**Albinism in *Bombina variegata* and *Thamnophis sirtalis***

Bansal, M., Bhat Chhetri, B., Ismail, A., O’Hara, R., & Uetz, P.

Introduction

The pigmentation of amphibians is controlled by four chromatophores, one of which are melanophores (Smith-Gill et al., 1972). Melanophores are responsible for the synthesis of melanin, which contributes to the pigmentation of dark colors. The melanin production in melanophores is dependent on the metabolism of tyrosinase, called the tyrosinase pathway. Oculocutaneous albinism (OCA) is a heterogeneous disorder that is characterized by a loss of pigmentation. Mutations in the *TYR* gene, which encodes for the protein tyrosinase, can inhibit melanin synthesis and cause OCA1a (Kamaraj & Purohit, 2014).

There have only been a few studies about the genetic mutations in the tyrosinase gene, and only a handful of studies on the mutations in amphibians. In Smith-Gill et al. (1972), it was concluded that failure in post-translational control contributed to the albino phenotype of *Rana pipiens* (leopard frog). This was due to the fact melanin could be produced in small amounts experimentally in some cells with the albino phenotype. Interestingly, the loss of genes that resulted in an albino phenotype had both tyrosinase and DOPA oxidase activity. This was in contrast with mammals, which showed no tyrosinase or DOPA oxidase activity in albino phenotypes (Smith-Gill et al., 1972).

Miura et al. (2018) investigated mutations in the tyrosinase gene in three frogs: *Pelophylax nigromaculatus* (pond frog), *Glandirana rugosa* (wrinkled frog), and *Fejervarya Kawamura* (rice frog). In total, five different populations were studied of which three were *P. nigromaculatus* and one each of *G. rugosa* and *F. Kawamura*. In two of the three *P. nigromaculatus* populations, there were two frameshift mutations because of the insertion of a thymine within exons 1 and 4, and the third population lacked three nucleotides that encoded a lysine in exon 1 (Figure 1). In the population of *G. rugosa*, a substitution from a [guanine](https://en.wikipedia.org/wiki/Guanine) to an [adenine](https://en.wikipedia.org/wiki/Adenine) created a missense mutation, in which there was an amino acid substitution from glycine to aspartic acid, and the mutation of *F. Kawamura* was also an amino acid substitution from glycine to arginine due to a substitution from a [guanine](https://en.wikipedia.org/wiki/Guanine) to an [adenine](https://en.wikipedia.org/wiki/Adenine) (Figure 1). The mutation for *F. Kawamura* and *G. rugosa* occurs in exons 1 and 3, respectively. The mutations of the third population of *P. nigromaculatus*, and the mutations of *G. rugosa* and *F. Kawamura* occurred in areas that are highly conserved among vertebrates which could result in a dysfunctional tyrosinase gene (Miura et al., 2018).

Timeline

Description automatically generated

*Figure 1:* Genetic mutations in the TYR gene cDNA that cause albinism in three frog species found by Miura, et al., 2018. Each colored box represents an exon. Deletion is indicated by the *delta*, insertion by the triangle, and substitution by the pipe.

We propose a study to determine if the mutation in the *TYR* gene causes the albino phenotype in *Bombina variegata* (yellow-bellied toad) and *Thamnophis sirtalis* (garter snake). To find meaningful results, access to both the wild type and mutant samples is needed for comparison, which *B. variegata* and *T. sirtalis* were accessible.

Materials and Methods

Albino and wild type *B. variegata* was received from Peter Uetz at Virginia Commonwealth University, Richmond, Virginia. Albino *T. sirtalis* shed skin sample was received from Barry Stephenson at Mercer University, Atlanta, Georgia (Stephenson & Drake, 2014). Wild type *Homo sapiens* samples served as controls and were collected from the authors.

Primers for *T. sirtalis* (XM\_014064877.1) and *H. sapiens* (M27160.1) were created by first aligning the cDNA of the TYR gene with other reptiles using Clustal Omega. Exon-intron boundaries were determined using the *H. sapiens* TYR transcript in Ensemble (ENST00000263321.6). Primers were then selected using Primer3Plus.

A picture containing text

Description automatically generated

Text

Description automatically generated

*Figure 2:* Forward and reverse primer pairs from *H. sapiens* and *T. sirtalis* tyrosinase gene DNA sequences.

*H. sapiens* controls were obtained by scraping the inside of the mouth with a toothpick. The shed skin of *T. sirtalis* was crushed using a micropipette tip. All samples were placed in 20μL of 1 M of NaOH and incubated at 65-75℃ for fifteen minutes. DNA was released and neutralized with 180μL 0.2M Tris-HCl (pH 7.4) and centrifuged at 14,100 g for 2 minutes. The supernatant containing DNA was collected for a total volume of 200 μL.

Amplification of the TYR gene fragment was performed by polymerase chain reaction (PCR). A 2X polymerase MasterMix Kit was used to creating the PCR mixture and all the materials were kept on ice before and after being used.

In the *T. sirtalis* and *H. sapiens* sample PCR tubes, 9.0μL of sterile water was added to 1.0μL of DNA sample. Following the addition of water, *T. sirtalis* (XM\_014064877.1) and *H. sapiens* (M27160.1) specific forward (1.5μL) and reverse (1.5μL) 25μM primers for the TYR gene were added to both the sample and the control PCR tubes respectively. Then 1.0μL of DNA sample from the DNA extraction was added to the PCR sample tubes. Finally, 12.5μL of 2X polymerase MasterMix from the kit was added to the PCR tubes. The final PCR mixture had a volume of 25μL.

The liquid in the PCR tubes was then mixed, avoiding bubble formation, and centrifuged for less than 20 seconds in the mini-PCR centrifuge to ensure that no liquid was left on the walls of the tube. The PCR machine (Bio-Rad T100 Thermal Cycler) was set to 95 °C (95° C 30 sec, 60 °C 15 sec, 72°C 30 sec) for 35 cycles than 4°C infinite. When placing the tubes into the machine, the PCR tubes were pushed down to make sure that the caps of the tube were secure and that the tubes were in contact with the walls of the machine wells. When the 35 cycles were completed, the reaction mixture in the tubes was moved and frozen at -20° C.

A gel electrophoresis analysis of the PCR reaction was performed. During this process, 25μL of each PCR reaction was loaded. The resulting mixtures were pipetted into wells 2-11 (*H. sapiens*) and wells 12-13 (*T. sirtalis*) of a 1% agarose gel containing 1X TAE buffer and ethidium bromide. Lastly, wells 1 and 14 contained 1KB ladders (Figure 4). The gel electrophoresis was conducted at a constant voltage of 100V for 35 minutes and then visualized with a gel imager.

These steps were repeated with some modifications to the protocol in the collection and treatment of the samples as well as the PCR cycle parameters. New *H. sapiens* samples were obtained from the authors by scraping the inside of the mouth with a toothpick and plucking a few hairs from the head. Only 10μL of H2O was added to the *H. sapiens* mouth swab sample, vortexing using the micropipette to break up the cells. The *H. sapiens* hair sample was treated with 10μL of 0.2% Tween detergent. Mycobacterium phage Trixie (NC\_023731.1) gene products 4, 74, 26, 42, 47, 50, 55, 61, 69, 78, and 88 were used as positive controls. Two *H. sapiens* samples with the previous protocol were used again. *T. sirtalis* sample was collected and treated again using the previous protocol.

In the *T. sirtalis*, *H. sapiens*, and Trixie sample PCR tubes, 9.0μL of sterile water was added to 1.0μL of DNA sample. Following the addition of water, *T. sirtalis* (XM\_014064877.1) and *H. sapiens* (M27160.1) specific forward (1.0μL) and reverse (1.0μL) 25μM primers were added to both the sample and the control PCR tubes respectively. For the Trixie gene products,  specific forward (1.25μL) and reverse (1.25μL) 20μM primers were added. Then 1.0μL of DNA sample from the DNA extraction was added to the PCR sample tubes. Finally, 12.5μL of 2X polymerase MasterMix from the kit was added to the PCR tubes. The final PCR mixture had a volume of 25μL.

The liquid in the PCR tubes was then mixed, avoiding bubble formation, and centrifuged for less than 20 seconds in the mini-PCR centrifuge to ensure that no liquid was left on the walls of the tube. The PCR machine (Bio-Rad T100 Thermal Cycler) was set to 95 °C (95° C 30 sec, 50 °C 15 sec, 72°C 30 sec) for 32 cycles than 4°C infinite. When placing the tubes into the machine, the PCR tubes were pushed down to make sure that the caps of the tube were secure and that the tubes were in contact with the walls of the machine wells.

A gel electrophoresis analysis of the PCR reaction was performed. During this process, 20μL of each PCR reaction was loaded. The resulting mixtures were pipetted into wells 2-9 (*H. sapiens*), wells 10-11 (*T. sirtalis*), and wells 13-24 (Trixie) of a 1% agarose gel containing 1X TAE buffer and ethidium bromide. Lastly, wells 1 and 12 contained 1KB ladders (Figure 6). The gel electrophoresis was conducted at a constant voltage of 100V for 35 minutes and then visualized with a gel imager.

Results

Alignments were performed using the cDNA tyrosinase sequences of frogs, snakes, and a human reference gene available in GenBank. Five different alignments were performed, the first two included only the snake and frog sequences, respectively. They showed high conservation among species. The next two alignments included the same snakes and frog sequences with a reference human sequence in each alignment and we observed high conservation between frogs and snakes in comparison to the human gene. The last alignment included all the frogs, snakes, and the human reference gene. The human gene was included to determine the intron-exon boundaries of the tyrosinase gene. Upon observing the alignment, it appears that the sequences are highly conserved so we assumed that the intron-exon boundaries of the human gene also applied to the frogs and snakes. Figure 3 shows the use of the human gene to highlight the exon boundaries and the alignment shows the high conservation of the human, frog, and snake cDNA sequences.

Table

Description automatically generated with low confidence

*Figure 3:* cDNA alignment of frogs, snakes and human tyrosinase gene.

In total 8 different tyrosinase primers were used, 4 primers were for the *H. sapiens* (M27160.1) (2 forward and 2 reverse) and the other 4 were for the *T. sirtalis* (XM\_014064877.1) (2 forward and 2 reverse). The *H. sapiens* primers were created using exon 1, where the forward primers were selected at the beginning of the first exon and the reverse primers were at the end of the first exon to amplify the DNA of the first exon. *T. sirtalis* primers were created using Primer3Plus in the first exon.

The first attempt of the PCR reaction was conducted using 5 human DNA samples and the *T. sirtalis* DNA. Since the annealing temperature was set to 60 °C and the melting temperature of the primers was around 57 °C, none of the samples showed any bands when the gel Electrophoresis analysis was conducted. The gel electrophoresis showed bands for some positive control samples provided by Dr. Uetz and the 1kb marker but didn't show any bands for any of our samples as shown in Figure 4.

A screenshot of a computer

Description automatically generated with low confidence

*Figure 4:* Gel Electrophoresis result after adjusting the protocol. Each well is labeled with our initials followed by the set of primers used for the sample. RO: Rowan O’Hara, MB: Muskan Bansal, LSO: Luiz Shozo Ozaki, AI: Ahmed Ismail, BBC: Bhoj Bhat Chhetri, GS: Garter Snake (*T. Sirtalis*).

A second PCR reaction was performed to correct the annealing temperature and lower it to 50 °C instead of 60 °C, because of the melting temperature of the primers. The concentration of the primers used was increased from 10 μM to 25 μM. The rest of the protocol remained the same to test whether the annealing temperature was the reason for the negative result. Unable to locate the *T. sirtalis* DNA sample, the gel electrophoresis was conducted using only our human samples (excluding Dr. Ozaki’s sample). A separate gel electrophoresis was performed using a 1% Agarose gel. Once again, the PCR product did not seem to have the target amplified gene. Only the primers are visible in the gel (Figure 5). This was determined by the size of the fragments being much too small to be the TYR gene.

A picture containing text, monitor, black

Description automatically generated

Figure 5: Same initial labels as Figure 4 and similar ladder marking.

These steps were repeated with some modifications to the protocol in the collection and treatment of the samples as well as the PCR cycle parameters as described above. The observed results of the third PCR attempt were the same as the previous two as there were no visible bands on the gel electrophoresis. Once again, it was determined that the only visible bands were too low for amplified tyrosinase DNA and are therefore only the primers as shown in Figure 6.

Graphical user interface

Description automatically generated with low confidence

Figure 6: Same labels as Figure 4 and similar ladder marking.

Discussion

In this study, we attempted to identify mutations in the tyrosinase gene from frogs and snakes to identify the associated mutations that may result in albinism. After difficulties in primer design due to a lack of genetic data from our respective species, we progressed our project by identifying degenerate amphibian tyrosinase primers and using exon alignment to design garter snake and human tyrosinase primers. Due to time constraints, we have struggled to complete a successful PCR to test the validity of our DNA extraction and primer design. We were able to validate our PCR methodology by extracting, amplifying, and testing for the presence of Trixie undigested plasmids as a positive control to our PCR protocol design. The PCR showed that the cut plasmids were present. We predict that the error may be due to DNA extraction or primer design. Our protocol involved using a nonionic detergent and removing cell debris by centrifugation. Further, we used an alcohol mixture to denature and precipitate proteins from the nucleic acid solution. As we applied a protocol presented by Luiz Shozo Ozaki that is used on mosquito samples, we may assess the quality and yield of DNA prior to PCR through spectrophotometry or gel electrophoresis. If there is low DNA yield or quality with the current protocol, other methods including silica-based technology, magnetic separation, anion exchange, and cesium chloride density gradients could be utilized (Gupta, 2019).

Primer design and choice could be a potential issue that is leading to negative results in our wet lab study. One potential method is to use other databases such as BLAST primers to identify primers with 40 to 60 percent GC content, increase the PCR primer lengths, and avoid dinucleotide repeats. Further, we can utilize degenerate primers or primers identified in other studies to test our protocol. In the further weeks, we hope to purchase and test degenerate amphibian primers on our frog samples.

In the future, we would like to design more degenerate primers for the respective cDNA of our sample species to be able to sequence the complete protein-coding nucleotide sequence and introns between the 5 exons of tyrosinase to capture full exons for each sample species. These can provide more accurate sequencing data to identify mutations that may result in albinism in these respective species.

We just received a response from the author of the *B. variegata* genome paper about the availability of the tyrosinase gene. We will design primers for the *B. variegata* species and conduct another PCR reaction. We will also consider the primers used in the Miura et al. 2018 as degenerate primers for the *B. variegata* sample since they were created for the frog sequences and the frog sequences are conserved.

References

Gupta, N. (2019). DNA extraction and polymerase chain reaction. *Journal of Cytology, 36*(2), 116–117. https://doi.org/10.4103/JOC.JOC\_110\_18

Kamaraj, & Purohit, R. (2014). Mutational Analysis of Oculocutaneous Albinism: A Compact Review. *BioMed Research International*, 2014, 905472–10. https://doi.org/10.1155/2014/905472

Madeira F, Pearce M, Tivey ARN, et al. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Research. 2022 Apr. DOI: 10.1093/nar/gkac240. PMID: 35412617.

Miura I, Tagami M, Fujitani T, Ogata M. Spontaneous tyrosinase mutations identified in albinos of three wild frog species. *Genes Genet Syst.* 2018 Feb 10;*92*(4):189-196. doi: 10.1266/ggs.16-00061. Epub 2017 June 30. PMID: 28674275.

Smith-Gill, S. J., Richards, C. M., & Nace, G. W. (1972). Genetic and metabolic bases of two “albino” phenotypes in the Leopard Frog, Rana Pipiens. *Journal of Experimental Zoology*, *180*(2), 157–167. https://doi.org/10.1002/jez.1401800203

 Stephenson, B. & Drace, K... (2014). A new report of albinism in the Common Garter Snake (Thamnophis sirtalis), and a review of existing records: is there a geographic bias in observations? *Herpetological Review.* 45. 569-577.