Genetic Investigation of Opsin Evolution in the genus *Sceloporus*

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Abstract

Sceloporus lizards are found all across the United States and Mexico. Males of this genus exhibit frequent sexual signaling, often via colorful abdomens but occasionally with sexual ornaments on other parts of the ventral surface of the body. These signals are energy expensive to maintain but convey important information to other males and to females about the health, strength, and fitness of the individual. It has been theorized that when women choose a mate based on a certain sexual ornament, the male offspring will inherit that same sexual ornament, while the female offspring will inherit the preference for the ornament. This sexual selection process allows certain signals that may not necessarily be beneficial for evolutionary fitness to remain in the gene pool because it plays such a large part in inter and intrasexual interactions. I wanted to determine to what degree visual opsins play a role in mate choice and perception of visual signals by identifying signatures of selection in opsins that may pertain to the colors of the sexual ornaments that different species exhibit. Identifying signatures of selection and tying those specific opsins to the colors that they perceive would lend evidence to the idea that visual perception has coevolved with sexual ornaments to allow species the best ability to interact with other individuals. I used preserved specimens of ten *Sceloporus* species and isolated and amplified the five visual opsin genes, as well as the 16S mitochondrial gene to vouch for the identity of the specimens. I followed QIAGEN DNeasy tissue extraction protocols and used custom primers for PCR, following thermocycles outlined in Zuniga-Vega et al. Clean PCR product was sequenced bidirectionally. The 16S sequences matched the sequences in GenBank and those were aligned to develop a phylogenetic tree, using *Uta stansburiana*, a close relative of

Sceloporus, as the outgroup. Unfortunately, I ran into PCR optimization issues with the vast majority of visual opsin genes. I was able to salvage a few Rh1 sequences and supplement those with some GenBank sequences to generate a gene tree for Rh1. Upon qualitative inspection of the Rh1 gene tree, it appears relatively consistent with the evolutionary tree developed from the 16S gene sequences. Because Rh1 is responsible for light perception in areas of low lighting, it does not necessarily tell us much about the coevolution of the opsin with sexual signals. It does, however, tell us quite a bit about the habitat and how often these species rely on their rods for dim light perception. A distinct clade of *S. merriami, S. marmoratus,* and *S. poinsettii* was generated from the tree and is consistent with the habitats of these species. These three species are usually found in rocky areas such as arid mountains or canyons where they can bask in the sun and have little to no foliage for shade. The other species on the tree are found in woodland areas or semiarid environments with low foliage that provide some sort of shade and darkness. It would make sense that Rh1 evolved much longer ago in these species to allow them the ability to best interact with the low light environment around them. Unfortunately, I did not generate enough data for the color sensing visual opsins to draw any conclusions about the coevolution of the visual opsins with sexual ornamentation. Further PCR optimization and gel purification needs to be completed to generate visual opsin sequences and test for selection.

Introduction

Sceloporus is a genus of lizard endemic to North America commonly known as spiny lizards. Males in this genus exhibit brightly colored patches (most often blue) on their abdomen that are used to convey information about the health of the male to any potential partners or adversaries (Zuniga-Vega, et al., 2021). Information that these color ornaments signal are factors

such as strength, reproductive ability, social “status”, immune system function, and quality of genes, which females use to assess the overall quality of their prospective mates (Jimenez-Arcos, Sanabria-Urban, & Cueva del Castillo, 2017). These ornaments are also used to intimidate in hostile male-male encounters where both lizards raise their body and puff out their chests to display their abdominal patches (Zuniga-Vega, et al., 2021). Larger and brighter abdominal patches signal a more successful male because an individual who can afford the energy cost associated with these color ornaments are perceived to be healthier overall (Morrison, Rand, & Frost-Mason, 1995). Research has shown that organisms can even decipher multivariate signals and what each part of their ornament may say about the phenotype of the individual exhibiting the signal (Tanner, Ward, Shaw, & Bee, 2017).

Sexual ornamentation is not a novel concept in the animal kingdom. Extensive research has been done on avian secondary sex characteristics and their relation to sexual selection. Bird sexual ornaments can manifest as songs, colorful feathering, ornate dances, and more (Catchpole, 1987). It is understood that when a female mates with a male with one of these elaborate ornaments, the male offspring will inherit the ornament and the female offspring will inherit the preference for that very ornament, creating a sort of positive reinforcement loop (Travers, 2017). This is known as runaway selection and is the mechanism behind these energy expensive ornaments that appear to be antithetical to natural selection due to the extensive energy cost associated with the upkeep of the secondary sex characteristics (Cuervo & Moller, 2000).

The duty of receiving these important sexual signals falls on the organism’s eyes. The vertebrate eye functions by concentrating light onto the retina, which is two layers of nerve tissue that captures light and converts it to an electrochemical signal that is sent to the brain via the optic nerve (Majumder, 2021). The first layer that light encounters is the pigmented layer which

supports and protects the photoreceptors from the damage that they would experience after years of direct light exposure (Mahabadi & Khalili, 2022). The second layer contains photoreceptors, rods and cones that convert light to electrical signals with the help of opsins (Mahabadi & Khalili, 2022). Opsins are highly conserved G-protein-coupled receptors that transduce from a resting state to an activated state upon light absorption which sets off a signaling cascade that eventually ends in the brain (Terakita, 2005). The opsin is essentially the functional unit that converts light to electrical signal. Members of the *Sceloporus* genus have five visual opsin genes: long-wavelength-sensitive opsin (LWS), short-wavelength-sensitive-1 opsin (SWS1), short-wavelength-sensitive-2 opsin (SWS2), rod opsin (Rh1), and rhodopsin-like-2 opsin (RH2) (van Hazel, Santini, Muller, & Chang, 2006). Each of these visual opsin classes are responsible for mediating a certain range of wavelengths. LWS, SWS1, SWS2, and Rh2 are known as photopsins or cone opsins and give organisms color vision during daylight (Terakita, 2005). Rh1 is the only member of the other class of opsins, known as scotopsins, and is expressed in the rods, allowing for visual perception in dim light (Trezise & Collin, 2005). Although *Sceloporus* species are considered diurnal, some species have been shown to have nocturnal tendencies which are aided by rhodopsin function in their retinas (Duncan, Gehlback, & Middendorf III, 2003).

Because sexual ornamentation is so prevalent in the genus *Sceloporus,* it is worth understanding the role that visual perception plays in the process of sexual selection and intrasexual interactions. Without viable color vision, individuals would not have the ability to perceive these ornate patches which would make them obsolete. For this reason, it is thought that photoreceptor evolution may mirror that of sexual ornamentation evolution. While sexual signals are selected for via sexual selection, opsins of the corresponding wavelengths may be selected

for by natural selection (Bloch, 2015). My research set out to investigate the evolutionary history of the five visual opsin genes in ten species of *Sceloporus* by analyzing 16S (to vouch for the taxonomic identity of the samples), LWS, SWS1, SWS2, Rh1, and Rh2 genes in *S. merriami, S. olivaceus, S. malachiticus, S. poinsettii, S. virgatus, S. cyanogenys, S. jarrovii, S. magister, S. slevini,* and *S. marmoratus* to identify selection signatures in these species.

Materials and Methods

My research focused on ten species of *Sceloporus* lizards: *S. merriami, S. olivaceus, S. malachiticus, S. poinsettii, S. virgatus, S. cyanogenys, S. jarrovii, S. magister, S. slevini, and S. marmoratus.* Samples were obtained on loan from the Louisiana State University Museum of Zoology frozen tissue collection. *S. merriami* was collected in Texas, USA; *S. olivaceus* in Zapata County, Texas, USA in 1989; *S. poinsettii* in Val Verde County, Texas, USA in 1989; *S. virgatus* in the Chiricahua Mountains, Arizona, USA; *S. cyanogenys* in Texas, USA; *S. jarrovii* in the Chiricahua Mountains, Arizona, USA in 1989; *S. magister* in Hidalgo County, New Mexico, USA in 1989; *S. Slevini* in the Chiricahua Mountains, Arizona, USA in 1989; and *S. marmoratus* in Bexar County, Texas, USA in 1989. There is no record of when or where *S. malachiticus* was collected. These ten species were chosen specifically because of the array of colorful ornaments they exhibit. Most of these species contain some variation of blue striping along their bellies, with the exceptions being *S. marmoratus* and *S. virgatus* which have rose colored bellies and blue throat patches respectively.

Tissue samples were digested and extracted following QIAGEN DNeasy tissue protocols. The tissue was incubated at 56°C with 180μL buffer ATL and 20μL proteinase K until

completely lysed (around 24 hours). Upon digestion, 200μL of buffer AL and 200μL of ethanol were added to the sample and the mixture was vortexed. This mixture was pipetted into a DNeasy spin column with a collection tube and centrifuged at 6000 x *g* for 1 minute. The spin column was placed into a new collection tube and 500μL of buffer AW1 was added before being centrifuged at 6,000 x *g* for 1 minute. The spin column was once again placed into a new collection tube and 500μL of buffer AW2 was added before being centrifuged at 20,000 x *g* for 3 minutes. The spin column was transferred to a microcentrifuge tube and the DNA was eluted by adding 200μL buffer AE directly to the center of the spin column membrane. The mixture sat at room temperature for one minute before being centrifuged at 6,000 x *g* for 1 minute. The DNA concentration of the extracted samples was quantified using a Qubit fluorometer with a broad range assay.

Samples underwent PCR using 25μL of Phusion Master Mix, 2.5μL of forward and reverse Invitrogen custom primers (drawn from Simoes et al.), 2μL of DNA template, and 18μL of DI water for a total volume of 50μL. I attempted to amplify LWS, SWS1, SWS2, Rh1, and Rh2 under the following thermocycles: 95°C for 10 minutes; 30 cycles of 95°C for 15 seconds (denaturation), 50-59°C for 30 seconds (annealing), and 72°C for 1.5 minutes (extension); and 72°C for 1.5 minutes (final extension). Amplifying the opsins took lots of optimization with the annealing temperature and magnesium levels in the master mix for each opsin. The 16S mitochondrial gene was amplified under the following thermocycles: 95°C for 10 minutes; 30 cycles of 95°C for 15 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 1 minute (extension); and 72°C for 1 minute for a final extension. PCR thermocycles were drawn from Zuniga-Vega et al., 2013. All PCR product was analyzed using a 1% agarose gel solution.

PCR product was purified following the QIAquick PCR Purification Kit protocols. PCR product was added to a microcentrifuge tube with 200μL of buffer PB and vortexed to mix. This mixture was transferred to a spin column with an attached collection tube and centrifuged at 16,000 x *g* for 1 minute. The spin column was transferred to another collection tube and 750μL of buffer PE was added. This was centrifuged at 16,000 x *g* for 1 minute, the flow through was discarded, and the tube was centrifuged for another 2 minutes at 16,000 x *g.* The spin column was placed in a new microcentrifuge tube and 50μL of buffer EB was added to the center of the spin column membrane. This sat at room temperature for 15 minutes before it was centrifuged at 16,000 x *g* for 1 minute. Due to poor PCR specificity in the opsin genes, most of the clean PCR product had to be spun down in a SpeedVac Concentrator to increase DNA concentration. The samples were sequenced bidirectionally in an automated DNA sequencer using the same primers used for PCR. Sequenced samples were analyzed using the National Library of Medicine's basic local alignment search tool (BLAST). I used Clustal to make an alignment of the 16S sequences, pulling data from GenBank for species that I did not have successful sequences for, as well as the 16S *Uta stansburiana* sequence to contextualize the data (Sievers, et al., 2011).

Results

All successful 16S mitochondrial gene sequences matched the sequences of the species I expected in GenBank with greater than 95% accuracy. I used IQ Tree to construct a phylogenetic tree for the *Sceloporus* species I was working with, using *Uta stansburiana* as the outgroup (Figure 1) (Trifinopoulos, Nguyen, von Haeseler, & Quang Minh, 2016). Due to extreme difficulty with PCR optimization, the only visual opsin data I have is a few successful Rh1

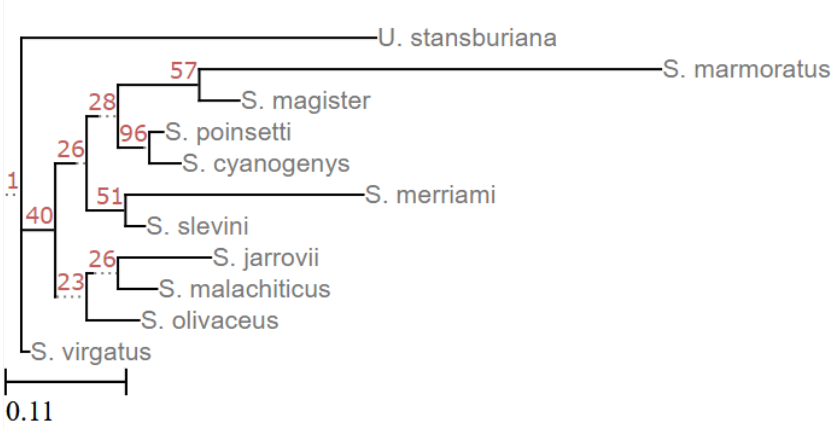

Figure 1. The figure shows the evolutionary relationship of the *Sceloporus* genus with support values shown in red.

sequences. The sequences I generated were supplemented with Rh1 sequences of *Uta stansburiana* and *Sceloporus undulatus* from GenBank. These sequences were aligned using Clustal and used to develop a gene tree for the Rh1 visual opsin gene, which is responsible for light perception in dim lighting (Figure 2) (Sievers, et al., 2011) (Trifinopoulos, Nguyen, von Haeseler, & Quang Minh, 2016). Unfortunately, due to PCR optimization difficulties, I was unable to generate any data for the color sensing visual opsin genes.

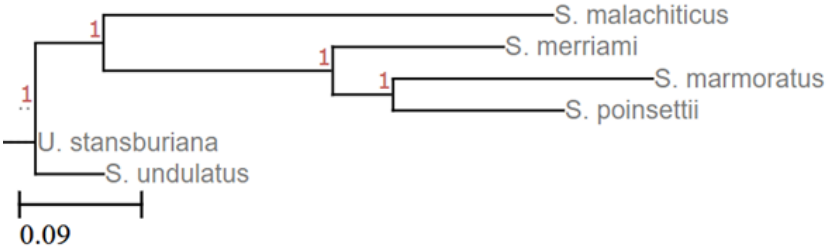

Figure 2. The figure shows the evolutionary history of the Rh1 gene in species of *Phrynosomatidae* with support values shown in red.

Discussion

Upon analysis of the Rh1 gene tree, it appears relatively consistent with the evolutionary tree generated via the 16S gene. Because Rh1, or rhodopsin, is sensitive to low levels of light, we would expect that species of the same or similar habitat would be more closely related on the Rh1 gene tree (Schneider, Rometsch, Torres-Dowdall, & Meyer, 2020). This is because the environment the lizard occupies determines the amount of light they receive, whether that be

through foliage or presence/absence of cloud coverage. A distinct clade emerged on the Rh1 tree containing *S. merriami, S. marmoratus,* and *S. poinsettii.* These three species are found in rocky, arid mountain ranges and canyons, areas that get lots of sunlight and have little to no shade due to lack of foliage. We would expect these species to have a closely related Rh1 gene due to the consistency of the environments they are found in. They do not necessarily have to see in the dark because they are diurnal organisms whose habitat is typically well lit. The other species on the tree (with the exception of the outgroup *U. stansburiana)*, *S. malachiticus* and *S. undulatus,* are found in woodland or semiarid regions that contain either tall trees or low foliage that can provide shade. These species experience varying degrees of darkness due to lack of sunlight in which they may have to rely on the Rh1 in their rods to perceive these low levels of light.

PCR optimization became the sticking point for my research. Despite hours spent in the lab attempting to perfect annealing temperature and Mg concentration, I was unable to get consistent banding on my gels. More time should be spent working to optimize PCR and successful bands should be gel purified to provide the cleanest, most concentrated PCR product for sequencing.

Sceloporus lizards represent a unique genus for the study of visual opsin evolution due to the presence of strong sexual signaling and the variation of phenotype and habitat. There is much work to be done to produce successful visual opsin sequences and quantitatively test for selection via dn/ds ratios between species. Studies in birds have shown that opsins have coevolved with colorful plumage, a sexual signal akin to the colorful abdomens of *Sceloporus* lizards (Borges, et al., 2015). I remain optimistic that successful visual opsin sequences will provide a link between opsin evolution and sexual ornament evolution.

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