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EXAMINATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN A PARTIAL CODING REGION OF THE MELANOCORTIN 1 RECEPTOR FROM MELANISTIC AND WILD-TYPE WHITE-TAILED DEER *(Odocoileus virginianus texanus*)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author WW executed primer design, data analysis and was the primary author of the manuscript. Author DMG provided critical guidance in the writing of the manuscript and prepared the revised manuscript. Author MRJF conceived of the study and acquired samples. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Melanism is well described in ruminant mammals, including deer. Melanism is expectedly rare in wild populations but remarkably prevalent in the white tailed deer population of central Texas. While many potential mutations could underlie the phenotype, for this population it is likely all share the same mutation. Among potential coding region mutations, the melanocortin 1 receptor is a prevalent source of melanism. The objective of this research was to determine whether we could discern single nucleotide polymorphisms in the melanocortin 1 receptor coding sequence correlated with melanism in white-tailed deer (*Odocoileus virginianus texanus*).
Methods: DNA was extracted from tissue obtained from wild-type and melanistic white-tailed deer. Primers designed based on an alignment of the genomic regions of the melanocortin 1 receptor genes for even-toed ungulates were used to amplify a segment of the coding sequence. The amplified product was bidirectionally sequenced and analyzed for the presence and nature of single nucleotide polymorphisms.
Results: Although several sites within the melanocortin 1 receptor gene were found to have single nucleotide polymorphisms, all implied synonymous substitutions, and none were significantly correlated with the incidence of melanism.
Conclusions: Mutations of the melanocortin 1 receptor gene's coding sequence do not account for the incidence of melanism in melanistic deer from the population sampled.

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Keywords: Melanism; white-tailed deer; *Odocoileus virginianus texanus*; melanocortin 1 receptor; single nucleotide polymorphisms.

ABBREVIATIONS

ASIP :agouti signaling inhibitory peptide
BLAST :basic local alignment sequence tool
CDS :coding sequence
MC1R :melanocortin 1 receptor
PCR :polymerase chain reaction
SNP :single nucleotide polymorphisms
tDNA :total DNA

1. INTRODUCTION

Melanism is a phenotype typically characterized by the extreme dominance of dark brown eumelanin over reddish yellow pheomelanin, resulting in a very dark pelage. While melanism has been observed in many mammalian species [1], it is an extremely rare phenotype in white-tailed deer (*Odocoileus virginianus*), with few detections of melanistic individuals across the continental United States. Of those sightings, only three have been documented in the literature, taking place in Pennsylvania, Wisconsin, and Texas [2-4]. The Texas population notably provides a seemingly anomalous prevalence of melanism, reaching up to 21% in some areas, enabling a study of melanism in the wild population [4,5].

The synthesis of eumelanin is influenced by melanocortin, which acts through the melanocortin 1 receptor (MC1R). MC1R is the product of the *extension* locus, which contains several known melanistic alleles in mice, cats, pigs, sheep, and various other mammalian species [6,7,8,9]. Many of the melanistic alleles at MC1R show mutations in the coding sequence, resulting in the receptor's constitutive activation or in dysfunction of the agouti signaling inhibitory peptide (ASIP)-binding site. ASIP normally inhibits MC1R activity, so disruption of its binding site lowers the amount of melanocortin required for MC1R activation, favoring melanism [7].

Cognizant of the importance of MC1R's activity in melanin synthesis, we set out to determine if any single nucleotide polymorphisms (SNPs) correlated with melanism by examining a large portion of the coding sequence of MC1R obtained from samples of melanistic and wild-type deer. Pigmentation has been recognized in numerous species as playing an important role in natural and sexual selection as well as speciation (see for example [10]), and in species hunted as trophies (as well as food), rare phenotypes may increase the desireability and value of specimens (see [2]). Thus, the objective of this research was to determine whether we could discern single nucleotide polymorphisms in the melanocortin 1 receptor coding sequence correlated with melanism in white-tailed deer (*Odocoileus virginianus texanus*).

2. METHODS

2.1 Primer Design

Primers were designed based on an alignment of the genomic regions of the MC1R genes for even-toed ungulates. Sequences for this alignment were obtained using BLAST using the *Bos taurus* MC1R gene against the NCBI database of whole genome sequences, limited to even-toed ungulates. This approach was intended to ensure maximum representation of sequences for primer design. Primer3 software in Geneious version 10.2 and manual inspection were used to develop the forward and reverse primers targeted against *Odocoileus virginianus texanus* MC1R. Primer sequences are displayed in Table 1 [8,9]. Primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA, USA), and their binding site on the MC1R gene from *Odocoileus virginianus texanus* is shown in Fig. 1.

Table 1. Primers targeted to *Odocoileus virginianus texanus* (White-tailed deer) MC1R gene

MC1R Primers	Sequence[a]
Forward	GGCTCTGTTCTCCCTGGACCGCAGCCCACC
Reverse	GCCCCACCATCTCCCCAGCCTCCTCATTC

[a]*Forward and reverse primers targeting the MC1R CDS listed 5'-> 3'*

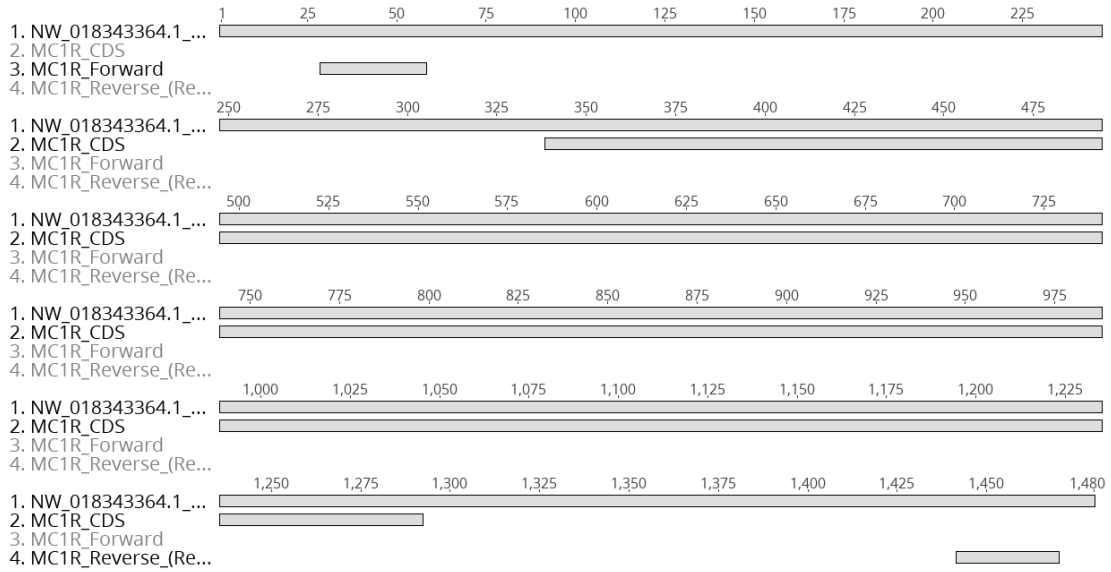

Fig. 1. Primer-binding locations

The forward primer binds at a site 280 nucleotides upstream of the CDS start codon, and the reverse primer binds at a site 148 nucleotides downstream of the CDS stop codon. The forward and reverse primers are 30 and 29 nucleotides long, respectively, and the CDS itself is 954 base pairs in length. Based on the position of the primers, the expected size of the amplification product is 1441 bp.

2.2 Samples and Extractions

Seven tissue samples from melanistic and ten samples from wild-type, white-tailed deer (*Odocoileus virginianus texanus*) were obtained from the MRJ Forstner Frozen Tissue Collection at Texas State University (San Marcos, TX). Salvage tissue samples of melanistic deer were obtained from multiple locations in Texas, and samples from wild-type deer were all obtained from the Kerr Wildlife Management Area: Deer Research Facility (see https://tpwd.texas.gov/huntwild/hunt/wma/find_a_wma/list/?id=12&section=deerPens). The deer at this research facility represent a pedigreed, closed herd maintained by Texas Parks and Wildlife since 1974. The sexes and counties of origin for the samples is shown in Table 2. Samples were extracted using the DNeasy Blood and Tissue Kit protocol (Qiagen, Germantown, MD). The tDNA quality was assessed with gel electrophoresis through 1% agarose at 75 volts for 45 minutes (Fig. 2).

Table 2. Sex and county of origin of tissue samples

MF_ID	Sex	County of origin (Texas)
Melanistic		
MF30222	Female	Gregg
MF28459	Male	Gregg
MF27010	Female	Gregg
MF25782	Male	Hays
MF25781	Male	Harrison
MF16973	Male	Blanco
Wildtype		
MF23711	Male	Kerr
MF23702	Male	Kerr
MF23698	Female	Kerr
MF23697	Female	Kerr
MF23696	Female	Kerr
MF23694	Female	Kerr
MF23692	Male	Kerr

Creation of optimized PCR protocol: Two gradient PCRs were performed using an Eppendorf Mastercycler (Hamburg, Germany) to determine the optimal annealing temperature for the target product. The two gradients selected were 50-58°C and 55-65°C, both in twelve, evenly spaced intervals. PCR was considered optimized when strong bands at the appropriate size were visualized and primer dimers and nonspecific products were minimized.

PCR cleanup: Shrimp alkaline phosphatase (0.4 µl), 0.1 µl exonuclease, and 1.5 µl water were combined with 5.0 µl PCR product in each well and incubated at 37°C for 20 minutes. This incubation was followed by incubation at 80°C for 15 minutes, yielding a clean PCR product ready for cycle sequencing.

Cycle sequencing: Big Dye (Applied Biosystems, Inc.) (2 µl), 6.5 µl nuclease-free water, and 0.5 µl of either forward or reverse primer were combined with 1 µl cleaned PCR product for cycle sequencing in an Eppendorf Mastercycler (Hamburg, Germany). Products were subjected to two rounds of cycle sequencing in an ABI 3500 XL (Thermo Fisher, Austin, TX, USA) to obtain forward and reverse reads.

Cycle sequencing cleanup: Columns for centrifugation were prepared with 300 µl Sephadex (G-50) solution. Once the Sephadex had solidified, the columns were weighed and centrifuged to remove excess water. Cycle sequencing products were then added to the column and the column centrifuged. Products in this collection tube were then dried overnight and analyzed in the Applied Biosystems 3500 Genetic Analyzer.

Assessment of sequence quality: FASTQ files were obtained from the automated sequencer and were analyzed in Geneious v10.2 (Biomatters Ltd, Auckland, New Zealand). Ends were auto-trimmed with an error rate set to 0.05, and forward and reverse reads were *de novo* assembled for each sample. A consensus sequence for each sample was then generated by extracting the consensus from each assembly after deferring to the highest quality base at that position. If the sequence had multiple successful reads through a position, a complete consensus was applied. Each sequence included in the analysis had a minimum of two reads (forward and reverse). Each sample's consensus sequence was aligned to the *Odocoileus virginianus texanus* genome NCBI sequence NW_018343364.1(NW) for assessment of potential SNPs associated with melanism [9].

Statistical analysis: Statistical analysis of SNP frequency was performed for the synonymous mutation sites using a Fisher's Exact Test [11] a *P*-value greater than .05 was considered not statistically significant.

3. RESULTS

A BLAST search of the whole genome sequences of even-toed ungulates using the MC1R gene from *Bos taurus* as a query sequence revealed 15 unique whole gene sequences, two of which had escaped auto-annotation as the MC1R gene due to being masked by the TUBB3 gene, ostensibly a reflection of mRNA sequences for TUBB3 with the MC1R sequence attached to the end. A sampling of PCR products amplified using these primers can be seen in Fig. 2. For the highest quality products, strong bands were visualized, and low amounts of primer dimers and nonspecific primer binding product were seen. The highest quality products were found at the high end of the tested temperatures, leading us to select an annealing temperature of 65°C for subsequent experiments. Using this annealing temperature, we obtained high quality sequences from six melanistic deer and seven wild-type deer. The high quality sequences for melanistic deer ranged in length from 887 bp to 1340 bp as follows: MF30222, 1185 bp; MF28459, 887 bp; MF27010, 938 bp; MF25782, 1340 bp; MF25781, 1167 bp; and MF16973, 1042 bp. The high quality sequences for wildtype deer ranged in length from 1213 bp to 1348 bp as follows: MF23711, 1341 bp; MF23702, 1314 bp; MF23698, 1348 bp; MF23697, 1214 bp; MF23696, 1341 bp; MF23694, 1213 bp; and MF23692, 1244 bp.

The DNA from melanistic deer yielded sequences that contained six sites for which three or more samples had a nucleotide that differed from the consensus; three of these six sites were outside the coding sequence, and three were within the coding sequence. The sites outside the coding sequence lacked coverage from all samples; those within the coding region all represent synonymous substitutions (Fig. 3). Statistical analysis of SNP frequency was performed for the synonymous mutation sites using a Fisher's Exact Test. The *P*-values for each site were .59 for position 162, .40 for position 693, and .27 for position 717. Although the reference sequence (NW) was used in the alignment, it was not included in the counts for the Fisher's Test.

4. DISCUSSION

Understanding the genetic basis of pigmentation is an important field of study, and melanism is often a very clear phenotype that can be attributed to a single allele [12–14]. SNPs in the MC1R coding region are known to be associated with melanism in several mammalian

species [13,14]; however, examples of melanistic animals in which no correlation was observed have also been documented [13]. More specifically, Schneider et al. [13] showed in a study of three South American felids, that MC1R variants were not associated with melanism in two of the three species studied. Although we uncovered several SNPs among the MC1R sequences from the white-tailed deer (*Odocoileus virginianus texanus*) samples sequenced, the P-values observed using the Fisher's test were uniformly greater than 0.05 when comparing melanistic to wildtype, suggesting that these SNPs were not correlated significantly with the incidence of melanism in this species. These results suggest that the incidence of melanism in white-tailed deer is not correlated with the *extension* locus through effects on the coding sequence of MC1R, but it does not rule out other mechanisms of action at the *extension* locus.

Our BLAST search of the WGS of even-toed ungulates using the MC1R gene revealed 15 unique WGS sequences, some of which had escaped auto-annotation of the MC1R gene due to being masked by the TUBB3 gene; this appears to be a consequence of an mRNA for TUBB3 with the MC1R sequence attached to the end [15]. This finding demonstrates the limitations of the auto-annotation process as well as supporting the application of the BLAST search method, which retrieved more MC1R sequences than simply querying the annotated database using the gene name as a search term.

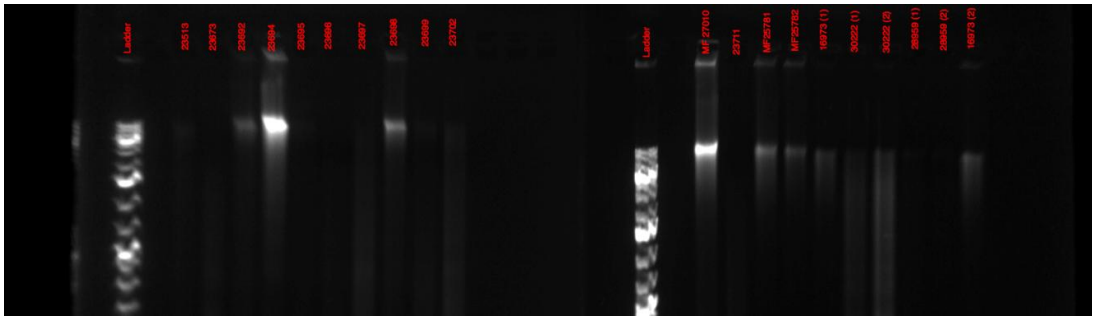

Fig. 2. Sample agarose gels showing quality of amplification products

Ladder = 1 kb DNA ladder. Sample identification numbers are indicated at the top of each well. Some of the samples included in this gel failed to yield quality product and were not further analyzed (23513, 23695, 23699 on the left gel and 16973 and 28959 on the right gel). Other samples (e.g. 23711) were re-extracted and re-amplified

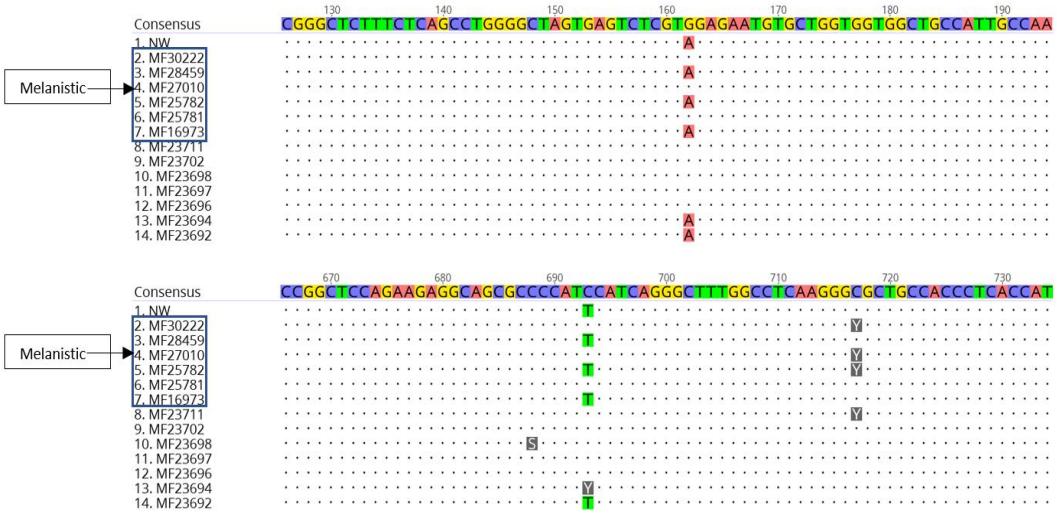

Fig. 3. Alignment of sequences showing variable sites

Sequence position 1 for the aligned sequences is the start codon of the CDS. Sites with more than two SNPs are noted at 162, 693, and 717. Melanistic samples (boxed) include (2) MF30222, (3) MF28459, (4) MF27010, (5) MF25782, (6) MF25781, and (7) MF16973; samples (8) MF23711, (9) MF23702, (10) MF23698, (11) MF23697, (12) MF23696, (13) MF23694, and (14) MF23692 are wild-type. (1) NW is the genomic reference (NCBI sequence NW_018343364.1)

5. LIMITATIONS

Three melanistic samples did not yield reliable coverage for the entire coding DNA sequence (CDS): MF28459 lacked 17 nucleotides from the beginning of the CDS and 54 nucleotides from the end of the CDS, and MF27010 and MF16973 lack 5 and 18 nucleotides respectively from the 3' end. However, all three maintain enough quality coverage for the variable sites identified among all samples.

Several of these SNPs could reflect sequencing artifacts as some SNP types are prone to errors, and confidence of making heterozygote calls can often be an issue [16]. Bleed-through of the signal from neighboring nucleotides is possible for sites 162 and 693. Site 162 has a G neighbor at site 163, and its consensus read as a G, so interference from the neighbor is possible. Site 693 has both a C (694) and a T (692) neighbor, and its consensus read as a C and minority read as a T, with MF23694 having a heterozygote C/T call, so interference from either neighboring nucleotide is possible. Bleed-through is not a possibility for site 717 as both of its neighbors are G and that site is consensus read as a C and minority read as a C/T heterozygote. Making a stringent determination of heterozygosity will require more sequences from additional individuals.

Of note is the possible impact of population stratification on the results. The alleles of the variable sites in the melanistic samples are perfectly evenly split, while in the wild-type one allele is more dominant. Logically, this could be caused by the fact that the wild-type samples all come from the same population at the Kerr Wildlife Management Area. The Kerr managed herd was sourced from the same central Texas population and is large which sought to minimize the bias. Nevertheless, an increased sample size, as well as sequences from a broader array of wild-type deer, would reduce the possibility of population stratification being a confounding factor.

The small sample size limits the conclusiveness of these results, and further examination will require sequencing of more melanistic *Odocoileus virginianus texanus*.

6. CONCLUSIONS

Mutations of the melanocortin 1 receptor gene's coding sequence do not account for the incidence of melanism in melanistic deer from the population sampled.

7. DECLARATIONS

7.1 Availability of Data and Material

Sequences were submitted to genbank under Bankit submission BankIt2183911, and accession numbers are as follows:

Sequence	Accesssion number
MF30222	MK415624
MF28459	MK415625
MF27010	MK415626
MF25782	MK415627
MF25781	MK415628
MF16973	MK415629
MF23711	MK415630
MF23702	MK415631
MF23698	MK415632
MF23697	MK415633
MF23696	MK415634
MF23694	MK415635
MF23692	MK415636

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. King RC, Mulligan PK, Stansfield WD. A dictionary of genetics. 8th ed. Oxford University Press; 2013.
DOI: 10.1093/acref/9780199766444.001.0001.
2. D'Angelo GJ, Baccus JT. First record of melanistic white-tailed deer in Pennsylvania. Am Midl Nat. 2007;157:401–403.
3. Baccus JT, Posey JC. Melanism in white-tailed deer in central Texas. Southwest Nat. 1999;44:184–192.
4. Posey JC. The incidence of melanism in white-tailed deer along the eastern edge of the

Balcones escarpment [Thesis]. San Marcos: Texas State University; 1991.
5. Nachman MW, Hoekstra HE, D 'Agostino SL, Kidwell MG. The genetic basis of adaptive melanism in pocket mice. Proc Natl Acad Sci United States Am. 2003;100:5268–5273.
6. Kijas JM, Wales R, Törnsten A, Chardon P, Moller M, Andersson L. Melanocortin receptor 1 (MC1R) mutations and coat color in pigs. Genetics. 1998;150:1177–85.
7. Schneider A, David VA, Johnson WE, O'Brien SJ, Barsh GS, Menotti-Raymond M, Eizirik E. How the leopard hides its spots: ASIP mutations and melanism in wild cats. PLoS One. 2012;7:e50386.
8. Kearse M, Moir R, Wilson A, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647–9.
9. NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2017;45:D12–D17.
10. Braasch I, Brunet F, Volff J-N, et al. Pigmentation pathway evolution after whole-genome duplication in fish. Genome Biology and Evolution. 2009;1:479–93.
11. Sawyer SA. Inferring selection and mutation from DNA sequences: The McDonald-Kreitman test revisited. In: Golding B, editor. Non-Neutral Evolution. Springer US. 1994;77–87.
12. Mills MG, Patterson LB. Not just black and white: pigment pattern development and evolution in vertebrates. Semin Cell Dev Biol. 2009;20:72–81.
13. Schneider A, Henegar C, Day K, et al. Recurrent evolution of melanism in South American felids. PLoS Genet. 2015;11:e1004892.
14. Nachman MW, Hoekstra HE, D'Agostino SL. The genetic basis of adaptive melanism in pocket mice. Proc Natl Acad Sci USA. 2003;100:5268–73.
15. Herraiz C, Olivares C, Castejón-Griñán M, Abrisqueta M, Jiménez-Cervantes C, García-Borrón JC. Functional characterization of MC1R-TUBB3 intergenic splice variants of the human melanocortin 1 receptor. PLoS One. 2015;10:e0144757.
16. Bowling JM, Bruner KL, Cmarik JL, Tibbetts C. Neighboring nucleotide interactions during DNA sequencing gel electrophoresis. Nucleic Acids Res. 1991;19:3089–3097.