Investigation of Pigmentation-Associated Polymorphisms in a Novel Indigenous American Population

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Acknowledgements

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Abstract

Skin pigmentation is one of the most variable phenotypes among all human populations, and as such, important advances have recently been made to understand the genetic basis underlying normal pigmentation variation among European, Asian, and African populations. However, very little is known about the genes involved in skin pigmentation among Indigenous Americans. Several genome-wide association studies have nominated single nucleotide polymorphism (SNP) markers as significantly contributing to differences between light and dark skinned populations. This study genotyped three polymorphisms that previously have been associated with pigmentation variation in other populations, but whose effects among Indigenous Americans is unknown. Genotypic data was combined with phenotypic melanin index data, and a simple additive regression model was sufficient in revealing significant genotype-phenotype associations. Results show that the *OCA2* rs74653330 polymorphism and the *SLC24A5* rs1426654 polymorphism both associate with pigmentation in this Indigenous American population, and further, they account for approximately 25% of the variation in melanin index. These results corroborate previous research indicating rs74653330 and rs1426654 as important ethnicity and pigmentation related SNPs. These findings are important in relation to ongoing research investigating the role of natural selection in shaping human skin pigmentation.

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Introduction

Our skin is truly what makes us human. The ability to touch, feel, reflect emotions, and meaningfully engage with others, in essence, our humanity, is possible only because of this incredible organ. Our skin is also one of the most fundamental ways in which individuals vary in their appearance, as pigmentation is a remarkably diverse phenotype among all human populations. Our species has long been fascinated with understanding the great variation in skin colors both between, and within, populations. In fact, the tomb of the ancient Egyptian pharaoh, Seti I, displays paintings of a light-skinned Asian man, a dark-skinned African man, and an Egyptian man with an intermediate skin tone (Quillen, 2010).

Biological anthropology is fundamentally concerned with understanding the evolution of the human species and the causes of modern human variation. Further, anthropologists are interested understanding the variable selective pressures that our early ancestors were exposed to as they migrated across the continents. It is for this reason that an evolutionary perspective is particularly apt to explain human pigmentation as it is an incredibly diverse phenotype that has directly been shaped by the hand of natural selection as early humans dispersed across the globe.

Evolutionary Context

Understanding the evolutionary history of human skin is imperative to understanding the diversity of its appearance. However, this task becomes complicated by the fact that skin is nearly impossible to study in the fossil record because it is a soft-

tissue organ that simply does not preserve over time (Jablonski, 2013). In light of these circumstances, we look to our closest living ancestor, the chimpanzee, in order to catch a glimpse into the evolution of our own skin. Traits shared between chimpanzees and humans also would be present in the common ancestor of the two species, and it is on this phenotype that selection would have acted to produce the modern human skin phenotype (Jablonski, 2013).

Chimpanzee skin and human skin share three important properties. First, both species have skin that is thoroughly covered in hair, although it is significantly thinner in humans. Second, both species have skin that is capable of producing sweat, and third, both species can produce melanin in their skin in response to exposure from ultraviolet radiation (Marks 2003; Jablonski 2013). By extrapolating from these shared traits, it is likely that the common ancestor had light skin that was covered with dark hair, with the ability to tan exposed areas of skin. This is the ancestral condition for the hominin lineage (Jablonski, 2013).

When the split occurred between the ancestors of modern chimpanzees and humans, approximately 5-7 million years ago, the chimpanzee lineage retained many similar features because they remained in the forested environment of equatorial Africa. However, the popularized savannah hypothesis states that many novel features in hominins resulted from a shrinking forest and an expanding savannah environment (deMenocal,1995). For example, bipedalism evolved as the most energetically efficient means of locomotion in an open grassland. The elongated legs of Turkana Boy, the *Homo erectus* juvenile discovered in Kenya, provide evidence for this increasingly active lifestyle (Moggie-Cecchi, 2001).

There is no consensus among anthropologists regarding why early *Homo* began to travel long distances and engage in increasingly active locomotion. Perhaps it was to hunt and collect meat, or maybe it was to facilitate social connections with nearby bands. Regardless, it is likely that selection favored hair loss at this point in evolution to expose both the eccrine sweat glands, and the pale skin, that were inherited from our chimpanzee common ancestor. Eccrine sweat glands provide a highly efficient means of maintaining a stable body temperature, especially for our ancestors living under the heat of the African sun (Jablonski, 2013).

At this stage, early *Homo* are highly efficient long distance travelers with an evolved ability to keep their exposed bodies cool. However, it was at this point in hominin evolution that ultraviolet radiation (UVR) became a negative pressure in the environment. UVR is an extreme selective force because it has the ability to damage DNA, which can result in deleterious mutations and prevent proper cell division (Jablonski, 2013). Further, UVR can degrade folate stores in the body. Folate is a vitamin that is necessary to produce DNA, and for this reason, it is absolutely imperative to promoting the proper cell division responsible for embryonic tissue growth and differentiation in a fetus.

Selection acts to increase fitness of individuals. One tenet of fitness is reproductive success, or the ability of an individual to pass their genes to the next generation. Owing to the fact that early *Homo* became more active, selection favored hair loss to expose eccrine sweat glands to keep the body cool. This also had the consequence of exposing the inherited condition of pale skin. Pale, early *Homo* would have had diminished reproductive success because UVR would effortlessly penetrate

the skin of expectant mothers, destroying their stores of folate, and hindering their ability to produce healthy babies.

Without the cultural means to protect themselves against exposure to UVR, early human ancestors had to adapt biologically (Jablonski, 2013). The folate hypothesis postulates that selection favored an increase of melanin in the skin in order to protect folate stores against degradation from UVR (Jablonski, 2004). Melanin is a dark pigment that has the capacity to act as a natural sunscreen and dissipate UVR that is absorbed by the skin. Consequently, phenotypically dark skin evolved as an artifact of selection favoring an increase of melanin to act as a barrier between UVR and folate stores in the body.

Archaeological data supports anatomically modern humans migrating out of Africa approximately 115,000 years ago (Jablonski, 2013). As these peoples began to encounter new habitats, their bodies had to adapt to various environmental pressures. Due to the spherical shape of the earth, there are greater levels of UVR near the equator as compared to the poles (Jablonski, 2013; Figure 1). As humans colonized these remote areas away from the equator, dark skin would no longer be beneficial. Rather, it would be detrimental.

Vitamin D is unique in that it is the only vitamin that humans are capable of synthesizing (Chaplin & Jablonski 2009). It is ingested in the diet from dark, leafy greens, but the primary source is a complex chemical process whereby bare skin that is exposed to UVR can convert sunshine into vitamin D. The issue with having dark skin in lower UVR areas, such as Europe and Siberia, is that melanin will prevent sunshine from penetrating the skin, and thus Vitamin D synthesis will not occur (Chaplin &

Jablonski 2009). Vitamin D is imperative for health because it facilitates the absorption of calcium from the diet in order to build a strong, healthy skeleton (Jablonski 2013). Children who lack proper amounts of Vitamin D develop nutritional rickets, whereby the softened bones of their legs buckle underneath the weight of their body. Additionally, pregnant women who lack sufficient levels of Vitamin D give birth to babies with weakened skeletons.

In order to allow enough sunshine to penetrate the skin to facilitate Vitamin D synthesis, selection favored a decrease in the amount of melanin in the skin (Chaplin & Jablonski, 2009). Given this, the Vitamin D hypothesis explains the evolution of light skin in populations living in low UVR regions as a consequence of the necessity of Vitamin D production.

The competing selective pressures to protect against UVR degradation of folate, while simultaneously allowing UVR penetration to synthesize sufficient quantities of Vitamin D, are responsible for the global gradient of skin colors observed among all populations today. It is for the given reasons that distributions of UVR and skin pigmentation follow a strikingly similar pattern (Figure 1).

Melanin Biology

Pigment of the skin is largely determined by the amount and type of melanin produced and stored in melanosomes, organelles located in specialized melanocyte cells of the epidermis (Norton et al., 2016). Melanin is a complex biopolymer that is found in two primary forms, brown-black eumelanin and red-yellow pheomelanin, and it is the ratio of these two types of melanin, as well as the number of melanosomes, that

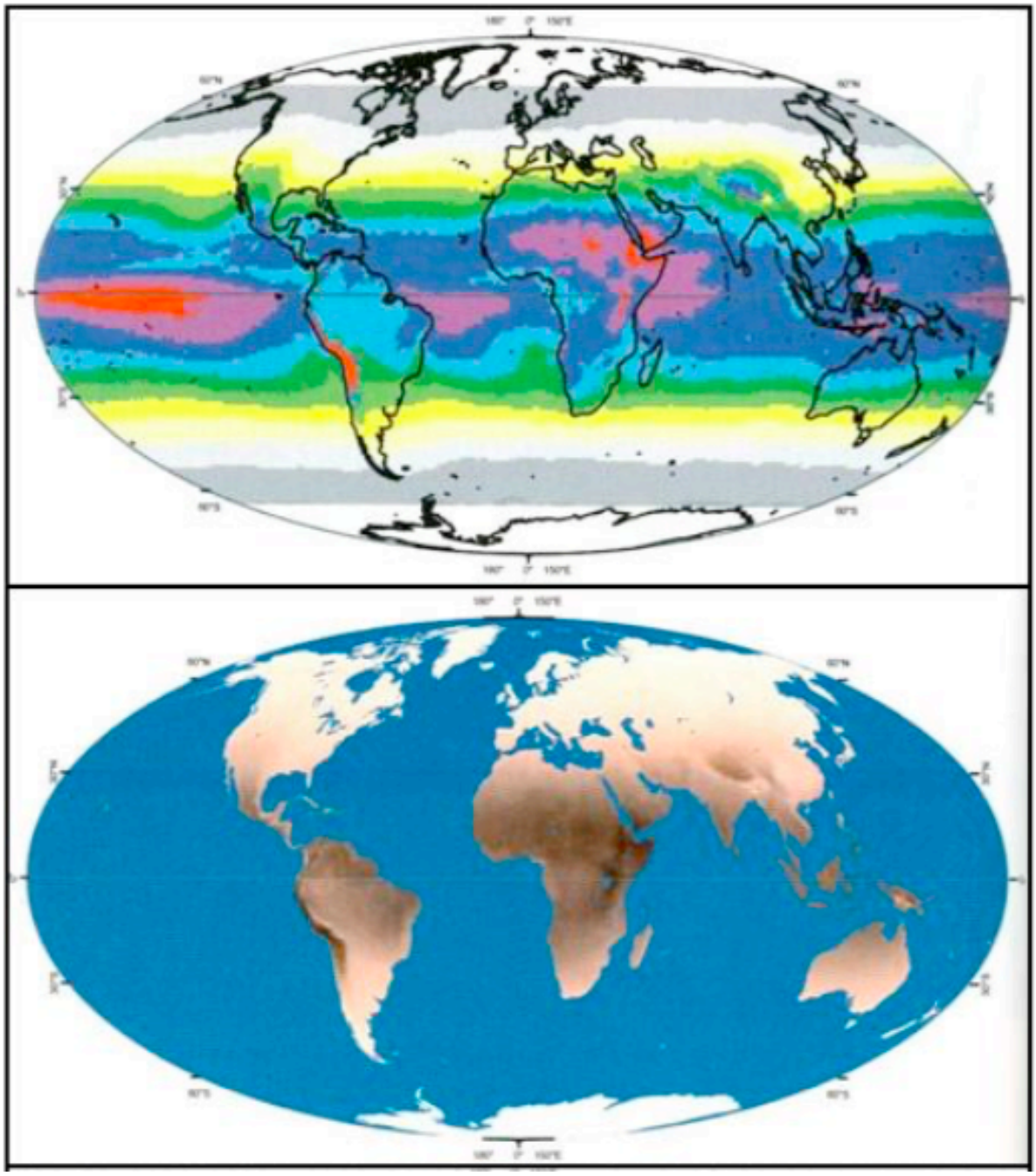


Figure 1: Worldwide distributions of UVR (top) and skin pigmentation (bottom). These distributions confirm the pattern of greater radiation near the equator, where dark skin is favored, and decreased amounts of UVR near the poles, where light skin is favored (*Image from Chaplin & Jablonski, 2000*).

contribute to the variation in skin color (Norton et al., 2016). As skin pigmentation varies among populations, so does melanosome distribution, as illustrated in Figure 2.

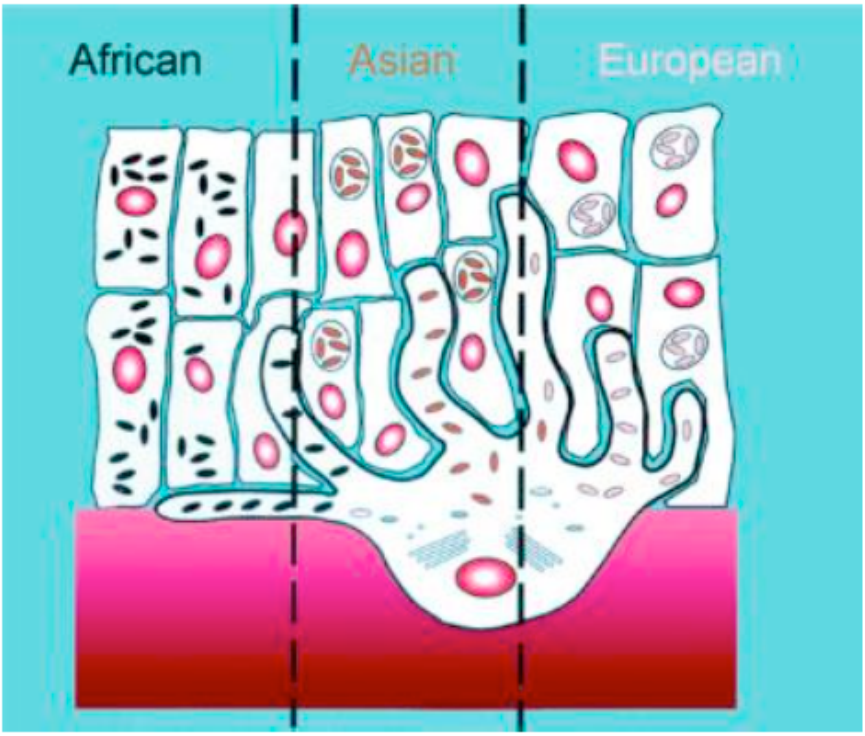


Figure 2: Distribution of melanosomes by population. In lightly pigmented Asian and European populations, melanosomes are smaller and less pigmented, while in darkly pigmented African populations, melanosomes are darker due to higher melanin concentration (*Image from Barsch, 2003)*.

Melanogenesis, or the synthesis of melanin, occurs within the melanosome organelle of melanocytes. This biochemical pathway is highly regulated and under complex genetic control by genes involving transcription factors, membrane proteins, enzymes, and several types of receptors and ligands (Sturm & Duffy, 2012). For these reasons, there are many genes potentially involved in the vast variation observed in human skin pigmentation (Quillen, 2010). Early studies identifying mutations that

eliminated melanin synthesis, resulting in diminished animal coat colors and human oculocutaneous albinism, led to the identification of *TYR*, *OCA2*, *TYRP1*, *SLC45A2* and *HPS6* as pigmentation-associated genes (Sturm & Duffy, 2012). *MC1R* has been demonstrated to interact with ligands on *ASIP* to affect the agouti protein and result in a non-agouti mouse coat color (Sturm, 2009). Another major pigmentation-associated gene is *SLC24A5*, for which both zebrafish and mouse knockouts express a lightened phenotype (Sturm, 2009). Constitutive skin pigmentation is a polygenic trait under the control of multiple loci, and in total, 76 genes have been identified as candidates to explain the diversity of human pigmentation (Quillen, 2010).

Importance of Studying Human Pigmentation

There are many reasons that clarify the importance of studying human skin pigmentation. Understanding the genetics of human pigmentation will enhance our understanding of the evolutionary history of our own species, and supplement our understanding of selection pressures encountered by our early ancestors. Additionally, skin pigmentation serves as an excellent model to characterize phenotypes resulting from an interaction between genetics and the environment.

Further, an evolutionary perspective can be particularly beneficial in combatting the social construction of 'race,' which aligns pigmentation and other physical traits with behavioral and cognitive abilities (Quillen, 2010). Differences in skin color are commonly (and incorrectly) understood as an indication of deeper biological differences among humans (Parra et al., 2004). However, population genetics has revealed that variation within populations is greater than variation between populations. There is no genetic or

biological basis to classify humans into discrete races, and further, 'races' are not genetically homogenous and lack clear-cut genetic boundaries (Yudell et al., 2016). Instead of using skin color as a proxy for the categorization of human populations, the evolutionary perspective is important in teaching that skin color is merely a matter of ancestry, geography, and natural selection. This perspective is invaluable insofar as it emphasizes that all humans are one species shaped by evolution in response to varying environmental pressures, plus random events like mutation and genetic drift, that can falsify racial thinking (Quillen, 2010).

Finally, understanding the genetics of pigmentation is of utmost importance to forensic investigators who are interested in determining, with a high degree of accuracy, the pigmentation phenotype of a perpetrator who leaves behind DNA evidence at a crime scene. The ability to genotype this genetic evidence and obtain a phenotypic description of the perpetrator's skin, hair and eye color would prove invaluable in apprehending suspects.

Objectives

The goal of this research is to understand how previously identified pigmentation-associated single nucleotide polymorphisms (SNPs) function in an Indigenous American population. A SNP is a single base change in a DNA sequence that occurs in a significant proportion of a large population and contributes to various characteristics, including skin pigmentation (Huang et al., 2015). To date, the vast majority of research into human pigmentation genetics has focused on African, European, and Asian populations, all but failing to consider populations in the Americas. This is an area rich

with potential for research as the current body of knowledge regarding the pigmentation of the earliest Americans is filled with several opposing hypotheses (Quillen, 2010).

When our ancestors entered the Americas for the first time, they transitioned from a region of low UVR to high UVR as they trekked southward towards the equator. It is almost certainly the exposure to these enhanced levels of UVR that account for the darkened skin phenotype of contemporary Indigenous Americans. However, debate ensues regarding the pigmentation levels of the first people to enter the New World. Logic holds that these peoples would have experienced selective pressure for light skin to enable survival in the low UVR regions of central and northeast Asia. However, others postulate that these people could already have darkened skin before entering the Americas if they consumed a diet rich with Vitamin D, like the Inuit, which would have removed the selective pressure for light skin.

This study aims to contribute to the lack of previous research in this understudied population, for the mechanism by which evolution acted to produce this phenotype remains unclear. To accomplish these goals, this study genotyped several polymorphisms that have been significantly associated with pigmentation in Old World populations, but have yet to be characterized in New World populations. These genotypes were analyzed for their association to skin pigmentation phenotype, in the hopes of contributing to the understanding of the genetic basis for the variation in pigmentation among Indigenous Americans. Additionally, this study aims to characterize melanin values for individuals with indigenous ancestry, as most studies reporting melanin values do so for admixed Hispanics.

MATERIALS AND METHODS

Study Participants

The data presented in this study were collected as part of a larger study, the aim of which was to understand the role that natural selection has played in allowing some populations to thrive at high altitude. Low-altitude Indigenous Americans were recruited in Palenque, Chiapas, Mexico (Figure 3), between June and August of 2015. Palenque was chosen because of its high concentration of indigenous peoples and its low elevation allowing it to serve as a low-altitude control for understanding Andean adaptations to high altitude (Bigham et al., 2013). Maya individuals were recruited as participants. In order to control for admixture, an individual was recruited to the study if all four of their grandparents spoke a Mayan dialect, such as Ch'ol, Tzotzil, Tzeltal, or Lacandon. Further, participants were asked to provide the names of their parents and any siblings in order to prevent any first degree relatives from participating in the study. In total, 101 participants were recruited from areas in and around Palenque. All individuals provided informed and written consent according to guidelines approved by the Institutional Review Board at the University of Michigan.

Height, weight, measurements of skin, eye, and hair pigmentation, three-dimensional photographs of facial morphology, hemoglobin, lung volume, and arterial oxygen saturation were collected from each participant.

Skin Pigmentation Measurement

The DSM II Dermaspectrometer (Cortex Technologies, Hadsund, Denmark) was used to obtain quantitative measurements of skin pigmentation, as spectrometry is

considered the “gold standard” for assessing pigmentation. Three independent measurements were consecutively taken from the inside of the upper left arm, the inside of the upper right arm, and the forehead. Melanin content on the inside of the arm is reflective of constitutive pigmentation given its low exposure to solar UV. Forehead measurements of melanin are a better value of facultative pigmentation, which is one’s skin color that results from exposure to UV light and other environmental factors. Melanin Index (M), RGB Wavelength, and CIELAB color dimensions were obtained from each reading. Here, I use M Index as the quantitative measure of skin pigmentation because melanin is the pigment that gives human skin it’s color. To calculate the M value for each participant, an average was taken from the six total measurements on the left and right arms. Higher M values correspond to darker skin, and lower M values correspond to lighter skin.

Figure 3: Location of the study recruitment site in Palenque, Chiapas, Mexico, shown enveloped within the larger area that the Maya inhabited during the peak of their civilization between ~2600 BC and ~1800 AD. Palenque is located approximately 140 meters above sea level and resides at a latitude of 17.48° North.

DNA Extraction

Participants donated 10ml of whole blood that was drawn from a forearm vein by a certified nurse. The blood was field stabilized with red blood cell (RBC) Lysis Solution and Cell Lysis Solution (Qiagen, Valencia, CA). Samples were then transported to the University of Michigan where DNA was extracted following the Puregene protocol according to the manufacturer's instructions (Qiagen, Valencia, CA).

Determination of Target SNPs

To date, there has been no published research showing an association between Indigenous American skin pigmentation and specific SNPs. In light of this, I selected SNPs to genotype that previously have been indicated to play a role in pigmentation in other populations, but whose effects in Indigenous Americans are unknown. Additionally, the SNPs that I selected were all nonsynonymous mutations that alter the amino acid sequence of the protein.

The first SNP that I selected was rs74653330 located in the *OCA2* gene on chromosome 15. *OCA2* has repeatedly been shown to play a role in pigmentation. This gene is involved in hair and skin color of European populations (Cook et al., 2009), and a polymorphism (rs12913832) in an upstream promoter region is significantly associated with European blue eye color (Sturm & Larsson, 2009). The *OCA2* polymorphism rs74653330 is particularly interesting to study given that this Alanine to Threonine mutation at position 481 (Ala481Thr) results in approximately 70% of the function of the wild type allele in melanogenesis (Yuasa et al., 2007). Further, Eaton et al. (2015) found this polymorphism to be significantly associated with normal skin

pigmentation variation in a sample of individuals living in Canada with East Asian ancestry.

The second SNP that I selected was rs1426654 in *SLC24A5* on chromosome 15. This SNP is a threonine to alanine nonsynonymous change at position 111 (Thr111Ala). Lamason et al. (2005) found that a mutation in *SLC24A5* significantly lightens the pigmentation of zebrafish, resulting in the *golden zebrafish* phenotype. The rs1426654 polymorphism is the orthologous human variant of the *golden* mutation. Subsequently, there have been many studies investigating the effects of this SNP on skin pigmentation (Dimisianos, 2008; Basu et al., 2013), but they have focused on European, Asian, and African populations. It has been noted in the literature that the detailed relationship between rs1426654 and pigmentation still needs more experimental evidence (Huang et al., 2015).

The third SNP that I selected was rs26722 in *SLC45A2* on chromosome 5. This gene carries the rs16891982 polymorphism that results in a leucine to phenylalanine protein change at position 374 (Leu374Phe). This polymorphism is significantly associated with olive skin and dark hair. Furthermore, it displays a strong north-south gradient in European populations (Sturm & Larsson 2009). rs26722 is another coding variant in this gene (Glu272Lys) that has been implicated to play a role in the degree of lightening of Caucasian populations (Graf et al., 2005). The rs26722 polymorphism was selected for genotyping in this study because the rs16891982 polymorphism has already been associated with light-skinned European ancestry (Soejima & Koda, 2007), and for this reason, was not hypothesized to yield significant results in an Indigenous American population.

Amplification of Target SNPs

Polymerase Chain Reaction (PCR) was performed to amplify the regions of the gene containing each of the polymorphisms (rs74653330, rs1426654, and rs26722).. PCR was performed in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA). Each PCR for the three SNP's was optimized and run under different optimized conditions. For the *OCA2* SNP rs74653330, the forward primer (5"-3") ATGTGGGCCTTTCACGATGT and the reverse primer (5"-3") GTGAAGAGGTGGCGTGATGA were used under the conditions of 2 μL of DNA, 5 μL of 10x PCR buffer, 3 μL of 50 mM MgCl2, 2 μL of 1.25mM dNTP's, 0.25 μL of 20 μM Taq DNA Polymerase, and 0.5 μL of each forward and reverse primer. All reagents were manufactured by Promega (Madison, WI) and the annealing phase occurred for 30 seconds at 60 °C. These conditions yielded a 314 bp fragment. For the *SLC24A5* SNP rs1426654, the forward primer (5"-3") ACCCTATGTTTGGCACTCAGG and the reverse primer (5"-3") TGTCCATCCCAGACGACGA were used under the conditions of 2 μL DNA, 5 μL of 10x PCR Buffer, 2.5 μL of 50 mM MgCl2, 4.0 μL of 1.25 mM dNTP's, 0.25 μL of 20 μM Taq DNA Polymerase, and 0.5 μL of each forward and reverse primer. All reagents used were Promega and the annealing phase occurred for 30 seconds at 58°C. These conditions yielded a 605 bp fragment. For the *SLC45A2* SNP rs26722, the forward primer (5'-3') TCAGTCACCAACCCAAGGAT and the reverse primer (5'-3') TGTCTTCAGGGAGTTTTCCCAC were used under the same reaction conditions as rs1426654. This SNP had an annealing temperature of 56 °C and yielded an 809 bp fragment.

Restriction Digestion of Target SNPs

Following PCR, digestion with a restriction enzyme was performed in order to cut the DNA fragments into differential band lengths to allow for genotyping on an agarose gel. The amplified segment of the *OCA2* SNP rs74653330 was digested with the restriction enzyme ApeKI, which cuts between G and C in the sequence (5”-3”) G↓CWGC, where the arrow denotes the enzyme cut site. The *SLC24A5* SNP rs1426654 was digested with the restriction enzyme HhaI, which cuts between G and C in the sequence (5”-3”) GCG↓C. The *SLC45A2* SNP rs26722 was digested with the TaqI restriction enzyme. This enzyme cuts between T and C in the sequence (5”-3”) T↓CGA.

Gel Electrophoresis Genotyping

All three polymorphisms were visualized on a 2% agarose gels with ethidium bromide staining. Ten μL of digestion product was combined with 10 μL of 2x OJ dye and run on an agarose gel for 230 volts for 45 minutes.

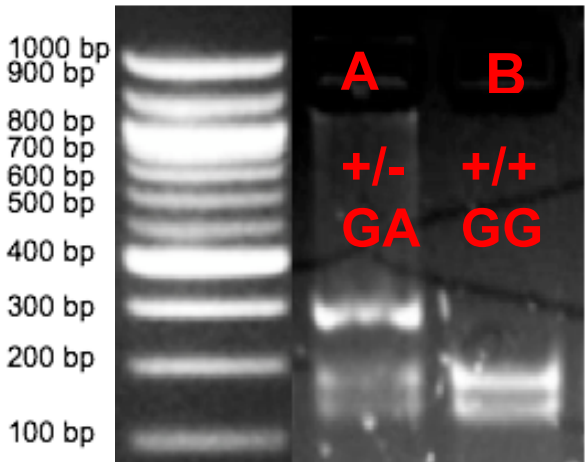
For the rs74653330 polymorphism, if an individual has the GG genotype, two G alleles will be cut resulting in bands of 134 bp and 180 bp (Figure 4B). If an individual has the GA genotype, 1 G allele will be cut, resulting in bands of 134 bp, 180 bp, and 314 bp (Figure 4A). If an individual has the AA genotype, the enzyme will not cut, resulting in a band of 314 bp. For the rs1426654 polymorphism, if an individual has the GG genotype, two G alleles will be cut resulting in bands of 276 bp and 329 bp (Figure 4D). If an individual has the GA genotype, 1 G allele will be cut, resulting in bands of 276 bp, 329 bp, and 615 bp (Figure 4C). If an individual has the AA genotype, the

enzyme will not cut, resulting in a band of 615 bp. Lastly, for the rs26722 polymorphism, if an individual has the TT genotype, the enzyme will not cut, resulting in a single band of 809 bp (Figure 4E). If an individual has the CT genotype, one C allele is cut, resulting in bands of 352 bp, 457 bp, and 809 bp (Figure 4F). If an individual has the CC genotype, 2 C alleles will be cut, resulting in bands of 352 bp and 457 bp (Figure 4G).

Statistical Analysis

Statistical analysis was performed using the R statistical package. Mean M index values were calculated for the entire cohort, as well as for each sex individually and for each specific genotype category. Welch's two sample t-test was performed to assess if there was a significant difference in melanin between the sexes. Linear additive regression model analysis was performed to search for associations between melanin and age, and melanin and genotype.

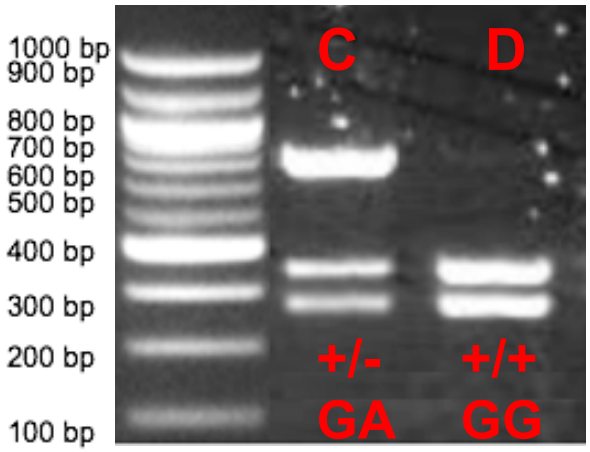
Figure 4: Gel Genotyping



***OCA2* SNP rs74653330**

A: One G allele is cut resulting in bands of 134 bp, 180 bp, and 314 bp.

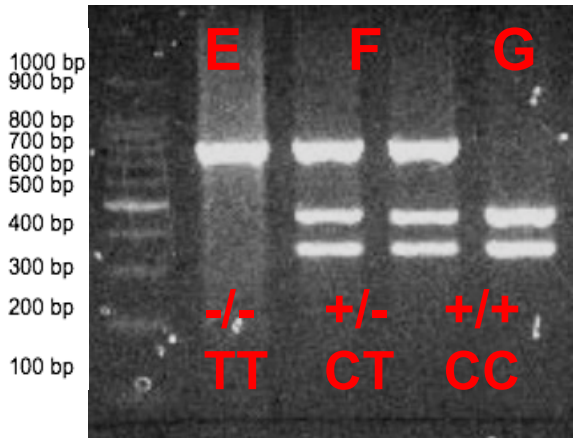
B: Two G alleles are cut resulting in bands of 134 bp and 180 bp.



***SLC24A5* SNP rs1426654**

C: One G allele is cut resulting in bands of 276 bp, 329 bp, and 615 bp.

D: Two G alleles are cut resulting in bands of 276 bp and 329 bp.



***SLC45A2* SNP rs26722**

E: No cuts result in one band of 809 bp.

F: One C allele is cut resulting in bands of 352 bp, 457 bp, and 809 bp.

G: Two C alleles are cut resulting in bands of 352 bp and 457 bp.

RESULTS

Population Data

Of the 101 participants recruited for the study, 50 were female, 51 were male, and they ranged in age from 18 to 35 years. Collectively, their melanin index (M) values ranged from (39.55 - 60.37), with an average M value of (49.04 ± 4.18) (Table 1). The mean M value for males was 48.5523, and the mean M value for females was 49.5306 (Figure 5). There was no significant difference in M values by sex (Table 2). Additionally, age did not correlate with M index for this sample of 18-35 year olds (R^2 = 6.88e-07, p=0.993). A table of age, melanin index values, and individual genotypes for each participant are provided in Appendix A.

Table 1: Study participant characteristics.

	M Range	**Mean M**
Entire Cohort	39.55-60.37	49.04 ± 4.1832
Males	39.55-57.56	48.55 ± 4.20
Females	39.64-60.37	49.53 ± 4.15

Table 2: Data from Welch's two-sample t-test to test for a significant difference in melanin index between males and females.

Mean Male M Value	**Mean Female M Value**	**t-statistic**	**p-value**	**Conclusion**
48.5523	49.5306	1.1773	0.2419	No significant difference

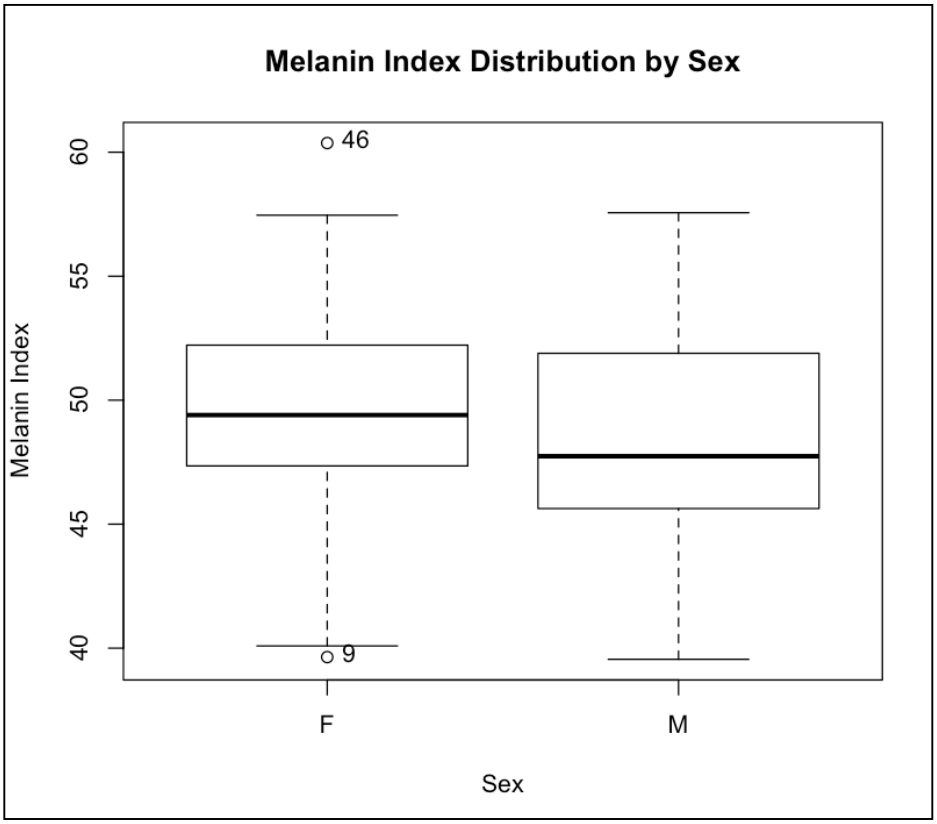


Figure 5: A boxplot shows similar melanin index distributions for both sexes.

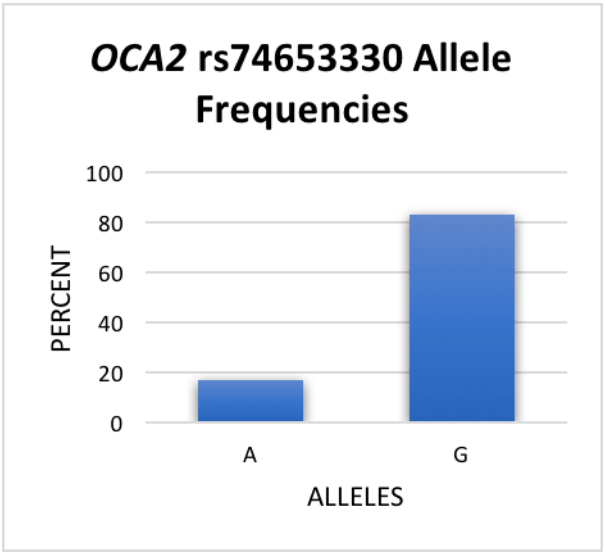
SNP Associations with Skin Pigmentation

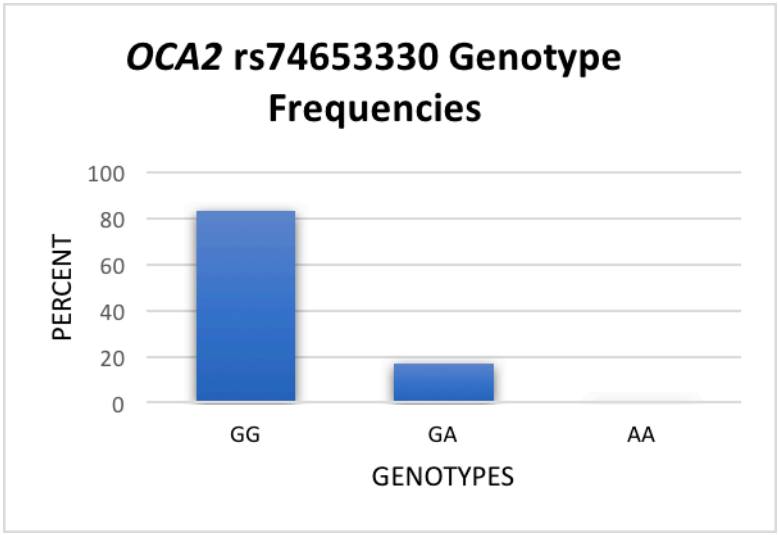
Genotypic and allelic frequencies were calculated for each polymorphism from the sample of 101 study participants (Figure 6 and Table 3). For the rs74653330 SNP, there were 17 GA heterozygotes, 84 GG homozygotes, and 0 AA homozygotes. For the rs1426654 SNP, there were 6 GA heterozygotes, 95 GG homozygotes, and 0 AA homozygotes. The rs26722 SNP showed the greatest genotypic variation with 46 CT heterozygotes, 9 CC homozygotes, and 46 TT homozygotes.

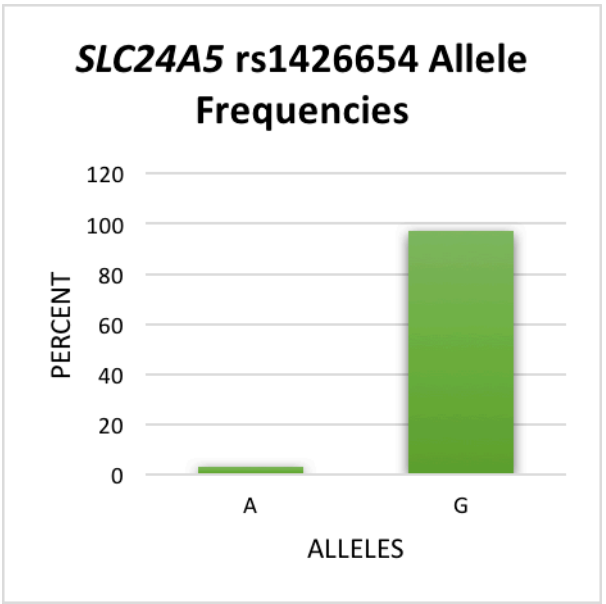
Table 3: Allele and genotype frequencies for each polymorphism genotyped in the Indigenous American cohort.

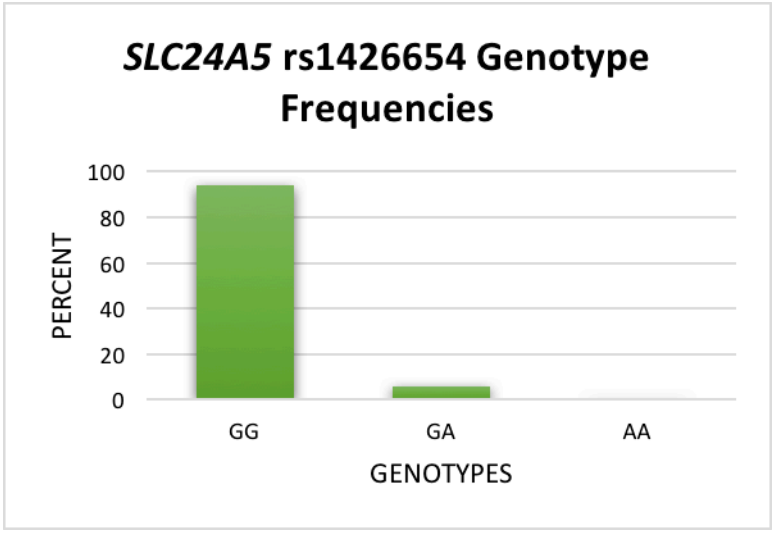
Gene	SNP ID	Alleles	Allele Frequencies	Genotype Frequencies
OCA2	rs74653330	A	8.4%	GA: 16.8%
		G	91.6%	GG: 83.2%
SLC24A5	rs1426654	A	3%	GA: 5.9%
		G	97%	GG: 94.1%
SLC45A2	rs26722	C	31.7%	CC: 8.9%
				CT: 45.54%
		T	68.3%	TT: 45.54%

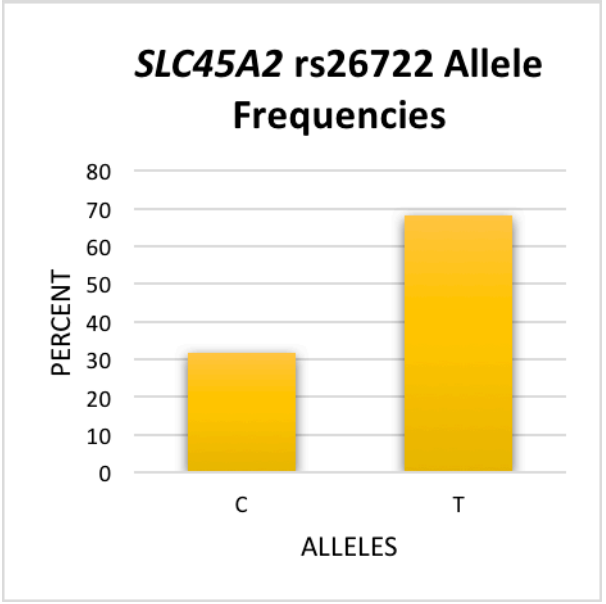
Figure 6: Allele and Genotype Frequencies

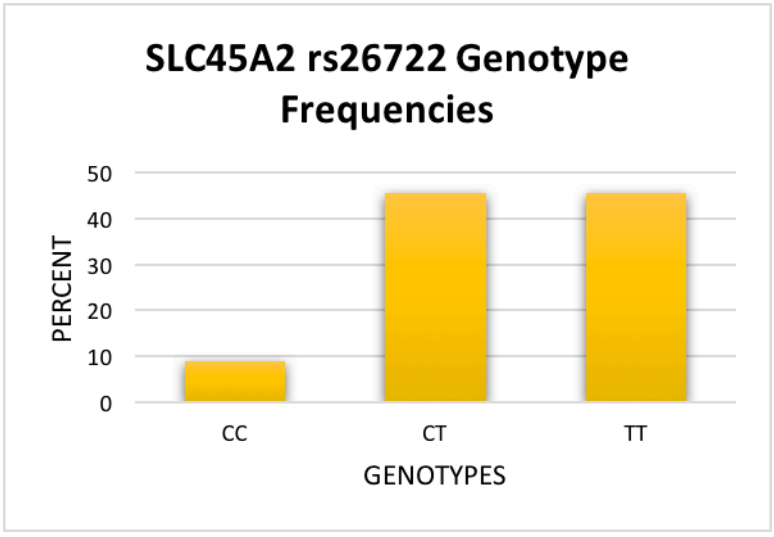












For the rs74653330 SNP, the mean M value for the GA genotype was 45.1435, while the mean M value for the GG genotype was 49.8245 (Figure 7A). In an additive regression model for the dominant allele G, the addition of a single A allele decreased the melanin index by approximately 5 units. Further, this polymorphism explains about 20% of the variation in melanin index. This correlation was found to be statistically significant at the 1% level after applying a Bonferroni correction for multiple tests.

The rs1426654 polymorphism also was found to be correlated with melanin index, although slightly less significantly. The mean M value for the GA genotype was 45.9300, while the mean M value for the GG genotype was 49.2384 (Figure 7B). In an additive regression model for the dominant allele G, the addition of an A allele decreased the melanin index by approximately 3 units. This association is significant at the 10% level, but after correction for multiple tests, this p-value is no longer significant.

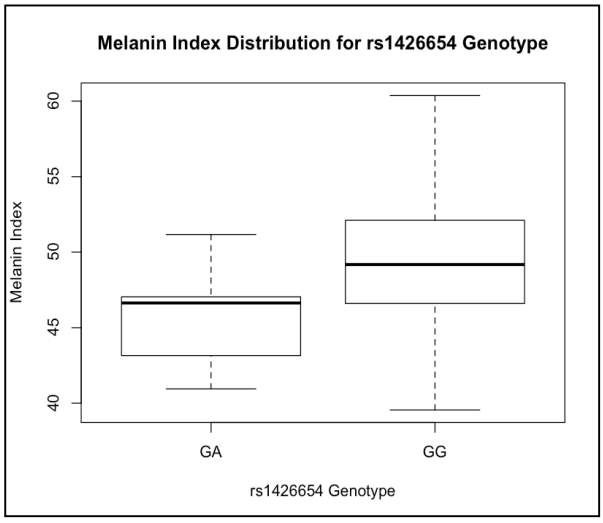
In contrast, the rs26722 polymorphism was not found to be significantly correlated with melanin. In a regression model for the dominant allele T, the addition of a single C allele only decreased melanin by 0.65 units. The mean M value for the CC genotype was 47.978, the mean M value for the CT genotype was 48.922, and the mean M value for the TT genotype was 49.358 (Figure 7C).

Table 4: Linear regression additive model for melanin index.

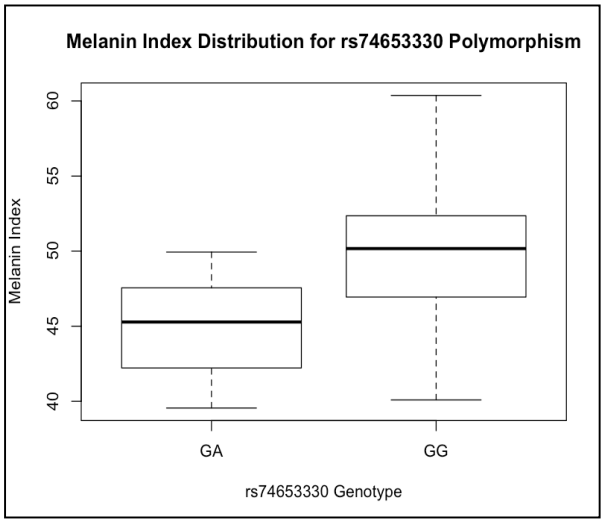
SNP	Gene	Alleles	Minor Allele	MAF	Estimate	R^2	p-value*
rs74653330	*OCA2*	GA	A	8.4	-4.87	0.20	0.00001
rs1426654	*SLC24A5*	GA	A	3	-3.17	0.05	0.07880
rs26722	*SLC45A2*	CT	C	31.7	-0.65	0.02	0.32500

$p<0.017$ for $\alpha = 0.05$, 3 tests

A.



B.



C.

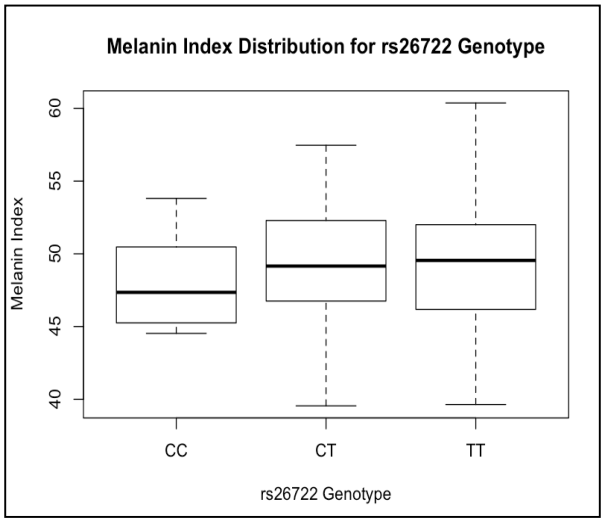


Figure 7: A boxplot showing the distribution of melanin index for each SNP genotype.

Sex, age, and the rs26722 polymorphism were not associated with M index for this Indigenous American population. However, two SNPs, rs74653330 and rs1426654, were found to significantly correlate with melanin index. A regression model created using both of these SNPs explained about 25% of the variation in melanin index (Table 5). Further, the rs1426654 SNP became more significant (p=0.00876) when in the

model included the rs74653330 polymorphism than compared to an independent model (p=0.0788).

Table 5: Regression data from a model with rs74653330 and rs1426654.

	Estimate	**p-value**	**R^2**	**p-value**
rs74653330	-4.0883	0.0199	0.2553	9.49e-06
rs1426654	-5.1509	1.15e-06		

DISCUSSION

Summary of Major Findings

This study found statistically significant associations between two polymorphisms, *OCA2* SNP rs74653330 and *SLC24A5* SNP rs1426654, and melanin values. Further, these two polymorphisms combined in a regression model explained approximately 25% of the variation in melanin index. These findings are significant because it is the first time that either SNP has been associated with skin pigmentation in an Indigenous American population, and further, they account for a considerable amount of variation in the phenotype.

Study Findings in the Context of Previous Research

Skin pigmentation is one of the most variable phenotypes among all populations, and it has long been accepted that these differences are adaptive. Overwhelming evidence points to darker skin phenotypes having been selected to protect folate stores against the deleterious effects of solar UVR exposure in tropical climates. Conversely,

lighter skin phenotypes were selected for in the northern hemisphere due to low environmental exposure to UVR, which is critical for the synthesis of Vitamin D (Sturm & Duffy, 2012). These competing selective pressures are responsible for the contemporary global gradient of skin color.

For these reasons, we expect populations residing near the equator to have darker skin, and thus higher M values, relative to populations who live at more extreme latitudes. One study reported individuals of African ancestry to have an average M value of 56.62 (Shriver & Parra, 2000). This is more darkly pigmented than individuals from European populations who were reported in another study have an average M value of 35.30 (Norton et al., 2016). We would expect individuals of Indigenous American ancestry to have M values intermediate between Europeans and Africans because this group resides at a latitude in between these two populations. Further, we would expect Indigenous Americans to have a lower M value than Africans because they have inhabited the region near the equator for less time, meaning that selection for dark skin has not acted upon this population for as long. The results of this study confirm these expectations, showing the average M value of this cohort to be 49.04 (Figure 11).

Norton et al. (2016) performed a similar study whereby the melanin index values of individuals who self-identify as Hispanic were obtained. The M values reported for this group (n=33) of admixed Hispanics was 39.9 ± 6.2. This is notably less than the non-admixed indigenous population from this study whose M values were recorded as 49.04 ± 4.18. The non-admixed sample also displayed less variation in melanin values. This illustrates the importance of considering variation in ancestry levels when designing anthropological studies.

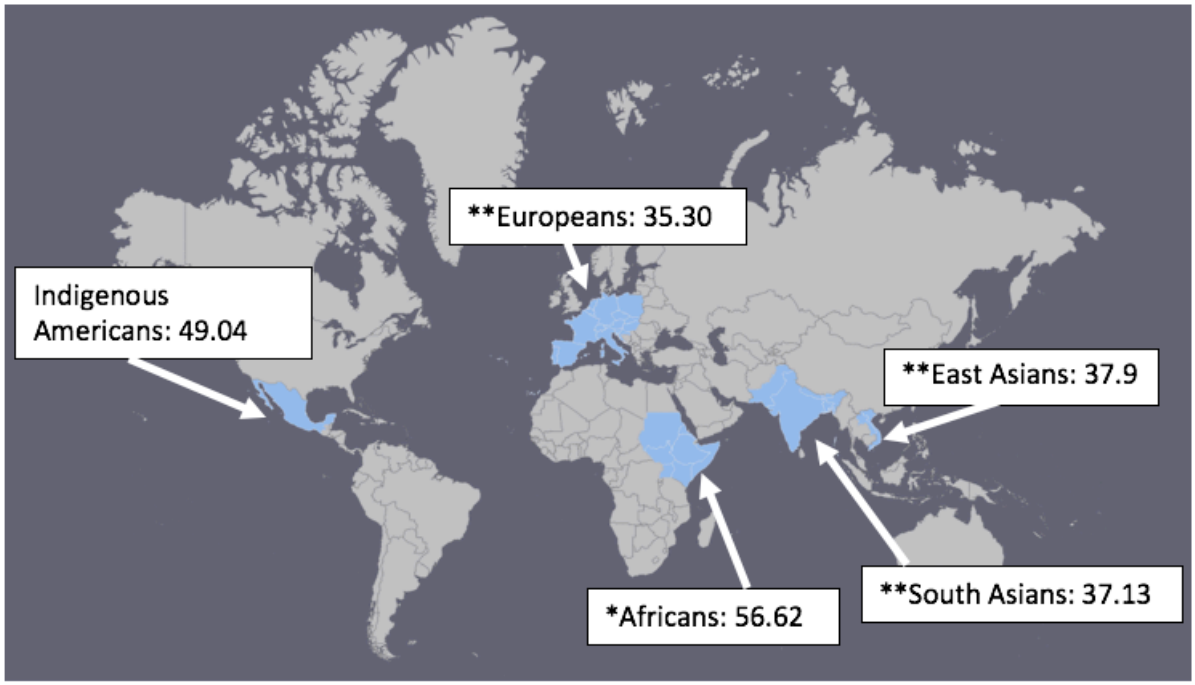


Figure 8: Map showing average melanin values for different populations. The Indigenous American data was obtained from this study.
* = Shriver, Parra, 2000; ** = Norton et al., 2016

When comparing males and females, several studies have supported sexual dimorphism with regard to skin pigmentation in many distinct populations. Darwin himself discussed his observation that men appear to be darker than women of the same population in *The Descent of Man* (Madrigal & Kelly, 2007). Males have been documented to have darker skin than their female counterparts in Island Melanesia (Norton et al., 2006), Belize (Byard & Lees, 1982), Papua New Guinea (Harvey, 1985), Korea (Roh et al., 2001), and among the Quechua peoples (Conway & Baker, 1972). Further, Jablonski and Chaplin (2000) selected data points from populations for which skin reflectance values had been published, and confirmed that females are consistently lighter than males.

In explaining these observations, Jablonski and Chaplin (2000) hypothesize that the lighter skin pigmentation of females is needed to permit relatively greater amounts of UV light to penetrate the skin, allowing for extra Vitamin D synthesis to support the additional calcium needs during pregnancy. This is supported by data that female pigmentation decreased around the time of menarche (Robins, 1991). It is also possible that pigmentation differences between the sexes are a result of differential behavior patterns. Males could experience greater exposure to UVR due to differences in clothing habits or activity patterns (Norton et al., 2006). Further, sexual selection has also been proposed to explain this phenomenon by postulating that the cross-cultural preference among men for lighter pigmented women is due to a perceived linkage between light-skinned women and fecundity (Madrigal & Kelly, 2007).

However, this study found no statistical difference in melanin indices between the sexes of individuals with Indigenous American ancestry. This finding directly contradicts previous research supporting sexual dimorphism of skin color, even in New World populations from Belize and Peru (Byard & Lees, 1982; Conway & Baker, 1972). There are multiple ways of interpreting this finding. Perhaps our sample size of 101 individuals, split almost equally with 50 females and 51 males, was too low to detect variation in melanin, and there truly is sexual dimorphism among Indigenous American populations. On the other hand, the studies supporting sexual dimorphism of skin color in other Indigenous American populations used spectrometry technology that is now almost 45 years old, and perhaps the modern spectrometer used in this study (DSM II Dermaspectrometer, Cortex Technologies, Hadsund, Denmark) had greater accuracy in determining melanin index values. This explanation would support the results from this

study in that there is no sexual dimorphism of skin color among Indigenous American populations. A possible explanation for this unexpected finding could be that among Indigenous American populations, such as the Maya, cultural practices allowed women to receive equal amounts of UVR as the men, so that they would have experienced equal exposure to the selective pressure of UVR.

The polymorphism that most significantly associated with melanin index was rs74653330 in *OCA2* (p=5.62e-06). This finding supports previous research highlighting the role of *OCA2* in pigmentation. For example, this locus is the major determinant of brown-blue eye color (Sturm & Frudakis, 2004) and multiple mutations in this locus result in oculocutaneous albinism type II (Yuasa et al., 2007). It is thought that the *OCA2* protein is a melanosomal membrane protein that has a potential role in trafficking other proteins to melanosomes (Hoyle et al., 2011).

The *OCA2* rs74653330 variant allele A, which is responsible for the Ala481Thr nonsynonymous mutation, has 70% of the function of the wild-type *OCA2* protein (Yuasa et al., 2007). This allele is at highest frequency in Northeast Asia (Sturm & Duffy, 2012). In particular, the variant allele is observed at 24% and 13% frequency among the Siberian Buryat and Khalha indigenous groups, respectively. Its frequency among the Han Chinese was 7.5%. Additionally, this variant allele failed to be detected in a sample of 3,000 Europeans (Yuasa et al., 2007). The results of the present study found the frequency of this allele to be 8.4% among Indigenous Americans. Combined with data demonstrating this allele's hypofunction compared to the wild type allele, it is theorized that this allele was selected for in a region of low UVR and spread northeast

(Yuasa et al., 2007). Lack of this allele in Europe also suggests that the evolution of light skin among East Asian and European populations was convergent.

This allele was detected in this study because it reflects the migration and gene flow of East Asian individuals into the New World. This study also supports the role of this allele in depigmentation because the presence of a single A allele decreased melanin by approximately 5 units. It is interesting to note that in a study of individuals of East Asian ancestry, the presence of the A allele decreased melanin by only 1.9 units (Eaton et al., 2015). These results support previous research indicating the rs74653330 derived allele as being under selection for depigmentation in low UVR regions.

The *SLC24A5* gene also plays an important role in pigmentation. Lamason et al. (2005) found that the *golden* mutation in this locus results in a lighter pigmentation phenotype in zebrafish (Figure 12). The authors found that the rs1426654 polymorphism is the orthologous human variant to this *SLC24A5* zebrafish mutation. This human SNP is responsible for the nonsynonymous Thr111Ala mutation. The A (Thr) variant is nearly fixed in European populations, while the G (Ala) variant ranges from 93%-100% frequency in East Asian and Indigenous American populations. This is supported by results from this study, where the G variant is present at 97% frequency.

Further, Lamason et al. (2009) suggest that the skin lightening effects of the A allele are dominant to the G allele. This hypothesis is confirmed by the results of this study, whereby the presence of an A allele decreased melanin by approximately 3 units ($p=0.0788$). These data also align with previous research in which the AA genotype is correlated with lighter traits, and the GG genotype is correlated with darker traits (Lima et al., 2015). In this study, while there were no AA homozygotes, individuals of the GA

genotype had an average melanin value of 45.93, while individuals of the GG genotype had an average melanin value of 49.2384. These findings are important in corroborating previous research identifying rs1426654 of *SLC24A5* as a significant ethnicity and pigmentation related SNP.

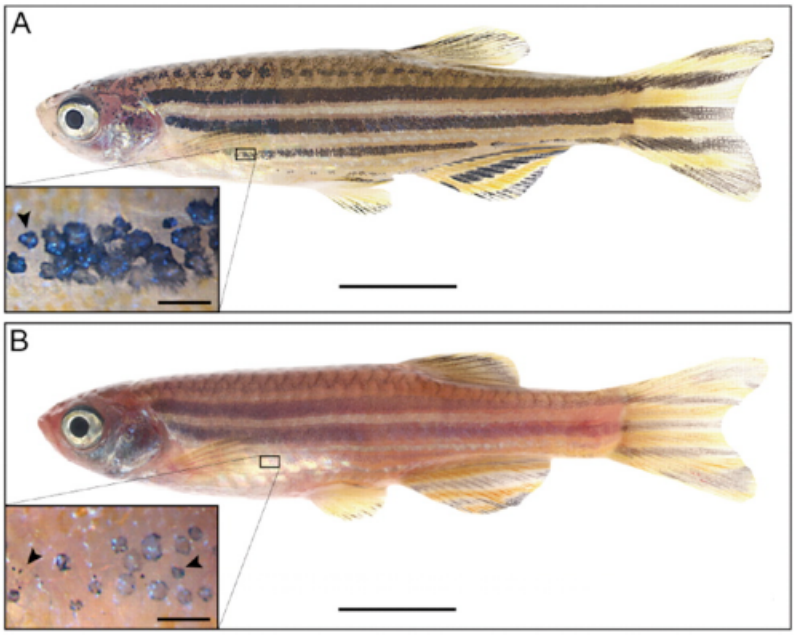


Figure 9: Lateral views of adult wild-type and *golden* zebrafish. Insets show that adult *golden* zebrafish have melanopores that are smaller, more pale, and transparent as a result of this mutation's reduction in the development of melanin. The *golden* mutation in zebrafish is orthologous to the human *SLC24A5* variant rs1426654 (Lamason et al., 2005).

Lastly, *SLC45A2* is considered to be one of the most important genes affecting human pigmentation because it encodes a transporter protein involved in melanin synthesis (Branicki, 2008). Given this gene's importance in influencing pigmentation, the rs26722 polymorphism at this locus was genotyped in this study. It was selected because of its Glu272Lys protein change and previous research linking this polymorphism to dark hair phenotypes among Europeans (Sturm & Duffy, 2012). However, in contrast to the promising results from the other polymorphisms in this

study, rs26722 was not found to associate with skin pigmentation in Indigenous Americans (p=0.325). rs26722 also does not associate with pigmentation among South Asians (Stokowski, 2007). In spite of this, these results are still important in supporting the convergent evolution of light skin between European and Asian populations, with *SLC45A2* being under selection in Europe, but not in Asia (Quillen et al., 2012).

A regression model was created combing both significant SNPs, rs74653330 and rs1426654. This model proved to be significant (p=9.49e-06) and explained approximately 25% of the variation in melanin index, highlighting the importance of both of these polymorphisms in explaining variation in skin pigmentation among Indigenous Americans. Further, the rs1426654 polymorphism became more significant when in a model with rs74653330 (p=1.15e-06) than in an independent model (p=0.0788). However, in explaining this result, it is not likely that these two SNPs function together as they are on different genes. Rather, it is a more probable scenario that the rs74653330 variable absorbed some of the residual variability, thus increasing the power of the statistical test on rs1426654.

Strengths and Weaknesses

This study derives strength from being the first to investigate an association between polymorphisms and pigmentation in an understudied Indigenous American population, as anthropological pigmentation studies have exclusively focused on European, African, and Asian populations. Recently, research has emerged to identify candidate genes under selection for pigmentation in Indigenous Americans (Quillen, 2010; Quillen et al., 2012). However, the present study still remains the first to

investigate the effect of specific SNPs in this population. Moreover, SNPs to investigate were strategically selected based on previous research in other populations, which contributed to the significance of the results. Another strength is that admixture was controlled for by selectively choosing participants whose grandparents all spoke a Mayan dialect. This quality control ensures that the results reflect the genetics of truly indigenous peoples. Further, this study supports the use of association studies to uncover meaningful genotype-phenotype relationships. Simple additive regression models were sufficient to detect significant associations between genetic and quantitative pigmentation data. Lastly, this study has published melanin index values for 101 Indigenous Americans (Table 9). This is significant as there are relatively few databases or papers providing access to spectrometer melanin index data for any population, much less for Indigenous Americans.

Despite the strengths of this study, considerations remain that should be accounted for in future study designs. First, I would advocate for a more robust study size, as there were only 101 individuals included for analysis in the present study. Obtaining a larger cohort would be important to provide greater statistical power. Additionally, I would include more variation in the population used for analysis. This study only included individuals from one group of Indigenous Americans, the Maya. In the future, other indigenous groups should be included in order to make more broad claims about Indigenous American pigmentation phenotypes. Last, population substructure was not accounted for as a covariate in any of the statistical analyses. This can be controlled for by including a principal component analysis (PCA), whose data

would help to control for the confounding effect of population substructure on the genetics of human skin pigmentation.

Conclusion

Skin pigmentation is a highly variable phenotype and remains one of the most fundamental ways in which individuals vary in their appearance. Understanding contemporary variation in human pigmentation requires an understanding of the selective pressures our early ancestors faced as they migrated across the globe, as well as an understanding of how selection targeted pigmentation-associated genes to increase the fitness of these peoples. The present study investigated the genetic basis of the skin pigmentation phenotype among Indigenous Americans. Two polymorphisms, rs74653330 and rs1426654, were both identified to significantly contribute to the variation in this phenotype. These results are important in relation to ongoing research investigating the role of natural selection in shaping human skin pigmentation.

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Appendix A

Sex, age, melanin index value, and genotype of each polymorphism for all participants.

SAMPLE ID	SEX	AGE	MELANIN INDEX (M)	RS3330 GENOTYPE	RS6654 GENOTYPE	RS6722 GENOTYPE
MEX0001	F	24	48.28	GG	GG	TT
MEX0002	M	27	45.17	GA	GG	TT
MEX0003	M	23	52.41	GG	GG	CT
MEX0004	M	19	44.44	GG	GG	TT
MEX0005	F	27	46.58	GA	GG	TT
MEX0006	M	28	44.92	GG	GG	CC
MEX0007	M	18	47.93	GG	GG	CT
MEX0008	M	22	47.56	GA	GG	TT
MEX0009	F	18	39.64	GA	GG	TT
MEX0010	M	25	47.04	GG	GA	CC
MEX0011	M	20	47.74	GG	GG	CT
MEX0012	M	24	39.55	GA	GG	CT
MEX0013	F	35	48.52	GG	GG	TT
MEX0014	M	21	49.13	GG	GG	CT
MEX0015	M	19	45.16	GG	GG	CT
MEX0016	F	22	49.94	GG	GG	TT
MEX0017	F	21	46.12	GG	GG	TT
MEX0018	M	29	52.00	GG	GG	TT
MEX0019	M	22	52.88	GG	GG	CT
MEX0020	M	24	51.80	GG	GG	TT
MEX0021	M	34	50.30	GG	GG	CT
MEX0022	M	20	50.04	GG	GG	TT
MEX0023	M	30	40.61	GA	GG	TT
MEX0024	M	26	43.73	GG	GG	TT
MEX0025	M	20	51.98	GG	GG	TT
MEX0026	M	29	41.21	GA	GG	TT
MEX0027	M	28	47.41	GG	GG	CT
MEX0028	F	27	52.29	GG	GG	CT
MEX0029	M	21	52.81	GG	GG	TT
MEX0030	F	21	48.93	GA	GG	TT
MEX0031	F	34	46.18	GG	GG	TT
MEX0032	F	18	49.15	GG	GG	TT
MEX0033	M	20	51.11	GG	GG	CT
MEX0034	F	28	49.13	GG	GG	CT
MEX0035	F	31	49.42	GG	GG	CT

MEX0036	F	32	49.18	GG	GG	CT
MEX0037	F	30	52.30	GG	GG	CT
MEX0038	M	20	46.66	GG	GA	CT
MEX0039	F	19	45.25	GA	GG	CC
MEX0040	M	21	43.66	GG	GG	CT
MEX0041	F	27	51.69	GG	GG	TT
MEX0042	F	21	52.43	GG	GG	TT
MEX0043	F	34	49.94	GA	GG	CT
MEX0044	F	18	52.83	GG	GG	TT
MEX0045	F	22	49.05	GG	GG	CT
MEX0046	F	22	60.37	GG	GG	TT
MEX0047	M	23	44.67	GG	GG	TT
MEX0048	F	27	56.16	GG	GG	TT
MEX0049	F	18	42.22	GA	GG	CT
MEX0050	M	24	46.19	GG	GG	TT
MEX0051	M	19	40.95	GG	GA	CT
MEX0052	M	20	46.98	GA	GG	CT
MEX0053	F	24	49.38	GG	GG	CT
MEX0054	F	27	46.84	GG	GG	CT
MEX0055	F	32	57.46	GG	GG	CT
MEX0056	F	32	40.09	GG	GG	CT
MEX0057	M	33	46.75	GG	GG	CT
MEX0058	F	32	52.22	GG	GG	TT
MEX0059	F	30	50.39	GG	GG	TT
MEX0060	F	24	51.38	GG	GG	TT
MEX0061	F	22	48.77	GA	GG	CT
MEX0062	F	35	50.83	GG	GG	CT
MEX0063	F	27	42.96	GG	GG	CT
MEX0064	M	23	49.97	GG	GG	CT
MEX0065	M	30	54.42	GG	GG	TT
MEX0066	M	18	50.45	GG	GG	TT
MEX0067	M	20	51.17	GG	GA	CT
MEX0068	M	18	57.56	GG	GG	TT
MEX0069	M	23	53.64	GG	GG	CT
MEX0070	M	19	46.61	GG	GA	CT
MEX0071	F	29	54.31	GG	GG	CT
MEX0072	F	18	54.22	GG	GG	CT
MEX0073	M	24	46.62	GG	GG	TT
MEX0074	F	19	54.49	GG	GG	TT
MEX0075	M	27	53.67	GG	GG	CT

MEX0076	F	23	43.15	GG	GA	CT
MEX0077	F	21	47.41	GA	GG	TT
MEX0078	M	19	47.75	GG	GG	CT
MEX0079	F	35	51.44	GG	GG	TT
MEX0080	M	18	53.49	GG	GG	CT
MEX0081	M	19	53.50	GG	GG	TT
MEX0082	F	19	47.35	GG	GG	CC
MEX0083	M	25	47.71	GG	GG	TT
MEX0084	M	31	44.65	GG	GG	TT
MEX0085	F	34	45.22	GG	GG	TT
MEX0086	M	28	47.33	GG	GG	TT
MEX0087	F	33	47.81	GA	GG	CC
MEX0088	F	29	53.81	GG	GG	CC
MEX0089	M	30	57.37	GG	GG	TT
MEX0090	F	23	44.53	GA	GG	CC
MEX0091	F	22	54.48	GG	GG	CT
MEX0092	M	19	50.63	GG	GG	CC
MEX0093	M	18	53.84	GG	GG	CT
MEX0094	F	19	51.56	GG	GG	TT
MEX0095	F	27	48.64	GG	GG	CT
MEX0096	F	18	51.30	GG	GG	CT
MEX0097	F	30	50.47	GG	GG	CC
MEX0098	M	21	51.74	GG	GG	TT
MEX0099	F	30	50.42	GG	GG	CT
MEX0100	M	18	45.98	GG	GG	TT
MEX0101	M	19	45.28	GA	GG	CT