four sex-linked loci together with advances in our understanding of the melanogenesis pathway and availability of the well annotated chicken genome limited the genome-wide pool of candidate genes to a single contrast for the *dilution* and the *B-locus* locus on the Z chromosome. The small sample association of the *dilution* allele with the His341Arg variant in exon four of the solute carrier protein *Slc45a2* was supported by an expanded independent sample by our collaborators that yielded a strong association ($p = 2.2 \times 10^{-16}$; Domyan et al. 2014). Variants in *Slc45a2* in multiple vertebrate species result in similar hypopigmentation phenotypes, including occulo-cutaneous albinism type 4 in humans, white tigers, sex-linked albinism in chicken and quail, and albino dogs (Newton et al. 2001; Gunnarsson et al. 2007; Dooley et al. 2013; Xu et al. 2013; Wijesena and Schmutz 2014), all presumably resulting from faulty trafficking of melanosomal proteins, like Tyrosinase (TYR) and TYRP1, and abnormal environment within the melanocyte organelles including the melanosomes, endoplasmic reticulum, and the trans-Golgi network that affects the maturation and function of these proteins (Brilliant 2001; Watabe et al. 2004; Dooley et al. 2013).

The cross-population association approach for the *B-locus* revealed a single causative mutation in *Tyrp1* that underlies the *Ash-red* phenotype; the occurrence of this mutation within a common haplotype in diverse breeds indicates a single origin. The introgression of this allele in a wide variety of breeds as consequence of breeders' fancies is facilitated by its dominant mode of inheritance.

The *Ash-red* phenotype results from predicted inefficient proteolytic cleavage and maturation of the TYRP1 protein that was later demonstrated by Domyan et al. (2014). Missense mutations in TYRP1 are expected to result in endoplasmic reticulum (ER) toxicity and limited interaction with Tyrosinase (TYR), thus inhibiting maturation and promoting degradation of the latter (Toyofuku et al. 2001; Kobayashi and Hearing 2007). In the mouse *Tyrp1*^{light} mutant, mutation of the amino acid residue flanking the pigeon Ala23 similarly disrupts signal peptide cleavage and transport of the mature protein, resulting in distally pigmented and proximally bleached pelage (Johnson and Jackson 1992). The bleaching effect in these mice is attributed to cytotoxic effects stemming from ER retention toxicity and disruption of melanosome integrity (Johnson and Jackson 1992). The bleached phenotype of the *Ash-red* allele may similarly result from cytotoxicity and deficiency of TYR.

Unlike the *Ash-red* allele, *brown* is a loss of function of *Tyrp1.* Unsurprisingly, multiple independent *brown* alleles have been identified as there are many ways to inactivate a gene; Domyan, et al. (2014) identified two additional *brown* alleles: a nonsense mutation in exon one and a frameshift deletion in exon three of the same gene. TYRP1 is required to temper the toxic metabolic byproducts of Tyrosinase-catalyzed melanogenesis in melanosomes. While missense mutations in TYRP1 inhibit maturation

of and limit availability of TYR (Toyofuku et al. 2001; Rad et al. 2004) , lack of TYRP1 does not inhibit eumelanin synthesis by limiting the availability of TYR.

Given the reported 3 cM distance of the *Stipper* locus from *B-locus* and similarities between the *Ash-red* phenotype and *Stipper* alleles, a formal exclusion of *Tyrp1* was required. I have shown here that the 77 kb tandem duplication centered on *Mlana* is in complete association with alleles of the *Stipper* locus ($p = 5.56 \times 10^{-15}$). The associated haplotype extends at most 3 kb from the left end of the duplication (i.e. toward *Tyrp1*) and 44 kb from the right end of the duplication. The 25 kb tandem duplication spans only *Mlana*, whereas the 77 kb tandem duplication also spans *Monocarboxylate transporter 2* (*Mct2* or *Slc16a7*) and portions of KIAA2026 and *Endoplasmic reticulum metallopeptidase 1.* MCT proteins are a poorly understood family of solute carriers that transport monocarboxylates such as pyruvate, lactate, and ketones across cell membranes for carbohydrate, lipid, and amino acid metabolism (Halestrap and Wilson 2012). *Mct2* has different expression profiles in the few species in which it has been studied and is not reported to be expressed in melanocytes (Halestrap and Wilson 2012). *Mlana* is a melanoma, melanocyte and retinal pigment epithelial cell marker (Coulie, Brichard, and Pel 1994; Aydin and Beermann 2009). Until recently the role of this gene in melanogenesis was unknown, but a knock-out mouse model has been used to demonstrate depigmentation phenotype resulting from loss of this gene (Aydin et al. 2012). The MLANA protein plays a key role in maturation of Premelanosome protein (PMEL), a melanosomal matrix protein, upon which the melanin pigments are deposited (Hoashi et al. 2005).

The 77 kb genetic lesion with *Mlana* at its center is the seed for the allelic continuum at *Stipper* locus. A parsimonious model of CNV expansion and copy-number correlation with allele severeity appears to hold among homozygotes of some *Stipper* alleles (Figure 4-16). However, lack of a clear copy-number correlation with *Stipper* allele severity among heterozygotes may be a consequence of phenotype ascertainment bias and genetic heterogeneity. Furthermore, *Mlana* copy-number in the de novo *Faded* hen is comparable to that of wild-type levels and 15 fold less than that of her sister. All of these observations together suggest that manifestation of *Stipper* allele phenotype/severity is likely to be regulated by non-genetic means.

Models to explain attributes of Stipper *mutants*

The chief sexually dimorphic characteristic at *Stipper* is the degree of plumage blanching and the degree of variegation between the sexes. As homozygous males have more severe phenotypes than heterozygotes and hemizygotes, this sexual dimorphism is attributed to dosage effects (Sell 2012). Pigeon fanciers report repeated de novo origins of less severe alleles, *Qualmond* and *Faded* from *Almond* stock at a frequency of roughly one in a hundred meioses. Several non-exclusive models account for multiple features of this locus. Simple dosage effects could manifest in different character states in each sex because in birds, unlike in mammals, global silencing of one sex chromosome in the homogametic sex is not known to occur (Lyon 1962; Teranishi et al. 2001). The extent and mechanisms of avian dosage compensation are still unclear, and are areas of active research, but recent results in chickens indicate that a chromosomal interval flanking *Dmrt1* (candidate avian sex determination locus) is hyper-methylated in males and hypo-methylated in females by the long non-coding RNA of the *Male hyper-methylation* locus (*MHM*) (Teranishi et al. 2001; Shang et al. 2010; Caetano et al. 2014; Graves 2014). This

mode of regional dosage compensation found in chickens does not appear to be found in all birds, as demonstrated by the lack of the *MHM* locus in zebra finches (Itoh et al. 2010).

The continuous nature of the plumage lightening phenotypes of various *Stipper* alleles is suggestive of dosage modulation by genetic or epigenetic means. The bioinformatic data leads one to expect a chromosome bearing a single 77 kb duplication to have four copies of *Mlana*. Similar to the bleached phenotype of *Ash-red* that is proposed to result from cytotoxic effects, amplification of *Mlana* could also affect melanocyte viability in a variety of ways. One intriguing possibility is raised by the fact that MLANA is a melanoma specific antigen recognized by cytotoxic T lymphocytes (CTL). It is thus conceivable that over-expression of *Mlana* triggers an auto-immune response resulting in immune-mediated depletion of melanocytes. Sufficient exposure to CTLs, which is known to select for cells that do not express the antigens, may relieve melanocyte depletion and restore melanogenesis in the pigmented flecks of feathers (Jäger et al. 1996).

An alternate model that I propose considers the requirement of the MLANA protein to participate in the maturation of the PMEL. In dogs, a SINE insertion in the terminal intron of *Pmel* is associated with the merle trait, which is a patchwork of wild-type and depigmented patches (Clark et al. 2006). Similarly loss of function mutations of *Pmel* in chicken, horse, and cattle result in varying degrees of hypopigmentation (Kerje et al. 2004; Brunberg et al. 2006). If the native state of the 77 kb *Mlana* tandem duplications in the germline is epigenetically silenced, then copy-number of *Mlana* may not be crucial to the phenotype as the data seem to suggest. In this scenario, silencing of *Mlana* can

have downstream effects on PMEL and melanosome morphology, thus resulting in the bleached phenotype, such that loss of silencing in the germline and soma results in de novo lower alleles and variegation (Aydin et al. 2012).

Other models: The long stretch of tandemly repeated homologous sequence is ripe for illegitimate recombination, and can also result in expansion or contraction of the motif in the germline, similar to well-known examples in dogs and humans (Fondon and Garner 2004; Choate et al. 2010; Gemayel et al. 2010). If such recombination occurred in the soma, it could adequately explain the observed somatic mosaicism and *cis*- vs. *trans*-allelic interactions between the *Stipper* and *B-locus.* A simple model of repeat expansion and contraction can however, entail a runaway process that can cause breakage of the chromosome. A “fragile Z” model might explain the variegation, but may not be adequate on its own to explain the *cis*- vs. *trans*- allelic interaction between alleles of the *Stipper* and *B-locus* in the soma unless *Tyrp1* is centromeric to *Mlana*. In this scenario, a Z-chromosomal fracture in melanocytes seeded by the 77 kb repeat expansion could spare the *Tyrp1* allele in phase or include it, thus accommodating the observed cis- trans- allelic interactions between the two loci. Further, this model can be untenable for germline instability due to developmental failures that could result from haplo-insufficiency. It is, however, conceivable that a combination of CNV expansion and somatic recombination can underlie the basis of allelic continua, de novo origins of alleles in pedigrees, and variegation.

Finally, variegation in the *Stipper* phenotypes is reminiscent of position effect variegation (PEV) in *Drosophila* (Talbert and Henikoff 2006). The genetic lesion caused by the *Mlana* centered duplications could be subject to or could itself trigger

heterochromatin spread from the near-by centromere, similar to the models demonstrated in the *Drosophila white* locus and the mouse *Agouti hypervariable* allele (Slotkin and Martienssen 2007).

Chapter 5 Conclusion

Repeated evolution of similar traits in multiple vertebrates, and here in domestic pigeon breeds, due to variation at the same nodes of the melanogenesis pathway is support for the conserved nature of the genetic tool kit. This is the same logic that has led several studies of natural populations to examine the few well understood genetic pathways and nodes therein. However, the number of such nodes that can be studied in natural populations is limited lack of polymorphism or by inability to conduct rigorous genetic studies. This limitation may never be overcome, but in domestic pigeons, we have the opportunity to better understand the genetic architecture and basis of gross morphological, behavioral, physiological, and color diversity.

In this volume I have mapped only a handful of pigmentation loci which together with several other Mendelian and non-Mendelian traits regulate pigment type and distribution on the feather. In addition to the traits mapped in this volume, we have mapped several other Mendelian traits including *albino, pink-eyed dilute,* and *Baldhead* all of which resemble traits occurring in other species, including the baldhead trait of the bald eagle. By accumulating such mapping results we are able to dissect pigmentation pathways and also have more powerful controls in future experiments in order to map complex traits, relating to body dimensions, behavior, physiology etc. Finally, these studies can reveal novel mechanisms unique to pigeons and to birds in general.

Appendix A

Ash-red association data

Association data for the C67G (scaffold 6-214991) – *Ash-red* associated variant and genotyping results to exclude flanking genes. Phenotype: A – *Ash-red,* b – *brown*, + - wild-type; remaining column shows SNP genotypes

		Sex	Phenotype	163495	185384	185438	185450	205707	205754	214991	228093	238615	1897293
11	Egyptian Swift	F	+					T/.	C/.	G/.			
14	F1 EbxBLTbcEB	F	A							C/.			
16	Oriental frill	F	+					T/.	C/.	G/.			
18	F1 EbxBLTbcEB	M	A							C/C			
19	F1 EbxBLTbcEB	M	A							C/C			
22	Fan Tail	F	+					T/.	C/.	G/.			
23	Berlin long-faced tumbler	M	+					G/G	T/T	G/G			
24	F1 EbxBLTbcEB	M	A	G/G	G/G	T/T	A/A	G/G	T/T	C/C	A/G	C/C	
25	F1 EbxBLTbcEB	M	A							C/C			
26	Berlin long-faced tumbler	M	+					G/G	T/T	G/G			
28	Unknown	M	+	A/A	G/A	C/C	G/G	T/T	C/C	G/G	A/G	G/G	
29	Oriental frill	M	+							G/G			
32	F1 EbxBLTbcEB	M	A							C/C			
33	Egyptian Swift	M	+					T/T	C/C	G/G			
35	Oriental frill	F	+					T/.	C/.	G/.			
39	Berlin long-faced tumbler	F	+					T/.	C/.	G/.			
52	Scandaroon	F	+					-	T/.	G/.			
62	Homer	F	+					-	C/.	G/.			
91	3/4 Berlin long face tumbler	M	+					T/T	C/C	G/G			
99	German Beauty Homer	M	+		G/G	C/C	G/G	T/T	T/C	G/G	G/G	G/G	A/A
102	Budapest short-faced tumbler	F	+					T/.	C/.	G/.			
104	Budapest short-faced tumbler	M	+							G/G			
105	Budapest short-faced tumbler	M	+					T/T	C/C	G/G			
112	German Beauty Homer	F	+					T/.	T/C?	G/.			
117	F1 Scanderoon x Roller	M	A		-	-	-	-	-	G/C	-	-	
118	F1 EbxBLT	F	A							C/.			
119	Egyptian Bagdad	M	A		G/G	T/T	A/A	G/G	T/T	C/C	-	C/C	G/-
142	Dragoon	F	+							G/.			
143	Dragoon	M	+					-	C/C	G/G			
154	Modena	F	A							C/.			A/.
155	Modena	M	+							G/G			
174	Modena	F	+							G/.			
180	Fan Tail	F	+						T/.	G/.			
186	F1 BLTxBSFT	F	+							G/.			
190	Mongrel	M	+							G/G			
199	Dragoon	M	+							G/G			
200	Roller	F	+					T/.	C/.	G/.			
201	Roller	M	A		G/G	C/C	G/G	G/G	T/T	C/C	A/G	C/C	A/A
202	Roller	F	b	A/.	G/.	C/.	G/.	T/.	C/.	G/.	-	G/.	
203	Roller	M	b					T/T	C/C	G/G			
205	Roller	M	A	G/G	G/G	T/T	A/A	G/G	T/T	C/C	-	C/C	
206	Roller	M	+					T/T	C/C	G/G			
207	Roller	M	+					T/T	C/C	G/G			
208	Roller	F	+	G/.	G/.	C/.	G/.	T/.	C/.	G/.	-	G/.	A/.
209	Roller	F	+					-	T/.	G/.			
210	Roller		+					T/T	C/C	G/G			
211	Roller	M	A	G/G	G/G	HET	HET	T/G	T/C	G/C	A/G	CG	A/G?
212	Roller	M	A	G/G	G/G	HET	HET	T/G	T/C	G/C	G/G	CG	A/G?
215	Mookee		+							G/-			
217	Mookee		+							G/-			
218	Mookee		+							G/-			
220	Mookee		+							G/-			
341	Roller	F	+		G/.	C/.	G/.	-	C/.	G/.	G/.	G/.	
368	F; h; 6206	F	A	G/.				G/.	T/.	C/.			G/.

		Sex	Phenotype	163495	185384	185438	185450	205707	205754	214991	228093	238615	1897293
369	M; h; 794	M	A	G/G						G/C			
370	M; h; 1550	M	A							C/C			
371	F; R; 9820	F	A							C/C			A-
373	F; h; 24	F	A	G/.				G/.	T/.	C/.		C/.	
374	F; h; 793	M	+					T/G	T/C	G/G		G/G	
376	F; h; 6315			A	G/.						C/.		
377	F; h; 7353	F	A	G/.				G/.	T/C?	C/.		C/.	G/.
378	F; h; 7389	F	+							G/.			
381	F; h; 1022	F	A						T/.	C/.		C/.	
382	M; h; 6230	M	+						T/C	G-		G/.	
383	M; h; 23801	M?	A							G/C		G/C	
384	M; h; 652	M?	+							G/G		G/G	
385	M; h; 23997	M	A	G/G				G/G	T/T	G/C		G/C	A/A
386	M; h; 14920	M	+					G/G	T/T	G/C		G/G	
544	feral	M	+							G/G			
545	feral	F	+							G/.			
546	feral	M	+							G/G			
547	feral	M	+							G/G			
548	feral	M	+							G/G			
549	feral	F	+							G/.			
550	feral	F	+							G/.			
551	feral	F	+							G/.			
552	feral	F	+							G/.			
553	feral	M	+							G/G			
554	feral	M	+							G/G			
555	feral	F	+							G/.			
EB2	Egyptian Bagdad	F	+							G/.			

Appendix B

Stipper association data

Association data for the 77 kb duplication. Results of the breakpoint spanning assay are indicated as '+' or '–', other genotypes are indicated by allele/amplicon size (in bp). Markers are indicated by position on Scaffold 6. *Stipper* alleles in decreasing order of dominance: *St – Stipper/Almond, Sa – Sandy, Q – Qualmond, F – Faded, Ch – Chalky, Fy – Frosty* (Peter 2015).

Cliv	sex	Kinship	Breed	Stipper	77Kbbkpt	3062767	3136032	3262170	3352116
1097	F	1	Texan pioneer	F	+		122/.		
1095	F	2	Texan pioneer	F	+	122/.	122/.		
1096	F	3	Texan pioneer	F	+		122/.	110/.	
0426	M	4	unknown	F	+				
1078	M	5	Racing homer	F	+				
0429	M	6	Racing homer	St	+	122/122	het	het	127/127
0423	M	7	unknown	St	+	122/122			127/127
1064	F	8	Racing homer	F	+		122/.	110/.	127/.
0479	F	9	Hollander mongrel	F	+		122/.		127/127
0478	M	10	unknown	F	+		122/122		127/127
1053	M	11	Racing homer	Fy	+		131/131	102/102	
0415	F	12	unknown	Q.	+	het			127/-
0417	F	12	unknown	Q.	+		122/.	110/.	
0498	M	13	Racing homer	Sa	+		122/122		127/127
0342	M	14	Roller	St	+		het	?	
1228	M	14	Roller	St	+	het		110/110	111/111
1144	M	15	Roller	St	+	122/122	het		
1061	M	16	Racing homer	St	+	122/122			
0204	M	17	Roller	St	+	111/111			
0425	M	18	unknown	Ch	+		122/122	110/110	127/127
0416	M	19	unknown	F	+	122/122	het	110/110	127/127
0006	F		Berlin long-faced tumbler	+	-				
0022	F		American fantail	+	-				
0023	M		Berlin long-faced tumbler	+	-	122/122			
0026	M		Berlin long-faced tumbler	+	-				
0027	M		Oriental Frill	+	-	111/111	131/131		111/111
0029	M		Oriental Frill	+	-	111/111			
0030	M		American fantail	+	-	122/122			
0047	F		Berlin long-faced tumbler	+	-				
0052	F		Scandaroon	+	-		131/.		127/.
0064	F		American fantail	+	-				
0099	M		German beauty homer	+	-				
0102	F		Budapest short-faced tumbler	+	-				
0105	M		Budapest short-faced tumbler	+	-		het		
0106	F		Budapest short-faced tumbler	+	-		131/.		
0112	F		German beauty homer	+	-		122/.		
0119	M		Egyptian Baghdad	+	-	122/122			
0142	F		Dragoon	+	-				
0200	F		Roller	+	-			110/.	
0201	M		Roller	+	-		122/122		
0202	F		Roller	+	-		122/.	102/.	
0203	M		Roller	+	-	111/111			
0206	M		Roller	+	-	122/122	het		

Cliv	sex	Kinship	Breed	Stipper	77Kbbkpt	3062767	3136032	3262170	3352116
0207	M		Roller	+	-				
0208	M		Roller	+	-				
0209	F		Roller	+	-				
0210	M		Roller	+	-			het	
0304			Scandaroon	+	-		131/-		
0307			Scandaroon	+	-				
0339			Roller	+	-				
0433			WOE Tumbler	+	-				
0449			American fantail	+	-				
0454			Lahore	+	-				
1056	F		Racing homer	+	-		122/.		127/.
1192	M		Horseman pouter	+	-				
1264	F		unknown	+	-				
1316	F		Roller	+	-			102/.	127/.
1317	F		Roller	+	-			111/.	

Bioinformatic discovery process for the *Mlana* centered tandem duplications

The following are the original notes and results from bioinformatic analysis of WGS libraries of 40 domestic pigeons from diverse breeds conducted by Dr. Fondon in which the duplication events affecting Mlana were initially discovered. The raw alignments have been omitted from the print version of this document for space considerations, and some minor edits (primarily formatting) have been applied for clarity.

```
scaffold6 100bp sliding window depth analysis

2/10/2016 - JWF

1,941,801-1,954,401  [CORRECTED:1983001-1995801 - 2/17/16 JWF] ~12.5kb duplication in libraries 83 and 92, and possibly 73
  (Saxon Monk, Starling, and Oriental Frill, respectively) - toy stencil?
- closest annotated gene is Receptor-type tyrosine-protein phosphatase delta (<2086710..>2115801)

2,504,701-2,510,001  [CORRECTED:2549801-2555101 - 2/17/16 JWF] very clean ~5kb duplication (possibly >2x - avg=2.4) in
  library 01 - English trumpeter(e2/e2)
- closest annotated gene is Lysine-specific demethylase 4C (<2613385..>2633653)

3,080,000-3,156,000  [CORRECTED:3140001-3217601 - 2/17/16 JWF] possible ~75kb duplication  (libraries 80,94,95,02,08),
- with amplification (~3x) of an internal range ~25kb in some of these libraries and ~2x in a few others (82,96,06,07)
- This is less than 20kb 3' of MLANA (3,173,417..3,178,870), and the also spans a hypothetical protein of unknown function.
- NOTE 2/17/16 JWF: After re-running with debugged script, the corrected coordinates place MLANA squarely inside the
  nested duplication. See details below.

4,473,000-4,706,000  [CORRECTED:4562101-4811301 - 2/17/16 JWF] possible ~230kb duplication in library 78 (Scandaroon) - Ku2?
- region is in gene desert near end of scaffold6
- nearest annotated intact gene in scaffold6 is Nuclear receptor ROR-beta complement(<4236888..>4276416)
- pseudogene annotated at complement(4780341..4781333) /locus_tag="A306_12054" /pseudo
- BLAST hits of last 900 bp of scaffold link to additional gene not in genome annotation –
  annexin A1(4855044-4856735 - somewhat mangled)

Strange... Mapped depth for all libraries drops to zero by 4,763,100 (scaffold length is 4,857,497)
Why are zero reads mapped for the last 95kb - sequence looks ok - not terribly gappy or obviously repetitive.
Is there a non-obvious repeat or assembly fork?
BLASTing last contig vs. Cliv1 - last 900 bp of scaffold6 is 100% identical to scaffold148:46296-47195 (+/+ orientation)
scaffold148 appears to be adjacent to scaffold6 - either assembly fork or direct repeat?
Found apparent bug in sliding window depth analysis - zero depth values are somehow being omitted - need to debug...  Done.
re-running over weekend, corrected coordinates entered above



2/17/2016 - JWF
```

After debugging script (corrected version is batchdepth_v07.pl) and re-running all candidate scaffolds, the coordinate ranges of the structural variants identified above need to be revised. Done.
The corrected coordinates place MLANA in the middle of the nested CNV.
77kb dup: ~3140001-3217601 [3139933-3217453]
nested within this 77kb dup is a 25kb dup (appears up ~3-4x in some libraries with both duplications)
25kb dup: ~3159601-3184501 [3159547-3185091]
and within this internal 25kb dup is MLANA (3173417-3178870)

Annotated genes in region:
KIAA2026:   3105202-3156452 + [uncharacterized]
MLANA: 3173417-3178870 - [melan-A / MART1]
A306_12036: 3184607-3188815 - [LOC102094287|monocarboxylate transporter 2 - similar to Slc16a family]
ERMP1:   3206127-3224299 + [endoplasmic reticulum metallopeptidase-1 - annotated frameshift is just an assembly error]

MLANA is the only gene duplicated whole by the 25 kb duplication. Flanking genes (KIAA2026 & A306_12036) have only 3' portions duplicated.
So no chimeric transcripts are expected, and the effects may be limited to simple amplification of MLANA.
The 77 kb duplication duplicates both MLANA and A306_12036 (monocarboxylate transporter 2) whole, and creates a fusion of the 5' end of ERMP1 and the 3' end of KIAA2026.
Both ERMP1 and KIAA2026 are + orientation, so a chimeric fusion product is plausible:
    ERMP1(3206127-3217453)::KIAA2026(3139933-3156452)
Need to determine if fusion interrupts exons of either gene, and if the reading frame is preserved.
Fusion product includes the first 8 exons of ERMP1(3206127-3217453):
 3206127-3206230   104
 3207403-3207704   302
 3209883-3209976   94
 3210324-3210429   106
 3211288-3211434   147
 3212905-3212997   93
 3214286-3214498   213
 3215396-3215616   221
    sum = 1280 bp   1280/3 = 426.67 codons

and the last 3 exons of KIAA2026(3139933-3156452):
 3145524-3145639   116
 3150399-3150586   188
 3152818-3156440   3623
    sum = 3927 bp   3927/3 = 1309 codons
The breakpoints lie deep within introns, so splicing is not expected to be disrupted.
However, the spliced fusion disrupts the reading frame for the last 3 exons of KIAA2026.
The fusion frameshift gives rise to 3 stop codons in the first of these 3 exons, so NMD is expected to eliminate chimeric transcripts.
So, no obvious effects of 77 kb dup beyond simple amplification of MLANA and A306_12036|monocarboxylate transporter 2 (but of course could be otherwise).

The duplications have obvious potential to account for some instability, especially further expansions and/or reversions.

How might amplification of MLANA give rise to Almond and other alleles of St?
Might be simple cytotoxicity. Could it be autoimmunity-mediated? Perhaps sort of a reverse vitiligo?
No shortage of potential models.

If the duplication is in tandem, then read pairs that straddle the breakpoint can be identified by inferred insert size.
Pulling out all read pairs with large insert size:

```
cut -f 1-10 mlana.scaffold6_3139001-3220000.SRR51* | sort -nk 9 | grep -v -e 'NNNNNNNNNNNNNNNNNNN' -e 'SRR511914' -e
'SRR511915' -e 'SRR511916' -e 'SRR511917' -e 'SRR511918' -e 'SRR511919' -e 'SRR511920' -e 'SRR516969' -e 'SRR516970' -e
'SRR516971' | head -n 208
cut -f 1-10 mlana.scaffold6_3139001-3220000.SRR51* | sort -nrk 9 | grep -v -e 'NNNNNNNNNNNNNNNNNNN' -e 'SRR511914' -e
'SRR511915' -e 'SRR511916' -e 'SRR511917' -e 'SRR511918' -e 'SRR511919' -e 'SRR511920' -e 'SRR516969' -e 'SRR516970' -e
'SRR516971' | head -n 208
```

77kb dup - breakpoint flanking read pairs, based on inferred insert size

library	#pairs
6980	9
6994	2
6995	6
7002	5
7008	5

Start by assembling the reads of breakpoint flanking read pairs to determine approximate breakpoint, then retrieve reads from all libraries in the breakpoint regions to reconstruct and characterize the duplications.
Retrieving all reads from all libraries w/ ~77 kb inferred insert size (except 511914-20 or C.rupestris).
Assemble left and right ends separately.
The flags of the breakpoint-straddling read pairs for "reads mapped in proper pairs" are not set, and one read of each pair is reverse-complemented, so likely tandem duplication.
Assemblies below.
The region between 3139933-3217453 is present as a 77,520 bp tandem duplication. The left breakpoint at 3139933 has a short "microhomology" (TCTCTTTTTTC) with a large CT-rich tandem repeat at the right breakpoint (3217469-3218093).
The reference sequence has this tandem repeat as 25 perfect copies of a 25mer (TTTCCCTTTTCCTTCCTTTTCCCCC), however the aligned reads indicate some departure from this, including some differences shared by breakpoint-spanning reads (though maybe not exclusively).
The fact that several read pairs from five different libraries straddle the breakpoint indicates that, at least for the breakpoint, the tandem repeat is not nearly as long as indicated in the reference genome assembly.
It should be straightforward to develop a PCR assay for the presence of this duplication breakpoint.

[RAW ALIGNMENTS OMITTED DUE TO SPACE/FORMATTING CONSIDERATIONS]

25kb dup: 3159547-3185091
Nested within this 77 kb dup appears to be an additional 25kb dup present in some libraries, such that the total copy number appears to exceed ~3x in libraries that have both duplications.
MLANA is entirely within this smaller duplication (3173417-3178870).
Retrieving all reads from all libraries w/ ~25 kb inferred insert size (except 511914-20 or C.rupestris).
Assemble left and right ends separately.
Use breakpoint-specific sequence ATACTGTGCAGCTTGAAGTTAAAAAGACATG to grep out breakpoint-spanning reads:
cut -f 1-10 mlana.scaffold6_3139001-3220000.SRR51* | grep -v -e 'NNNNNNNNNNNNNNNNNNN' -e 'SRR511914' -e 'SRR511915' -e 'SRR511916' -e 'SRR511917' -e 'SRR511918' -e 'SRR511919' -e 'SRR511920' -e 'SRR516969' -e 'SRR516970' -e 'SRR516971' | sort -nk 4 | grep -e 'GTGCAGCTTGAAGTTAAAAAGACATG'
Folding all breakpoint-straddling read pairs and breakpoint-spanning reads into a single alignment. Done (assemblies below).

[RAW ALIGNMENTS OMITTED DUE TO SPACE/FORMATTING CONSIDERATIONS]

Tandem duplicated region: 3159547-3185091 (25,545 bp, but this includes 5bp microhomology [TGAAG], so really only 25,540 bp duplicated)

```
                                3159547
                                   |
left reference:         ctgcatctttaccTGAAGTTAAAAAGACATG
                                   ||||||||||||||||||
breakpoint:             ATACTGTGCAGCTTGAAGTTAAAAAGACATG
                        ||||||||||||||||||
right reference:        ATACTGTGCAGCTTGAAGcacattccaacct
                                      |
                                   3185091
```

# of independent supporting reads (either breakpoint-spanning reads or breakpoint-straddling read pairs):

lib	dup 25kb	dup 77kb		
6979	18		BLT	
6982	13		Chinese_owl	[e+/e1]
6996	17		Frillback	[BA/BA]
7006	12		Archangel	[e+/e2][BA/B+]
7007	32		Cumulet	[e1/e1](breed typically white due to homozygous Grizzle)
6980	7	9	Birm roller	[sy/+][d/d]
6994	11	2	African_owl	
6995	45	6	Ice_pigeon	
7002	18	5	Lahore	[BA/B+]
7008	38	5	Egyptian_swift	[sy/sy]

77kb dup occurs in subset of libraries with 25 kb dup, so the 25 kb dup came first, and the 77 kb dup is derived from it.

Appendix C

Primer sequences

cliv_mc1r_x1_f1	CTGCAGGTGAGCATGTCAAT
cliv_mc1r_x1_r1	CCTCACTGCCAGGGTGTC
cliv_mc1r_x1_f3	CAGCCCGTTGCAGCGT
cliv_mc1r_x1_f4	CCACAGCCCGTTGCAGC
cliv_mc1r_x1_f2	CATGTGGCGGACGATGCT
cliv_mc1r_x2.1_f1	TGAGTTGCAGATGATGAGGA
cliv_mc1r_x2.1_r1	AGCAACCTGGTGGAGACACT
cliv_mc1r_x2.1_f2	GAGGGGGTCAACCACTGAG
cliv_mc1r_x2.1_r2	ACTCTTCATGCTGCTGATGG
cliv_mc1r_x2.2_f1	CGGTACCAGCCACAGCAT
cliv_mc1r_x2.2_r1	CATGTCCAGCCAGCAGAAG
cliv_mc1r_x2.2_r2	CAGAAGCAGCCCGTCTACC
cliv0_mc1r_scaf123_5700865_F	CTTGACACTACGTGCTGTGG
cliv0_mc1r_scaf123_5700102_R	ACATGCTGCGGAGGTGGT
cliv1_s123_mc1r_5699797_R	CCATTATCGGTGTCCCACTG
cliv1_s123_mc1r_5700195_R	AGAGGAGGATGGCATTGTTG
cliv1_s123_mc1r_5700214_F	CAACAATGCCATCCTCCTCT
cliv1_s123_mc1r_5700316_F	GCGCTACCACAGCATCAT
cliv1_s123_mc1r_5700784_F	GTGCCCTGGAGCTGAGGT
cliv0_slc45a2_7_f1	ACCTCGCCCATGTGATGT
cliv0_slc45a2_7_r1	CATGCTGCGTTAGGATTGAA
cliv0_slc45a2_6.2_f1	TAGCGATGCTCTTGGTTGTG
cliv0_slc45a2_6.2_r1	TTAATTGTTGTTACTTCAGTCATGG
cliv0_slc45a2_6.1_f1	CCATGACTGAAGTAACAACAATTA
cliv0_slc45a2_6.1_r1	GCCCGTTGTCAACAGCTACT
cliv0_slc45a2_x5_f1	TCCACCAGAGCTGAGGAGAT
cliv0_slc45a2_x5_r1	AGCGTCAGGATCAGTCCATC
cliv0_slc45a2_x5_f2	TCACATGGAGACAGTATTTCTGTTTAG
cliv0_slc45a2_x5_r2	AGCGTCAGGATCAGTCCATC
cliv0_slc45a2_x3_L	CCTTTTTCCCACCCCTGTAG
cliv0_slc45a2_x3_R	TGCAAACAGTCAGGATCAGC
cliv0_slc45a2_x4_f2	CAGAAGTGCTGTATTCCACGAC
cliv0_slc45a2_x4_r2	CTGAGAGCTTCATCTGGAACAG
cliv0_slc45a2_x4_f1	ATATCCCCAGCTCTCCCAGA
cliv0_slc45a2_x4_r1	CCAGAGCCAAAGCAGAAAAT
cliv0_slc45a2_x3_f1	CCTGGGATGTGTGAATCTGA
cliv0_slc45a2_x3_r1	TTTGAGAAACTACAGCTACAGCA
cliv0_slc45a2_x2_f1	CAAACAGCGGAAGTGTCTGA
cliv0_slc45a2_x2_r1	GCTAATACAGCTGGAGGGAAGA
cliv0_slc45a2_x1_f1	CACACTGTTGACCCATCCTG
cliv0_slc45a2_x1_r1	ATGGGCGTACACACCGTTAT
cliv_tyrp1_1_f1	AGCAGCTGCCTTGATAAATGTT
cliv_tyrp1_1_r1	GGGAGGGTAGCCTGAAGGTTT
cliv_tyrp1_1_r2	GGGTAGCCTGAAGGTTTATGTCA
cliv0_tyrp1_x1_r3	CAGGAGAGAAGTCAGTGTGC
cliv0_tyrp1_x1_r4	AGCTAGGTGTCCCACCACAG
cliv0_tyrp1_x1_r5	AGTATGGCAGTGCAGGGTTC
cliv_tyrp1_2_f1	GAAAGAGCTGCTACCATTTTTACCT
cliv_tyrp1_2_r1	GCTCCAATAAAAATTGGATGAATG
cliv_tyrp1_2_f2	AGAAAGAGCTGCTACCATTTTTACC
cliv0_tyrp1_x2_L	TGCCCTGTACCCAGAAAAGT
cliv0_tyrp1_x2_R	TTTCCTCTGTATCCGCACAG
cliv_tyrp1_3_f1	TGTCTGGGAAAAGCCATTGCA
cliv_tyrp1_3_r1	GGCATCAGCGTGAAAAATGA
cliv_tyrp1_3_r2	CAAAGGCATCAGCGTGAAAA
cliv_tyrp1_4_f1	CCAAAGCCAATACTTAATGCTGA
cliv_tyrp1_4_r1	CACACAGGACCCCATGAAGA
cliv_tyrp1_4_r2	GGACCCCATGAAGATCCAGA
cliv_tyrp1_5_f1	GCATCTTGCTGTCCCACAAA
cliv_tyrp1_5_r1	AAGCAGGGAAACACAGCAAC
cliv_tyrp1_5_r2	GCAGGGAAACACAGCAACTT

cliv_tyrp1_6_f1	GCTGTACCAAATCCCAAACACA
cliv_tyrp1_6_r1	GACAGAGCAGAGCCCTCCA
cliv_tyrp1_6_f2	CCAAATCCCAAACACAACTCA
cliv_tyrp1_7_f1	TGAACCCCAAAGAGTTAGTGGAA
cliv_tyrp1_7_r1	TCACATTTCGGAGCAAATATTATCA
cliv_tyrp1_7_f2	GTGAACCCCAAAGAGTTAGTGGA
cliv_tyrp1_7_r2	TTCTGCTTCACAATTCCTTGAATAA
cliv0_tyrp1_x1_sacI_204_R	ACACACCACACCGGTCAGTA
cliv0_tyrp1_x1_sacI_204_F	TGGAAGGAGCTGGGTCTG
cliv0_tyrp1_x1_sacI_133_R	ACAACACATGCCGCTCCT
cliv0_tyrp1_x1_sacI_133_F	ATCCACCATCATGCAGCTC
cliv1_tyrp1_scaf6_198711_F	GGAGCTTCACCAAACAATCC
cliv1_tyrp1_scaf6_197764_R	TCACTTGTAGATGCCGGAATAA
cliv1_tyrp1_scaf6_189329_F	TGCGACTGACCCAGATGTTA
cliv1_tyrp1_scaf6_188787_R	TCCAGCAGTCGTTTTCTTCA
cliv1_tyrp1_scaf6_227446_F1	AGGTTTCCCAGTTGAAGCTGA
cliv1_tyrp1_scaf6_227199_R1	TGTGTTAAATGTTGTCCCATTCA
cliv1_tyrp1_scaf6_228058_R	TTAGGCCTTCAGGAGGTGTC
cliv1_tyrp1_scaf6_228294_F	GCTCAGGATCTCACCCAAAA
cliv1_tyrp1_scaf6_228168_F	GATAGAGGAGACCATGGGGTAG
cliv1_tyrp1_scaf6_227969_R	GCAGCTGGGCAATGTAAAAC
cliv1_tyrp1_scaff6_238276_F	TCCAGTTACACAGGTCTCAAGC
cliv1_tyrp1_scaff6_238482_R	GAAGAGTGTAGTGGAGTAACAGATGC
cliv1_tyrp1_scaf6_203185_F	TGACCTGCTGAATGTTCTTAGG
cliv1_tyrp1_scaf6_202935_R	TATGGCCTGCTCAAAACATGA
cliv1_tyrp1_scaf6_192691_F	TTTGGGTAAAAATTGTGCGTAA
cliv1_tyrp1_scaf6_192466_R	TTCCATCCCTCCCAGTTCTA
cliv1_tyrp1_scaf6_192051_F	GGAGAGGTTGCACAAGGACT
cliv1_tyrp1_scaf6_191837_R	ACCTGGCACAAATCTTCTGG
cliv1_tyrp1_scaf6_203526_F	TCATCTGACCAAGCATGTGG
cliv1_tyrp1_scaf6_203305_R	GCAAGAGTGACAGAAAGAAGCA
cliv1_tyrp1_scaf6_187144_R	TTGCATAGTAAAACCTTAGGACA
cliv1_tyrp1_scaf6_187540_F	GCTTGCTGGAAAGACTGAAAG
cliv1_tyrp1_scaf6_185569_F	TGACCCCTACAAATCCAAGG
cliv1_tyrp1_scaf6_185205_R	CCACATCAACCGAGCAGATT
cliv1_tyrp1_scaf6_227969_R	GCAGCTGGGCAATGTAAAAC
cliv1_tyrp1_scaf6_228294_F	GCTCAGGATCTCACCCAAAA
cliv0_tyrp1_scaf6_156912_L	ATGGGCTGGAACAGAATCAA
cliv0_tyrp1_scaf6_157194_R	CAACAGCTGGAGCACTGGTA
cliv0_tyrp1_159001_F	ATAGGAGGCAGAGAGGCACA
cliv0_tyrp1_158404_R	GGCCCAATCCCATTAGTACA
cliv0_tyrp1_scaf6_163654_F	TCGGCTGTAGTGCTTTCTGA
cliv0_tyrp1_scaf6_163385_R	AGCAGTGCCAAAATGTAGGG
cliv0_tyrp1_scaf6_61132_R	TTTGATGCTCCTGACTGCAC
cliv0_tyrp1_scaf6_60583_L	TGTCAGCATTCTCATTTTACTGG
cliv0_tyrp1_scaf6_61393_L	CAACATAAACTCTTGGGGCTAA
cliv0_tyrp1_scaf6_62068_R	CCCCCAAGTACATTTTGACC
cliv0_tyrp1_scaf6_63286_L	CATGGGCAGCAGTACTCAGA
cliv0_tyrp1_scaf6_63660_R	ATCCCCCTGCCTAAATCAAC
cliv0_tyrp1_scaf6_67959_R	TCCTGGTACCATGCATTCTT
cliv0_tyrp1_scaf6_67367_L	GCCTTTTGTCTGCCTTTGTAG
cliv0_tyrp1_scaf6_1897129_L	TTGCAGTACAATGGAACAGCTT
cliv0_tyrp1_scaf6_1897568_R	AGATGTGGTTGGCAACTTCC
cliv0_tyrp1_scaf6_1896845_L	AGATCAGTTCTCAGCAATTTCC
cliv0_tyrp1_scaf6_1897150_R	AAGCTGTTCCATTGTACTGCAA
cliv0_tyrp1_scaf6_1876269_L	GAGCTTGTGTTGCAGCGTAG
cliv0_tyrp1_scaf6_1876745_R	CCCAGACTAAACACGGTAATGC
cliv1_tyrp1_s6_180600_R1	AGGCCATTCAGCCTCTTGAT
cliv1_tyrp1_s6_180721_F1	GACAAGCCTAAGGCAGTTTTCA
cliv1_s120_nfib_1714059_L	TTGTCAGTCAGGTTGCGTGT
cliv1_s120_nfib_1714265_R	GTGTAGGTCCCCCAGGAGTT
cliv1_s120_sh3gl2_36126_L	GACAATGCAAAGGCAGCTTTA
cliv1_s120_sh3gl2_36509_R	CCTTCATTACAAATATTCTGCATCC

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cliv1_s6_tyrp1_442569_L              GGCATTCTTGATATCTGTCTTGG
cliv1_s6_tyrp1_443016_R              AGGGTGAAACAGGTGAAGGA
cliv1_s249_map1b_23336_L1            TTGTCTGTTGGAATGGTTTCTG
cliv1_s249_map1b_24110_R1            GGTCACCTGAAGCCAAGG
cliv1_s6_4268018_rorb_L1             TGGTGAAAACAGAATTGTCTCG
cliv1_s6_4268355_rorb_R1             TCTGGGTTAGACATGACTGGAA
cliv1_s301_45082_L1                  CCAGGATGTGACCCAGAAAA
cliv1_s301_45422_R1                  CTGCTGTCTGCTGGTTGTTC
cliv1_s120_1702951_nfib_L1           TGGTGGAACAACACTGGAAA
cliv1_s120_1703236_nfib_R1           TCCTATCCCTGTGAGATGTCCT
cliv1_s249_1883007_slc1a1_L1         AGCTTTAGCGCTCAGGTCAA
cliv1_s249_1883471_slc1a1_R1         CCGGGAGAAGAAGAGAGCTT
cliv1_s120_53430_L                   CCCAAAGGTAATTAATGTTCCAG
cliv1_s120_53660_R                   CAGTGACCACTCCAGCAGAC

cliv0_MLANA_x1_f1                    CATTTAAACAGCACAACAGATCC
cliv0_MLANA_x1_r1                    TTACACCAGCCCTCTGTCGT
cliv0_mlana_x2_R1                    TTGAGGGCTCGATATGGTTC
cliv0_mlana_x2_F1                    TCCCTCCCTAAAATGTTCCTG
cliv0_mlana_x3_R1                    GGTGTGTTTCATGTTGTGCAG
cliv0_mlana_x3_F1                    GGATACCAATGCAATCTTTTGA
cliv0_mlana_x4_R1                    GGCAATGAAGACCAATTTAGAGA
cliv0_mlana_x4_F1                    TTAAGCGCTATGTGCTCCAA
cliv0_mlana_x5_R1                    GATGCAAGATGCCCAGACTT
cliv0_mlana_x5_F1                    AAAAATGAAGACATAATGAATGAGC
cliv1_mlana_77Kbbkpt_3217387_L       GTTGGTGGCTCAAGATTTCTG
cliv1_mlana_77Kbbkpt_3140013_R       CCTGGGCACACAAGGTAAG
cliv1_mlana_refM13_3139921_L  [GCAAAAGAGTAGC]GCTCCTCCACTATCTCTTTTTTCATCTAG
cliv1_mlana_77Kbbkpt_3139933_L       TTCCCCCTTTCTCTTTTTTCATCTAG
cliv1_mlana_77Kbbkpt_3139933_L2      TTTTCCCCCTTTCTCTTTTTTCATCTAG
cliv1_s6_3139933_77Kbbkpt_L3tl [TCTTGTACAGTCTCAACAGTCGC]TTCCCCCTTTCTCTTTTTTCATCTAG
cliv1_s6_3159547_25kbtail_L1  [AGTAGACCGTACACACAGTC]GCAGCTTGAAGTTAAAAAGACATGT
cliv1_s6_ 3159539_25kbref_L1         tctttaccTGAAGTTAAAAAGACAT
cliv1_s6_3159682_25kbcmn_R           CTGAATATCTATGTAGAATATCTGAAGG
Unique_secondary_primer              TCTTGTACAGTCTCAACAGTCGC
cliv1_s6_3262135_mlana77kbRt_L       CATTTCTAAAATACTGCAGTTTGAC
cliv1_s6_3262135_mlana77kbRt_R       AGCAGTTTGTAATGAATTCTGAGT
cliv1_s6_3264351_mlana77kbrt_L       TCAAGCACAAGGCAAGTCAC
cliv1_s6_3264490_mlana77kbrt_L       TCATTTTCCAAATATGATTATTTCACC
cliv1_s6_2849146_mlana77kblt_L       CCAGTATTTGCGCTCCAGAT
cliv1_s6_2849320_mlana77kblt_R       TCAGGACAACTAATAATTCCTCAGTG
cliv1_s6_3131056_mlana77kblt_L       GAGAAACAGATCACAAGAGTGTCC
cliv1_s6_3131197_mlana77kblt_R       CTCTCAAAGACTGTCATTCTCTCCT
cliv1_s6_3174436_mlana_x4_R1         CACAGAATTAAAGTTCCTGTATTCC
cliv1_s6_3174526_mlana_x4_F1         TAGTGTGGGCACAATACGAA
cliv1_s6_3135939_77Kblt_L            TGCACTGCACCTCAGTACAAG
cliv1_s6_3136075_77Kblt_R            GGAAACAAAGGGCAGTTAGG
cliv1_s6_3062697_77Kblt_L            TAACTGAATTTAAAGGCTTTCC
cliv1_s6_3062818_77Kblt_R            TCAGTTTACTCTGGTAAGTGAACA
cliv1_s6_3352055_77Kblt_L            CAAATGCCTGACCATATTTCC
cliv1_s6_3352165_77Kblt_R            TTGCCCCACTGCGTATTT

cliv1_s6.3298629_Zctrl_qPCR_L        GTAGCCAGTCACCTGGAAGC
cliv1_s6.3298718_Zctrl_qPCR_R        GGTGAGGTATCCCCAGGATT
cliv1_s6_3174436_mlana_x4_R1         CACAGAATTAAAGTTCCTGTATTCC
cliv1_s6_3174526_mlana_x4_F1         TAGTGTGGGCACAATACGAA
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Biographical Information

Shreyas Krishnan started his career in science early during his undergraduate years, volunteering with a group that scoured the beaches of Madras (India) for sea turtle nests, with the objective of relocation to protected zones. During this time he regularly visited the local herpetology zoo library at the Madras Crocodile Bank Trust, where he returned to work several years later. After a three year undergraduate program in Zoology he proceeded to Pondicherry University for a Master of Science in Ecology. During this time he developed the ideas that motivate his research, from travels through the Deccan and Western Ghats. Following this Shreyas was hired by the herpetology zoo to man a United Nations Environment Program station that conducted population assessments of major sea turtle nesting beaches on the remote island of Great Nicobar. During this period he developed the next layer of questions and proposed a community ecological level study that he commenced not too long before he was invited to work with Dr. Jonathan A. Campbell at the University of Texas at Arlington (UTA). For his Master's thesis work Shreyas won several grants to continue his research in the Andaman and Nicobar Islands. After his Master's degree at UTA, Shreyas forayed into alternate careers, with the objective of returning to his PhD in a few years. This was perhaps his best decision, as he was introduced to Dr. John W Fondon III when he returned in 2010. The opportunity to investigate the very questions that motivated him, but in a tractable system that allowed rigorous genetic dissection was second to none. Shreyas is now a geneticist and evolutionary biologist, planning to continue the work he began during his PhD and also return to address questions that teased him early on.