THE GENETIC BASIS OF PIGMENTATION VARIATION IN DOMESTICATED ZEBRA FINCHES

by

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Introduction

The zebra finch (*Taeniopygia guttata*) is the most common estrildid finch native to Australia with subpopulations in Southeast Asia as well as domesticated populations throughout the world. When it comes to avian genomic studies, the model species has always been the chicken (*Gallus gallus*) because of its importance in agriculture and wide availability. However, recently the zebra finch was the second avian species to have its complete genome sequenced (Buggiotti, 2007). Taking advantage of this available genomic data, I was able to focus on genes involved in coloration and pigmentation of the domesticated zebra finches.

Historically, biologists have approached evolutionary biology via two parallel concepts, one looking at the genotypic level and a second looking at the phenotypic level (Hoekstra, 2006). This is exactly why pigmentation is such an ideal trait to study; it is a particularly promising system through which an understanding of the connection between genotype and phenotype can be established. By linking genetic changes to variations in a trait of adaptive significance, one can acquire exciting insight into how adaptation proceeds at the molecular level and also shed light on the evolutionary process in general (Hoekstra, 2006).

Melanin pigments are the most abundant and widespread pigments and confer most of the black, brown, grey and rufous colors in zebra finches. Melanin found in the maturing follicles of these birds is synthesized in special pigment cells called melanocytes. Once synthesized, melanin pigments offer birds a series of biological, physical and chemical advantages (Kobayashi et al. 1998). Because of melanin's chemical structure, conjugated double bonds can act as scavengers for free radicals and protect cells and tissues from oxidative damage. Melanin also has strong intermolecular properties, resisting breakage and wear and tear through strengthened tissue. The

UV-absorbing properties of melanin make melanin pigments ideal photoprotectants (Sarangarajan et al. 2001).

From what is understood of melanogenesis, in an event of DNA damage, upregulation of the melanocyte-stimulating hormone (MSH) signaling leads to the activation of the trans-membrane MC1R (melanocortin-1 receptor) receptor of melanocytes. Activated MC1R induces upregulatation of a range of genes required for melanogenesis via G-protein signaling. OCA2 (oculocutaneous albinism type 2) and SLC45A2 (solute carrier family 45 member 2) encode proteins required for correct packaging of melanin synthesis enzymes such as tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1) into melanosomes. TYR, TYRP1, and downstream enzymes then metabolize tyrosine into melanin (Sarangarajan et al. 2001).

Although the bulk of our knowledge about pigmentation pathway stems from studies done on laboratory mice and other mammals, the melanin pathway is highly conserved among vertebrates. Hence, minute alterations in the pathway of melanocyte production can generate dramatic pigmentation variations among vertebrates (Hoekstra, 2006).

TYRP1

Tyrosinase-related protein (TYRP1) is a membrane bound protein expressed in melanocytes that acts to stabilize tyrosinase, an essential enzyme involved in rate-determination in melanogenesis (Bennett et al. 2003). The TYRP1 gene likely arose from a gene duplication event of an ancestral tyrosinase gene followed by a duplication event of an early tyrosinase related protein, as evidenced by the presence of tyrosinase and TYRP genes found in urochordates and fish, respectively (Kelsh et al. 2000). Although TYRP genes have arisen in a number of vertebrate lineages, their function in melanogenesis has been conserved, despite

divergence in size and intron length among species (Kelsh et al. 2000). TYRP1 mutations occur in a variety of species, including birds, humans and other mammals, and result in similar but not identical phenotypes (Kobayashi et al. 1998). In humans, mutations in TYRP1 generally result in reddish or reddish-blond hair. In Solomon Islanders, a population that differs from the general islander trend of 'dark hair-dark pigmentation' due to higher ultraviolet radiation of the equator, a TYRP1 mutation has been found to cause blond features in the otherwise dark colored individuals (Eimear et al. 2012). In this study, 43 blond and 42 dark-haired Solomon Islanders were sequenced; the mapping interval contained only the TYRP1 gene and results yielded a single nucleotide polymorphism with a frequency of 0.93 and 0.31 in blond and dark-haired individuals, respectively. This SNP yielded a C-to-T transition at chromosome 9p23 corresponding to an arginine-to-cysteine mutation in Exon 2 of the TYRP1 gene at amino acid position 93. The study then advanced by genotyping 918 Solomon Islanders, calculating the TYRP1 mutation to be at a frequency of 26% . Similarly, 941 individuals from 52 worldwide populations were genotyped for the same mutation, revealing the genotype to be rare or completely absent outside the Solomon Islands. It was concluded that the major cause of blond hair in Solomon Islanders was mostly due to a non-synonymous mutation at a highly conserved region of the TYRP 1 gene. This missense mutation was predicted to affect the catalytic activity of TYRP1, causing blond hair through a recessive mode of inheritance (Eimear et al. 2012).

To further investigate pigment dilutions and TYRP 1, a study on Soay sheep was reviewed (Gratten et al. 2007). This study targetted the TYRP1 gene based on previous genetic mapping work and mapped the coat color locus to a limited window on chromosome 2 (Gratten et al. 2007). The study was conducted on the coat color of a free-living population of Soay Sheep, which depends on inheritance of a single autosomal gene with two alleles, where dark is

completely dominant to light. Sequence diversity in the TYRP1 coding region yielded a total of six polymorphisms, two of which were non-synonymous. The first of these was a G-T transversion at nucleotide position 869 on Exon 4 of the TYRP1 gene. This delineated a perfect association with coat color where the recessive light sheep were homozygous for T at the 869th position, as compared to the dark morphs, which were either homozygous for G or heterozygous. This fit the most likely model of inheritance in which dark (G allele) was concluded to be dominant to light (T allele). This G-T transversion at the 869th nucleotide position caused a cysteine to phenylalanine amino acid change, replacing a highly conservative cysteine residue. The study further suggested that the 869th position may be under strong functional constraint and that dark coat color is ancestral to light (Gratten et al. 2007). The study also compared its functionally significant SNP finding with the notion of cysteine residues and their involvement in the formation of internal disulphide bridges, integral for successful transportation, processing and maturation of synthesized proteins in the cytoplasm (Costin et al. 2003). Cysteine residues are essential for correct protein folding; hence, it makes sense to speculate that all known alterations to the tyrosinase family members should involve either a gain or a loss of a cysteine residue (Murisier et al. 2006). To test the legitimacy of the association between coat color and TYRP1, the study employed pedigree analysis of 547 phenotyped sheep. Analysis of these pedigree data revealed an almost perfect association with coat color where the TYRP1 869G→T transversion cosegregated with coat color throughout the Soay sheep pedigree (Gratten et al. 2007).

In addition to the work with mammals, there has been some recent work studying the function of TYRP1 in birds. SNP- coloration associations have been identified by both Buggiotti (2007) and Domyan et al. (2014) in the TYRP1 gene with plumage color variation in flycatchers

and domestic pigeons. The very recent study on domesticated pigeons was the first of its kind to shed light on the mutations of TYRP1, isolating it as the only locus in the entire genome to be significantly associated with base color variation. I particularly focused on one of the base color mutations that give the domesticated pigeon its unique red hue. The study divided the pigeons by conferring them to one of three "base" colors: wild-type blue/black (B+), ash-red (BA) and brown (b). The BA is dominant to B+ and b, and b is recessive to the others. It was found that all blue/black pigeons were homozygous G (B+ allele) on the TYRP1 gene, whereas the ash-red pigeons were hetero- or homozygous for C (BA allele), consistent with the dominant mode of inheritance of ash-red. The ash-red pigment mutation causes an alanine-to-proline substitution at Exon 1 of the TYRP1 gene corresponding to a single peptide cleavage site (Domyan et al. 2014). In addition, a perfect association was found between the dominant ash-red mutation and the phenotype in 150 birds from 56 breeds from all three variants of base color. Results from association suggested that the ash-red mutation occurred only once through selective breeding, spreading species wide. Real time PCR analysis revealed that the location of ash-red mutation at a highly conserved cleavage site was most likely the reason for this mutation due to cleavage efficiency. This cleavage efficiency alters the normal TYR functionality in the process of melanogenesis, resulting in an increased ratio of pheomelanin to eumelanin production, resulting in the reddish hue observed by the BA version of TYRP1 protein (Domyan et al. 2014).

SLC45A2

Solute carrier family 45 member 2 (SLC45A2) is a transporter protein that mediates melanin synthesis. Mutations in SLC45A2 have been shown to disrupt tyrosinase processing and trafficking at the molecular level but the function of this gene is not fully understood (Sato et al. 2010). In a study conducted by Gunnarsson et al. (2012), mutations in the SLC45A2 gene were

shown to cause imperfect albinism both in chicken and the Japanese quail as well as their mutated phenotypes, silver and cinnamon, respectively. The study described five different SLC45A2 mutations associated with plumage color in both chicken and quail. Two of the mutations were null mutations causing sex-linked imperfect albinism. The deletion of the base pair T on the 106th position in Exon 1 of the SLC45A2 gene resulted in a frameshift and a premature stop codon. In their studies, the researchers found that melanocytes from the albino birds had morphologically strange melanosomal/lysosomal organelles, positive for both tyrosinase and acid phosphatase activities, and a number of morphologically normal premelanosomes were found to be lacking both enzymes. Hence, concluding that in the albino melanocyte, tyrosinase was not being efficiently shuttled to the premelanosomes (Gunnarsson et al. 2012). As stated above, during the process of melanogenesis, the normal function of SLC45A2 protein is believed to be involved in directing the tyrosinase to these premelanosomes (Domyan et al. 2014). Thus, the loss-of-function mutation of albinism is fully consistent with its observed variant phenotype (Gunnarsson et al. 2012). Other interesting results generated from this study were that of two different missense mutations associated with the sex-linked silver mutation of the chicken. One of these led to a L-to-M transversion at Exon 3 of the SLC45A2 gene in a highly conserved region and delineated a perfect association with the presence of silver across different breeds of chicken. Similar to the ash-red mutation in the domesticated pigeon, there has been strong selection at the 'Silver' locus of the chicken. The researchers stumbled across this conclusion when they identified the degree to which the marked difference in the various polymorphisms between populations carrying the Silver allele proved to be a hallmark of a "selective sweep for a favorable mutation" (Gunnarsson et al. 2012).

Perhaps the most interesting feature of the SLC45A2 mutation described in this study is

its specific inhibition of the expression of red pheomelanin in birds carrying Silver. This phenotypic effect is well documented in other animals with a characterization of diluted red pigment with no or only a minor effect on black pigment. The study concludes by speculating that the amino acid cysteine is essential for the synthesis of red pigment whereas very little tyrosinase activity is required. Hinting that one of the functions of SLC45A2 is to transport cysteine into the melanosome and that this function is disrupted by the mutations associated with Silver (Gunnarsson et al. 2012).

Similar to the chicken and quail study, Xu et al. (2013) proposed that SLC45A2 is a sucrose/proton co-transporter and mediates melanin synthesis by regulating melanosomal pH and/or osmotic balance. In this very exciting project, Xu and colleagues found a single amino acid change in the SLC45A2 gene to be the cause of the white tiger phenotype, a vast deviant from its ancestral Bengal tiger. They recruited nine heterozygous oranges (Ww) and seven homozygous (ww) white tigers. Whole genome sequencing was performed and a total of 509,220 SNP markers were identified and aligned with the tiger reference genome. Only certain scaffolds fitting various criteria, especially those of a recessive mode of inheritance and indication of reserved synteny were scanned for SNPs among samples and parent genomes. Seven genes displaying polymorphsims in the coding regions between white and ancestral Bengal tigers were identified. Only one nonsynonymous substitution in two of these genes was considered functionally significant, delineating a C-to-T transition in Exon 7 of the SLC45A2 gene, which corresponded to an alanine-to-valine substitution at amino acid position number 477. This transversion by the name of A477 is highly conserved among vertebrates, and the same mutation at the same ‘A477’ position has been reported only once in vertebrates (Xu et al. 2013).

The SLC45A2 nonsynonymous mutation in the white tiger primarily inhibits the

synthesis of red/yellow pheomelanin, with no or only minor effects on the black eumelanin. This very specific phenotypic characteristic was previously identified in the analyzed study on silver chickens (Gunnarsson et al. 2012). Similar to the chicken study, Xu et al. (2013) speculated that the cysteine residue is essential for pheomelanin production, and that these mutations act via disrputing osmatic balance and sucrose transportation.

Zebra Finch Morphs

For my research, I focused on an array of morphs of the zebra finch. The first of which is the ancestral lineage, the australian finch (*T. g. castanotis*) which are mainly grey with characteristic black 'tear drop' eye stripes and 'zebra-like' black and white barring on the body and upper tail. The throat and upper breast are light grey with a broad black band outlining the upper chest. The sides of the belly are chestnut with many white spots. The remainder of the belly and the under-tail are pearl white. The male is distinguished from the female by its chestnut cheek patches, a characteristic that gave the species an alternative name of Chestnut-eared Finch. Both sexes have red eyes and bill. The legs and feet are a mixture of orange and yellow (Zann, 1996).

The second morph of interest to this research was the Timor zebra Finch subspecies (*T. g. guttata*), a subspecies of the australian finch. Although the characteristics of the Australian and Timor finches are fairly similar, there are some fundamental differences. In appearance the timor is much smaller than the other finches, and even slightly smaller than the ancestral australian zebra. The typical chestnut cheek patches and flank markings are present in the males, as are the barred tails but in both sexes. The area under the beak is grey, lacking the typical characteristic zebra stripes of the australian finch, and the breast band is much thinner (Zann, 1996).

For this project, the first mutated morph of the zebra finch we examined was the lightback finch. The mode of inheritance for this mutation is sex-linked. The morphology of the male delineates with the head, wings and back diluted to a silvery gray color. The belly and tail covers are bright white. The cheek patches are diluted to an apricot color and the flanks are diluted to a light orange color. The black markings (tear mark, tail and breast bars) remain at full strength, just like the ancestral australian. The female presents with a gray head, back and wings diluted as in the male. The belly and tail cover are also bright white with the tear marks and tail bars remaining at full strength (Beckham, 2011).

The second mutated morph relevant to this research was the Regular Chestnut Flanked White mutation (CFW-R). The mode of inheritance for this mutation is sex-linked. CFW-R males have a white body with traditional markings remaining at nearly full strength. These markings include the cheek patches and flanks, tear and breast marks. The tails of the male are a diluted gray color. In essence, CFW-R birds with strong markings are very rare as their markings are often diluted. The orange cheeks are especially difficult to see in the males. The females, like the males, have a white body with dark tear marks under the eyes. The tail is also a diluted gray color. Females often have more black flecking on the top of the head than do males (Beckham, 2011).

Similar to the CFW-R, the Continental Chesnut Flanked White (CFW-C) zebra finch is a variation of the CFW-R. Like the CFW-R, the male CFW-C is a mostly white bird with the orange cheek and flank and black tear, breast and tail marks intact. Unlike the CFW-R, the CFW-C males have a cream color to their back with much darker tail, sometimes even black. On the best CFW-C, the orange markings are still not as strong as the best CFW-R males. They also have a ruby color to the eyes, which is much more noticeable in chicks, but can still be seen in

the adults. The CFW-C females, like the male, are similar to their Regular counterparts, but have a cream back, black tail bars and red eyes. The CFW-C females are also free of any black flecking on the head (Beckham, 2011).

Regular and Continental CFW's along with the lightback morph are all part of a multiple allelic series developed through years of selective breeding (Beckham, 2011). In a multiple allelic series, the CFW mutation shares the same locus on the Z chromosome as the lightback mutation and both varieties of CFW. Because of this common chromosomal location, the mutations cannot be combined. It is important to recognize the sex-linkage in birds: the male zebra finches are labeled ZZ and females are labeled ZW. In these birds, females always express the sex-linked allele. Thus, females are either CFW of one variety or another or lightback, whereas the males can carry either combination of mutations on their ZZ chromosomes (Zann, 1996). The 'Continental' CFW mutation is recessive to the 'Regular' CFW, and both the CFW varieties are recessive to the lightback. Given the sex-linked nature of the CFW and lightback mutations, it was speculated that genes controlling pigmentation would most likely be found on the Z chromosome of the zebra finch. Upon scanning the Z chromosome within the completely sequenced genome of the zebra finch, our two candidate pigmentation genes, SLC45A2 and TYRP1 were identified and targeted.

Methods

Genomic DNA samples from fourteen individuals for SLC45A2 gene and seven individuals for TYRP1 gene were used from the collection in the Balakrishnan Lab at East Carolina University. The individuals sequenced for the SLC45A2 gene: theree domestic australian males, three timor finch males, two CFW-C males, two CFW-C females, one lightback female and three CFW-R females. The individuals sequenced for the TYRP1 gene included: two CFW-C females, two CFW-C males, two CFW-R females and one lightback female. TYRP1 and SLC45A2 genes were amplified using polymerase chain reaction (PCR) with a 25µl reaction containing 16.4 µl of water, 2.5 µl of 10x buffer, 2.5 µl dNTP, 0.25 µl Taq polymerase, 1.0 µl DNA and 1.25 µl each of forward and reverse primers. TYRP1 Exon 1 F/R through Exon 7 F/R and SLC45A2 Exon 1 F/R through Exon 7 F/R primers were used on their respective sets of DNA samples at a concentration of 10µm. Approximately 500 bp's, depending on the quality of reaction, of Exon 1-7 of TYRP1 and SLC45A2 were amplified. PCR reactions were performed in an Eppendorf thermocycler with the following cycling parameters: 94 °C for 2 minutes; 35 cycles of 94 °C for 30 seconds, 60°C for 45 seconds and 72°C for 1 minute, and 72°C for 5 minutes. PCR products were run on a 1% agarose gel to ensure the completion and strength of reaction. PCR clean was carried out using a QIAquick PCR purification kit. After elution in various buffers and centrifugation, the PCR products were ready for sequencing. Purified sequencing reaction products were run on an automated DNA sequencer. Sequence data from each individual was compared on Genious to determine overall sequence variation among the morphs.

Results

Data collection was limited to certain exons of the two genes based on the clarity of sequences revealed by the Genious software. For SLC45A2, exon 5 was the only exon that generated noisy sequence results, unable to render quality data. Exons 2, 3 and 6 generated satisfactory reads but were of no significance due to their lack of SNP's. For the TYRP1 gene a 300 base pair long clean read of Exon 6 showed no evidence of any functionally significant SNP's. Similarly, a 500 base pair read of Exon 7 was perfectly conserved among the seven individuals. Exon 1-5 of TYRP1 consistently obscured meaningful data collection by generating low percentage and quality of reads.

Exon 1-SLC45A2

A single variable nucleotide site was found at Exon 1 of SLC45A2 in four out of the fourteen individuals.

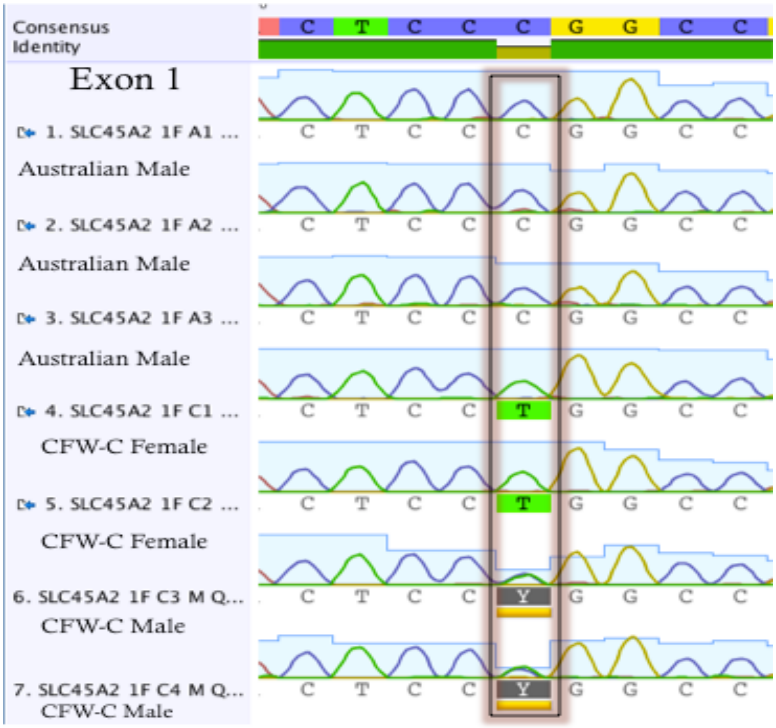

Figure 1: Variable nucleotide sequence of SLC45A2 Exon 1

Figure 1 illustrates a section of SLC45A2 Exon 1 for where the highlighted nucleotides are an indication of deviance amongst the fixed nucleotides in this very clean sequence read. The figure represents a nucleotide substitution between the domestic australian finch and the male and female CFW-C morphs. As evident by the content in the red box, all three of the australian males have a cytosine as their corresponding nucleotide. However, movement along the box indicates that both CFW-C females posses a C-to-T nucleotide substitution. The "Y" for the CFW-C males is the software's code for indicating that the nucleotide can either be a cytosine or a thymine at that given position. As evident by the peaks for both of the CFW-C males, the amplitude of the green peak is equal to that of the purple peak, meaning at that position of Exon 1, CFW-C males are heterozygous for the mutation causing the C-to-T nucleotide substitution. To help visualize nucleotide sequence data, the software Se-Al was used to generate amino acid sequences from the nucleotide sequences in Figure 1.

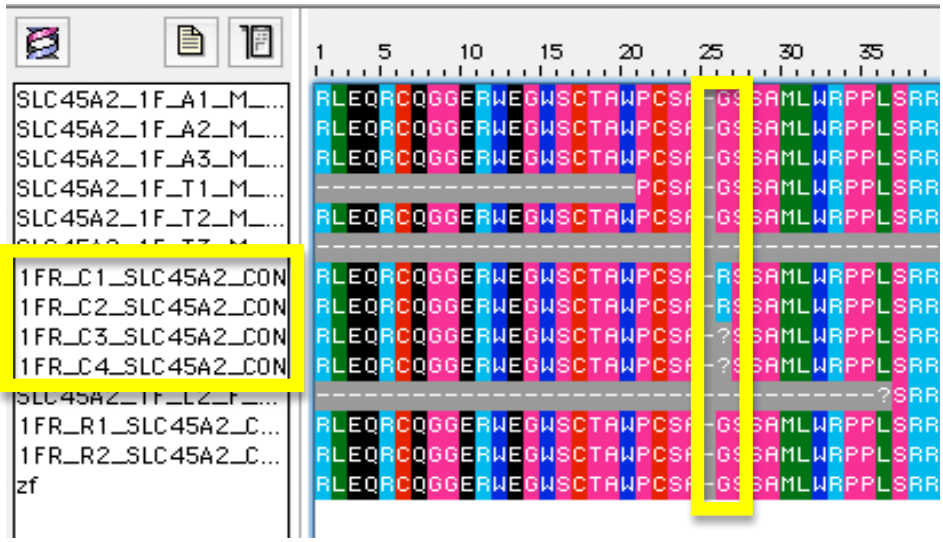

Figure 2: Amino Acid sequence from Figure 1

Figure 2 is a translated version of figure 1, only difference being that it is depicting amino acid sequences. As evident by the yellow box, CFW-C males and females are the only section of the

box where there is a discrepancy among amino acids. This mismatching is the result of the C-to-T nucleotide substitution observed above, translating into the substitution of glycine (G) with arginine (R) for the CFW-C females. The CFW-C males possess a "?" at their respective sites due to the heterozygous nature of the mutation, causing the C-to-T transition observed in Figure 1.

Exon 2- SLC45A2

Similar to Exon 1, a single variable nucleotide site was found at Exon 2 of SLC45A2 in one out of the fourteen individuals sequenced for the gene.

Figure 3: Variable nucleotide sequence of SLC45A2 Exon 2

Exon 2 had one nucleotide transition; the lightback female morph displayed a C-to-T nucleotide substitution. The sequence was translated by Se-Al, generating an amino acid sequence represented by Figure 4. The cytosine to thymine nucleotide shift in Exon 2 resulted in an amino

acid substitution of alanine (A) to threonine (T) for the lightback female morph.

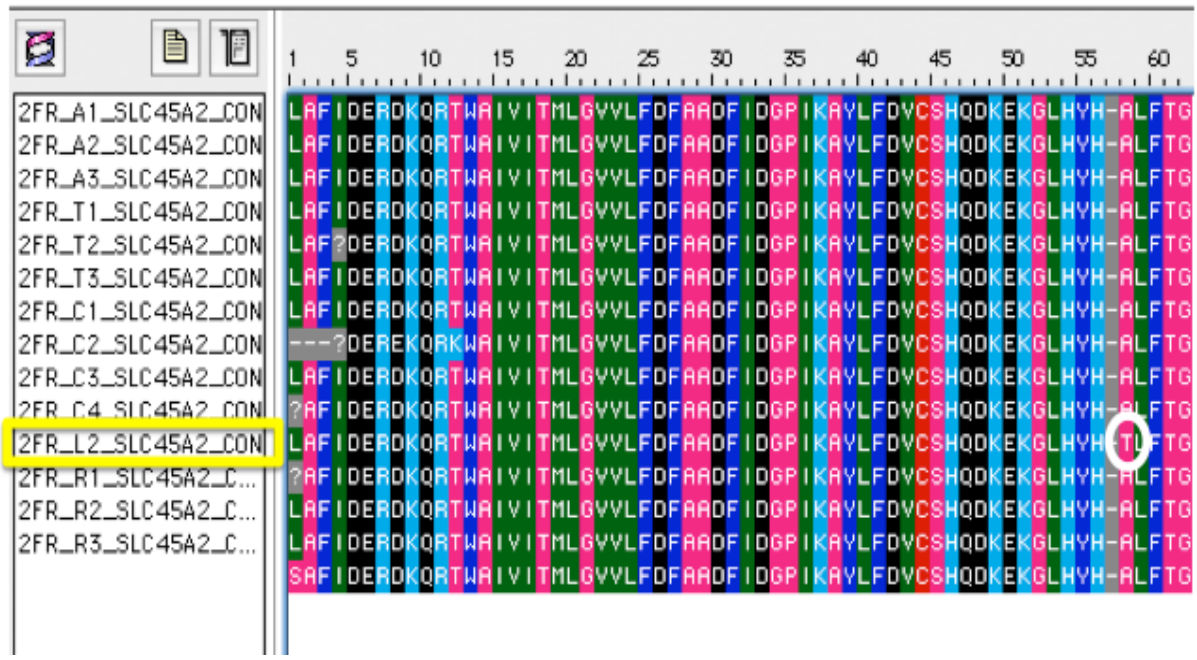

Figure 4: Amino Acid sequence from Figure 3

Exon 7 – SLC45A2

A single variable nucleotide site was also detected in Exon 7 of SLC45A2 in three out of the fourteen individuals. All three timor zebra finch males possessed a thymine nucleotide for a specific site of exon 7, whereas the remaining individuals had a cytosine as their corresponding nucleotide. This C-to-T substitution yielded an amino acid sequence where the timor males had an isoleucine(I) at the 45th amino acid position as compared to the rest of the individuals who had a valine(V) at the same position. Hence, in exon 7 we observed a V-to-I amino acid substitution for the male timor subspecies.

Exon 7 – SLC45A2

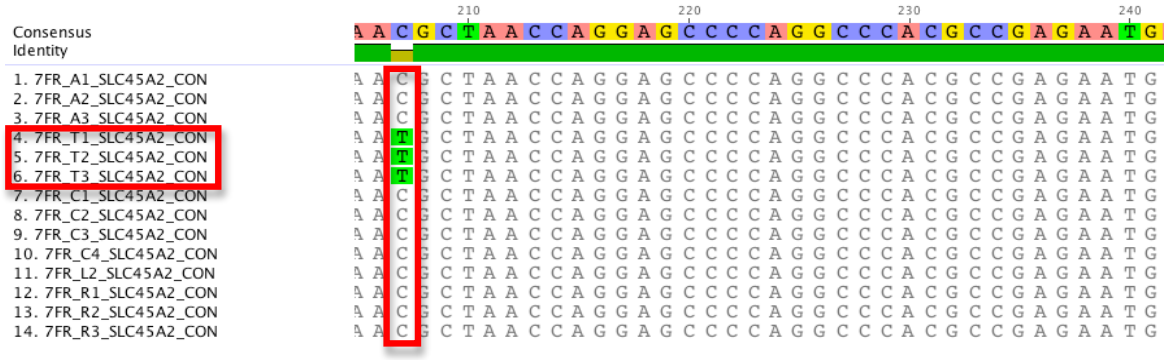

Figure 5: Variable nucleotide sequence of SLC45A2 Exon 7

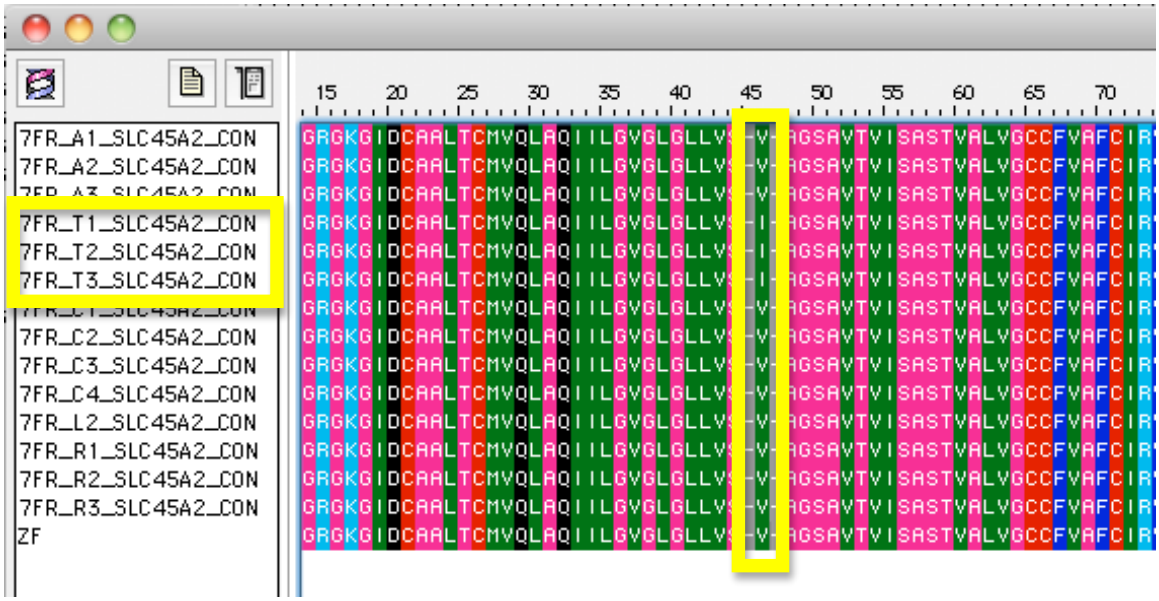

Figure 6: Amino Acid sequence from Figure 5

Discussion

Extensive pigmentation variation occurs in both natural and domesticated populations in the form of different colored morphs (Buggiotti, 2007). As a developing model system for genomic studies, the domesticated zebra finch has proved to be an important resource in the study of evolutionary biology and provides an ideal avian model system in which to explore the roles of TYRP1 and SLC45A2 in plumage coloration. As evident by the phenotypic comparison of the ancestral australian zebra finch to domesticated forms, the quality of melanin-based coat color differed extensively. Thus, in my survey I identified two potentially interesting polymorphisms that could contribute to the observed color differences. In exon 1 of the SLC45A2 gene we found two heterozygous males of the CFW-C mutation. This meets the expectation of what one would expect for a dominant mutation in a homogametic (ZZ) male. We also found an intriguing mutation in exon 2, however it was only supported by a single genomic DNA sample of the lightback female. Though the substitution may seem important, especially compared to the highly conserved nature of the sequences obtained from exon 2, without additional lightback samples we cannot confirm the importance of this C-to-T substitution in the lightback morph. Similar to the results on white tigers (Xu et al. 2013), exon 7 of SLC45A2 in the timor subspecies showed a C-to-T non-synonymous nucleotide substitution on the 207th base pair. The same non-synonymous nucleotide substitution was observed by Xu et al. (2013), but at a different site with different amino acid translations. Data from TYRP1 sequences showed no significant variations among morphs, therefore suggesting that SLC45A2 may contribute to the observed phenotypic differences.

Although we found two mutations in SLC45A2 that are of potential interest, further work needs to be done to confirm the functional significance. Future projects should focus on

sequencing these genes in additional, independently derived samples. Furthermore, experimental crosses can be developed for the identification of coat color pigmentation genes in the domesticated zebra finch. Once an adequate sample of birds exhibiting wide variety of coat color are analyzed, their SNP markers can be identified and aligned to the zebra finch reference genome for further analysis on whether mutations in SLC45A2 or TYRP1 are indeed functionally significant.

Based on other research on our two candidate genes, we were expecting to find substitutions relating to the much-speculated importance of cysteine residues and its involvement with the pigmentation genes on a molecular level (Gunnarsson et al. 2012). The results we obtained, however, did not corroborate previous studies in this regard. Rather, we found substitutions to arginine, threonine and isoleucine from glycine, alanine and valine, respectively. It remains to be determined what the functional implications of these amino acid changes might be. In this respect, our study sets the stage for analyses of pigmentation and its genetic basis in this important avian model.

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