PONTIFICIA UNIVERSIDAD CATÓLICA DEL ECUADOR

FACULTAD DE CIENCIAS EXACTAS Y NATURALES

ESCUELA DE CIENCIAS BIOLÓGICAS

Cloning of the opsin genes present in *Bothrops atrox* (Viperidae) retina

Disertación previa a la obtención del título de Licenciada en Ciencias Biológicas

MICAELA STACEY SOLÍS

Quito, 2017

Certifico que la Disertación de Licenciatura en Ciencias Biológicas de la Srta. Micaela Stacey Solís ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Atentamente,

Dra. Christiana Katti

Directora de la Disertación

Quito, 10 de febrero de 2017

A mi familia y los amigos que son familia.

AGRADECIMIENTOS

Al Proyecto Prometeo por el financiamiento.

Al museo de Herpetología QCAZ por la ayuda con los especímenes estudiados y el uso de las instalaciones.

A Christiana Katti por abrirme las puertas, su constante apoyo y sus enseñanzas.

A mi madre Cristina Solís, a mi padre Luis Stacey y a mi hermano Antonio Stacey por el apoyo y amor incondicional.

A toda mi familia.

A Kathya Bustamante, Estefany Guerra y Andrea Vela por estar siempre a mi lado.

A todos los amigos que han estado en las buenas y en las malas.

A María José Navarrete y Marcel Caminer por la ayuda en los análisis.

INDEX

LIST OF FIGURES

LIST OF TABLES

RESUMEN

El sistema visual de vertebrados ha evolucionado a lo largo de millones de años para adaptarse a los hábitos nocturnos o diurnos, así como a diferentes ambientes donde el rango espectral variable y la intensidad de la luz están presentes. Aunque se sabe mucho sobre el sistema visual de los vertebrados en general, los estudios son escasos en reptiles y particularmente escasos en serpientes. Los reptiles muestran una gran diversidad con respecto a la estructura de su retina que puede estar conformada solamente de conos, dominadas por bastones o ser retina dobles; Sin embargo, las mismas cinco opsinas se encuentran en estos animales a pesar del cambio en la conformación de la retina. Debido a sus orígenes nocturnos y fosoriales, las serpientes sólo poseen tres de estas opsinas visuales: RH1 (rhodopsin-like 1) presente en los bastones, LWS (long-wavelenght sensitive) y SWS1 (short-wavelenght sensitive 1) presentes en conos. A pesar de que el estudio de opsinas en serpientes ha sido ampliamente ignorado en el pasado, ha retomado claramente su importancia en los últimos años, en los cuales han surgido nuevos estudios que reconsideran la teoría de Transmutación propuesta por Walls en 1934. En este estudio, hemos clonado totalmente las opsinas RH1 y LWS, así como una parte de la opsina SWS1 presente en *Bothrops atrox*. Estas secuencias fueron utilizadas para predecir la Absorbancia máxima (λ_{max}) de cada proteína. Este es el primer estudio que se centra en las opsinas de un viperido, donde se obtuvieron las primeras secuencias completas disponibles para dicha familia de serpientes, que ha sido pobremente estudiada hasta ahora

Palabras clave: Absorbancia máxima (λ_{max}), Luz, Opsina, Proteína, Retina, Serpiente

ABSTRACT

The visual system of vertebrates has evolved over millions of years to adapt to nocturnal or diurnal habits as well as different environments where varying spectral range and intensity of light are present. Although much is known about the visual system of vertebrates in general, studies about reptiles, and snakes in particular, are scarce. Reptiles display great diversity regarding their retinal structure, and different types of retinas exist (pure cone, mostly rod or duplex retina); however, the same five opsins are found in all of these animals. Because of their nocturnal and fossorial origins, snakes only have three of these visual opsins, namely: RH1 (rhodopsin-like 1) present in rods, LWS (long-

wavelength sensitive), and SWS1 (short-wavelength sensitive 1) that are present in cones. The study of snakes´ opsins has been largely ignored in the past; however, its importance has become clear within the past few years, and studies which reconsider Wall´s transmutation theory, proposed back in 1934, have emerged. In this study, we cloned the full length of RH1 and LWS opsins, as well of a part of the SWS1 opsin present in *Bothrops atrox* and have used these sequences to predict the Maximal absorbance (λ_{max}) for each protein. This is the first report focusing on viper opsins; we report the first full length sequences available for opsins of this family, which has been poorly studied so far.

Keywords: Light, Maximal absorbance (λ_{max}), Opsins, Retina, Snake

1. INTRODUCTION

Light is involved in a variety of biological processes related to animals life and behavior, and the capacity to perceive light can affect features such as reproduction, ability to evade predators, hunt, as well as biological clocks, among other functions (Cermakian and Sassone-Corsi, 2002; Doyle and Menaker, 2007). Vertebrates and invertebrates perceive light stimuli using photo sensitive cells which form highly specialized structures, located mainly, although not exclusively, within the eye. In vertebrates, the eyes as well as specialized areas of the brain, such as the parietal eye found in reptiles and other non-mammalian vertebrates, contain light receptors (Davies, Hankins and Foster, 2010; Shichida and Matsuyama, 2009). These have evolved over millions of years based on sensory information provided by different environments such as varying spectral range and intensity of light, as well as nocturnal or diurnal habits (Atick, 1992; Nilson, 2013; Mustafi, Engel and Palczewski, 2009).

Vertebrates possess two different types of traditional photoreceptive cells, the cones and the rods, which can be differentiated by their distinct morphologies and functions. Cones are shorter, about half the length of rods, and have a conical shape. Rods, on the other hand, are more slender and elongated. The inner segments of both photoreceptor types, contain all the usual cytoplasmic components of a cell such as the nucleus, mitochondria and other organelles, and their outer segments, contain the cell´s photosensitive membranes (Carter-Dawson and Lavail, 1979; Hill, Wyse, Anderson and Anderson, 2004).

Furthermore, rods and cones also differ in function. Rods are highly sensitive to light and can detect as little as one photon, which makes them ideal receptors in dim light or scotopic (low light) conditions. Since rods are highly sensitive to light they adapt slowly to darkness (Shichida and Matsuyama, 2009), and exhibit a relatively low spatial resolution (Hecht and Mintz, 1939). In contrast, cones are less sensitive, require many photons to be activated, and therefore, facilitate vision under photopic conditions (well-lit environments). These cells are also responsible for color perception and the existence of different cone types provides animals with color vision (Shichida and Matsuyama, 2009).

In reptiles, as in many vertebrates, studies have shown that different types of retinas exist. Considering their morphology (i.e. which type of photoreceptor they consist of), most reptile retinas are duplex, and therefore consist of both rods and cones. However, the reptile retina can vary from rod-dominated, such as the retina of the crocodile *Alligator mississippiensis* (Janke and Arnason, 1997), to pure cone retina such as the retina of the turtle *Trachemys scripta elegans* (Loew and Govardovskii, 2001). Squamata is a very diverse reptile order, and its members possess retinas of variable nature as a result of constant evolution to different environments and diurnal/nocturnal-habits (Röll, 2000; Crescitelli, 1972).

Retinal morphology of various species belonging to the class reptilia supports the theory of transmutation postulated by Walls (1934). Walls argued that at some point during evolution early tetrapods shifted their lifestyles from nocturnal to diurnal and depended solely on photopic vision, resulting in a complete loss of their rods. After the evolution of different types of predator animals, such as birds and mammals, early tetrapods were forced to become nocturnal again, and therefore animals that had lost all of their rods had to modify their remaining photoreceptors i.e. cones, to re-establish photosensitive cells to detect low light levels, i.e. rods. Therefore, Walls suggested that in lineages which had lost one of their photoreceptor types, rods and cones could evolve into one another through series of intermediate morphotypes (Walls. 1934). Within the last couple of years, a small number of studies had emerged considering this theory in light of the snake visual system (Simões et al., 2016a; Schott et al., 2016). However, not many studies focused specifically on the snake visual system have been published over the years, especially considering that reptiles are the only class whose photoreceptors undergo transmutation.

Snakes are divided into two major infraorders: Scolecophidia and Alethinophidia. Scolecophidia includes blind snakes and thread snakes, while Alethinophidia includes all other snakes. Alethinophidia can be further separated into two superfamilies; Henophidia which includes boids (pythons and boas) and caenophidia which is more diverse including vipers, elapids, and colubrids (Scanlon and Lee, 2011; Hsiang et al., 2015).

Scolecophidians are fossorial snakes, and have reduced eyes covered with scales in some cases; therefore their visual systems have not been studied extensively. The few published reports show that scolecophidians have pure rod retinas and possess only one functional visual opsin, rhodopsin (RH1), while SWS1 and LWS opsins are lost in this group (Simões et al., 2015).

Henophidia includes many nonvenomous, crepuscular/nocturnal and/or burrowing snakes (Conant and Collins, 1998) and is considered the more primitive superfamily of the infraorder Alethenophidia. We believe that the pattern of the retinal photoreceptors of boids and pythons is ancestral to the pattern of the more advanced snakes since Henophidians have evolved earlier than Caenophidians (Crescitelli, 1972). Henophidians are mostly nocturnal and therefore have duplex retinas, which are greatly dominated by rods (90% rods-10% cones). Species belonging to this group, such as *Python regius* and *Boa constrictor*, although primarily nocturnal, can be active both during the day and during the night and have through evolution modified their retinal rod to cone ratio to accommodate their behavior. They developed a more cone-rich retina which indicates that the visual system is plastic and responds to the organism´s needs (Sillman, Johnson and Loew, 2001; Sillman, Carver and Loew, 1999).

Caenophidia is a much more diverse group than Scolecophidia and Henophidia and includes various diurnal and nocturnal species; therefore, the retinal composition of the species it encompasses is quite variable to respond to the particular needs of each organism. Studies suggest that diurnal Caenophidians, such as colubrids, are more derived (advanced) than nocturnal ones like some vipers and tend to have pure cone retinas with no evidence of rod photoreceptors (Hauzman et al., 2014). Similarly to colubrids, elapids also seem to lack rods and present many morphologically distinct types of cones (Hart, Coimbra, Collin and Westhoff, 2012). Vipers, another category of Caenophidian snakes are known for being nocturnal, but their visual system has not

been studied in detail. A new study by Simões et al. (2016b) is the only published report providing insight to opsins present in vipers.

In reptiles, as in all vertebrates, cones and rods contain visual pigments composed by a protein (opsin) which is permanently linked to a chromophore (Bowmaker, 2008). Opsins are members of a family of G-protein coupled receptors and allow vision mostly within the visible light spectrum, and in some cases within ultraviolet or infra-red wavelengths (Bowmaker, 2008). Opsins are found in membranes of the outer segments of photoreceptors, and respond to light by changing their conformation, and eventually causing biochemical reactions that result in chemical and electrical signals which finally get to the brain, thus transmitting visual information (Van Hazel, Santini, Müller and Chang, 2006). The sum of these chemical and electrical reactions is a process called phototransduction and it takes place in the outer segments of the photoreceptors (Miller, 1981; Yau and Hardie, 2009).

There are 5 types of opsins in reptiles, one of these, rhodopsin is exclusively expressed in rods (RH1, rhodopsin-like 1) whereas four additional opsins are expressed in cones responsible for photopic and color vision. The following cone opsins are present in reptiles: LWS (long-wavelength sensitive), SWS2 (short-wavelength sensitive 2), SWS1 (short-wavelength sensitive 1) and RH2 (rhodopsin-like 2). RH2 opsin is found in reptilian cones which, despite functioning as cones, resemble rods in their morphology. Reptile opsins are encoded by the genes *rh1, lws, sws2, sws1* and *rh2* respectively, the sequence of which differs slightly among different species. Sequence variability, especially that of amino acids found at spectral tuning sites, can cause a change in opsin sensitivities (Yokoyama, 2000; Van Hazel et al. 2006; Yokoyama, Yang and Starmer, 2008a; Simões et al. 2015). Sensitivity to different wavelengths of light is a key characteristic of opsins. Each opsin type has an absorbance range, including a maximal absortion wavelength (λ_{max}). Each opsin type displays a characteristic λ_{max}; for example, UV-sensitive opsins absorb maximally around ~360nm, whereas red light sensitive opsins absorb maximally at ~560nm (Yokoyama et al. 2008a). It is believed that because of the fossorial origin of snakes, the morphology, function and even the presence of opsins in their eyes is different from the rest of reptiles, and possess only the following three types of opsins: RH1, LWS and SWS1 (Caprette, Lee, Shine, Monkay and Downhower, 2004). The λ_{max} of the three different opsins have the following ranges: SWS1 λ_{max}: ~350-440 nm, RH1 λ_{max}:

~460-530 nm and LWS/MWS λ_{max}: ~495-575 nm (Bowmaker and Hunt, 1999; Osorio and Vorobyev, 2005).

Our study is focused on the opsins expressed in the retina of *Bothrops atrox* and their λ_{max}. *B. atrox* is a nocturnal, terrestrial viper (Viperidae) which is widely distributed in tropical forests in Central and South America (Stocker and Barlow, 1976). This species plays an important ecological role in tropical forests, and it is one of the most poisonous snakes of the Amazon region causing considerable human mortality and morbidity within its distribution range (Oliveira and Martins, 2002). Here we report the cloning of full length *B. atrox* RH1 and LWS, as well as partial sequence cloning of the SWS1. We use these sequences to predict λ_{max} of each protein. To the best of our knowledge, this is the first study focused exclusively on the visual system of a viper, as well as one of the first studies regarding the vision of vertebrates in Ecuador.

2. MATERIALS AND METHODS

2.1. ANIMALS AND COLLECTION OF SAMPLES

We used adult *Botrhops atrox* snake collected in Zamora Chinchipe. This specimen is registered in the Herpetology museum of Pontificia Universidad Católica del Ecuador with the QCAZ 13857. The animal of interest was euthanized using Roxicain before eye enucleation following the sacrifice protocol stablished by Simmons and Muñoz-Saba. (2005). After enucleation the retina or the entire posterior eye cup was separated from the rest of tissues, and samples were immersed briefly in liquid nitrogen for rapid freezing and eventually transferred at -80°C for long term storage.

2.2. RNA EXTRACTION

The Trizol Plus RNA purification system (Invitrogen) was used for RNA extraction, using the manufacturer's instructions. Briefly, 1ml of Trizol was added to frozen samples and those were triturated using a pestle. The samples were subsequently centrifuged at 13,000 x g for 10 minutes at 4°C and the supernatant, which contained the RNA, was separated from the pellet and incubated briefly at room temperature (RT) before adding 0.2ml of chloroform, shaking vigorously and incubating at RT for an additional 2-3 minutes. Samples were then centrifuged at 13,000 x g for 15 minutes al 4°C, vielding three separate phases: phenol-chloroform phase (bottom), an interphase and a colorless aqueous phase (top) where all the ARN is located. The aqueous phase was mixed with an equal volume of 70% of EtOH and the mixture was transferred onto a column and centrifuged to bind the RNA on the column. The RNA was washed, treated with DNase (to remove any remnants of genomic DNA) and eluted in 50µl of nuclease-free water. The RNA yield and quality were analyzed using a Nanodrop 1000^{TM} UV-Vis spectrophotometer (Thermo-Fisher Scientific), aliquoted in 1-2µg fractions, and stored at -80°C for future usage.

2.3. cDNA SYNTHESIS

For cDNA synthesis the GoScript system (Promega) was used, following the manufacturer's instructions. A primer containing a known sequence (to be used later on in RACE reactions as an anchor) and an oligo-dT tail, and 1-2µg of total RNA were used in each reaction. After the reverse transcription, the enzyme was heat-inactivated

and the template RNA was removed using RNase H (Promega). The resulting cDNA was purified using the QIAquick PCR purification system (Qiagen) and was eluted in 50µl of nuclease-free water.

2.4. POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION AND RAPID AMPLIFICATION OF cDNA ENDS (RACE):

The genes of interest (visual opsins) were amplified using GoTaq Flexi DNA polymerase (Promega) in various nested PCR reactions following the manufacturer's instructions. We used degenerated as well as gene specific primers to amplify visual opsins. Degenerated primers were designed based on known sequences of previously cloned opsins (Davies *et al.,* 2009) to amplify the sequence of the three visual opsin genes that can potentially be expressed in snakes: SWS1, LWS and RH1. After the initial amplifications, gene specific primers were designed to amplify the extreme ends of the genes of interest. To verify that *B. atrox*, as other snakes, only contains the three aforementioned opsins, we attempted to amplify RH2 and SWS2 (which should be present in lizards but not snakes). The results from these attempts were negative and were therefore abandoned. All primers used are shown in Table 1. In each reaction, we used 1-2µl of cDNA replaced by water in the negative control samples.

For the first and second rounds of the PCR we used the following conditions for the amplification: 94°C for 10 minutes; 48 cycles at 94°C for 30 seconds, 45-55°C for 1 minute, 72°C for 1,5 minutes; and an extension at 72°C for 10 minutes to complete all amplicons (Davies et al., 2009).

The 5' and 3' ends of each opsin sequence were obtained using Rapid Amplification of cDNA Ends (RACE) (Schramm, Bruchhaus and Roeder, 2000). To obtain the 3' end cDNA was used as before, using appropriate primers (see table 1 for details). In contrast, to amplify the 5' end of the opsins, terminal deoxynucleotidyl transferase (TdT) was used after the reverse transcription to add cytosine nucleotides to the 5' end of the newly synthetized cDNA. These nucleotides were used as an anchor in the PCR reactions. For the amplifications we used primers with a known sequence (anchor) and a series of guanines (to bind to the cytocines added to the template). Subsequent PCR reactions were performed using the conditions described above.

2.5. ELECTROPHORESIS

Samples were analyzed using electrophoresis. Specifically, we used 1.2% agarose gels, which were dyed using SYBR Safe (Thermo Fisher Scientific) to visualize the amplified DNA. The bands were visualized using an automated gel imaging Gel Doc™ XR+ Imaging System (BIO RAD) under UV light, and were excised and purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol. After purification, the amplicons were eluted in 50µl of nuclease-free water and sequenced directly (Macrogen, Korea) to reveal the sequences of the genes of interest.

2.6. SEQUENCE ANALYSIS

Fragments of the genes of interest were aligned using the Phylogenetic Data Editor (PhyDe) and Bioedit programs to obtain a consensus sequence for each gene. Every piece of the sequence was amplified and sequenced, to increase confidence in the sequence obtained. Nucleotide sequences were then translated to amino acid using the freeware San Diego Biology WorkBench and the largest open reading frame for each protein was selected for further analysis. We used the resulting amino acid sequences to predict spectral sensitivity for each opsin and compared them with known sequences from the other vipers, as well as bovine rhodopsin.

3. RESULTS

Using cDNA generated from freshly sampled *B. atrox*, sequences of all three visual opsins present in snakes (RH1, LWS, and SWS1) were successfully amplified. Amplification of RH2 and SWS2 was attempted unsuccessfully. For each gene amplified, various PCR reactions were performed using different degenerate and gene-specific primer combinations. We amplified and sequenced the full-length RH1 opsin (a total of 1247 nucleotides) and LWS opsin (a total of 1322 nucleotides). SWS1 opsin was partially sequenced with a total of 551 nucleotides.

The opsin nucleotide sequences were translated to amino acid sequences and used to predict spectral sensitivity for each opsin, and to compare them with known sequences from other vipers.

The amino acid sequences available for three African vipers (*Bitis nasicornis, Echis ocellatus and Causus rhombeatus*) (Simões et al., 2016b) were obtained from GenBank (*Bitis nasicornis* (Accession numbers: AOF40381, AOF40293, AOF40388), *Echis ocellatus* (Accession numbers: AOF40382, AOF40294, AOF40389), *Causus rhombeatus* (Accession numbers: AOF40384, AOF40295, AOF40390)) and compared/aligned to those generated for *B. atrox* as well as bovine rhodopsin from *Bos Taurus* (Accession: NP_001014890) (Kazmin et al., 2015) (see Figures 1, 2 and 3). Bovine rhodopsin is one of the first discovered and best studied opsins and, as a convention, it is used as reference to determine the amino acids present at the tuning sites, which can determine spectral sensitivity causing shifts in the visual pigments.

Opsin spectral sensitivity can vary due to amino acid substitutions in crucial protein sequences positions, which are known to shift the absorption maximum (Yokoyama, 2000). These amino acid sites are called spectral tuning sites. We examined the sequences of the three opsins obtained from *B. atrox* and determined the amino acids present in these crucial positions to predict their spectral sensitivity (see Figures 1, 2 and 3). In the case of RH1, up to 12 spectral tuning sites have been reported in vertebrates. The following 7 amino acids were shown to be of particular importance in changing RH1 and RH2 spectral sensitivity: 83/ 122/ 207/ 211/ 265/ 292/ 295 (Davies et al., 2007; Hunt, Fitzgibbon, Slobodyanyuk and Bowmaker, 1996; Yokoyama, Tada, Zhang and Britt, 2008b). In snakes sites 83 and 292 have been shown to vary. *B. atrox* has D83 and A292 (Figure 1), as many other snakes (Simões et al., 2016b). Comparing

the combination of D83 and A292 to different vertebrates and other snakes, we predict a λ_{max} of 500-505 nm for RH1 from *B. atrox* (Davies et al., 2007; Hunt et al., 1996; Simões et al., 2016b; Yokoyama et al., 2008b). Although in many vertebrates the above combination has produced the stated spectral sensitivity, there´s evidence from more basal snakes, which have a spectral sensitivity of 497-499 nm although they possess a N83 instead of a D83 (Davies et al., 2009). Given this evidence, and since we know that a D83N change can blue shift the maximal absorbance (λ_{max}) by about 6 nm, it is possible that the sensitivity of the RH1 opsin we have cloned is a bit shifted to the red and more towards 503-505 nm.

We also cloned the full length LWS opsin from *B. atrox* and compared it to sequences from other snakes as well as bovine rhodopsin (Figure 2. See above for explanation) to determine amino acids found in the spectral tuning sites. There are five tuning sites for LWS (relative to rhodopsin) found at positions 164/ 181/ 261/ 269/ 292 (Yokoyama, 2000; Davies et al., 2007; Yokoyama et al., 2008b). Many vertebrates, including some snakes, have the combination SHYTA in these critical amino acids giving them a λ_{max} close to 560 nm (Yokoyama, 2000; Davies et al., 2007; Davies et al., 2009; Reviewed by Hunt and Colling, 2014). *B. atrox* on the other hand, as the three vipers examined by Simões et al. (2016b), possesses the following amino acids in the crucial positions for spectral tuning A164/ H181/ Y261/ T269/ A292 (AHYTA). Given this combination, the predicted λ_{max} for this protein could be around 553 nm (Asenjo, Rim and Oprian, 1994; Yokoyama et al., 2008b). However MSP (Microspectrophotometry) on a LWS opsin from *Python regius* and *Xenopeltis unicolor* had shown their λ_{max} to be closer to 550 nm in spite of possessing the combination SHYTA at the spectral tuning sites (Davies et al., 2009). This may indicate that other amino acids, unknown thus far, may be involved in the spectral tuning of this protein. It is known that the S164A substitution causes a blue shift of 7 nm, and if the case were the same as *P. regius* for *B. atrox*, then the spectral sensitivity of its LWS could also be closer to 543 nm.

As mentioned above we did not manage to clone the full length of SWS1 opsin, the protein which permits animals to see in UV/Violet wavelengths, probably due to its low expression in the retina (see discussion for details). It has been previously shown that the sensitivity of SWS1 depends primarily on the amino acid found in position 86 (Cowing, Poopalasundaram, Wilkie, Robinson and Bowmaker, 2002). When an F is

found in position 86, the spectral absorbance is UV-sensitive, whereas the protein is violet- sensitive if it possess Y86, S86 or V86 (Cowing et al. 2002; Parry, Poopalasundaram, Bowmaker and Hunt, 2004; Yokoyama, Takenaka, Agnew and Shoshani, 2005). In general, the protein has a λ_{max} close to 360 nm when UV sensitive and above 410 nm when violet sensitive (reviewed in Hunt and Peichl, 2014). *B. atrox* SWS1 has a F86 and therefore its $\lambda_{(max)}$ is predicted to be around 360 nm, similar to all the other vipers examined so far (Simões et al., 2016b). The presence of a UV-sensitive protein in *B. atrox* is also consistent with a previous study that showed that UV-sensible cones are common among nocturnal mammals and reptiles (Veilleux and Cumming, 2012).

4. DISCUSSION

B. atrox, similarly to the ancestral snake (and almost all snakes) (Davies et al., 2009; Simões et al., 2015), has lost two of the five opsin genes that are expressed in other reptiles, and only has RH1, LWS and SWS1. This loss is believed to be the result of the nocturnal and fossorial nature of many snakes, and presumably of the ancestral snake as well (Davies et al., 2009). Here we describe the cloning of all visual opsins from *B. atrox*; the genes encoding these opsins were amplified using standard methods and the resulting protein sequences were used to predict the sensitivity (λ_{max}) of these visual pigments.

B. atrox is a nocturnal predatory snake found in tropical forest grounds, where even moonlight and/or starlight are restricted by the dense forest foliage. The exact retinal morphology and rod to cone ratio is unknown in *B. atrox* retina, but as many nocturnal animals, and given its predatory habits, may have experienced strong selective pressures to maximize photon absorption in dim light environments (Lythgoe, 1984; Partridge and Cummings, 1999; Cummings and Partridge, 2001). Individuals belonging to this species need to move, hunt and/or mate in nocturnal conditions (with restricted moonlight/starlight), which could explain their need for a more rod-rich retina and the presence of only a small population of cone photoreceptors in their retina (Conant and Collins, 1998).

Opsins, proteic pigments that enable vision under different illuminations have been extensively studied. Opsin sequences are known to be highly conserved and amino acid substitutions at crucial positions affect their sensitivity to light. We have identified these substitutions at specific amino acid positions within *B. atrox* opsins, allowing us to predict their spectral sensitivities (λ_{max}) of the opsins expressed in the retina of this viper. Our predictions of λ_{max} for the B. atrox opsins (RH1 ~503-505 nm, LWS ~553 nm and SWS1 ~360 nm) resemble those found by other investigators when examining nocturnal vertebrates (Veilleux and Cumming, 2012; Simões et al., 2016b).

The variety of habitats that snakes had to adjust to during evolution, as well as their purported photic lifestyle change from nocturnal to diurnal and vice versa (Walls, 1934), may constitute the underlying causes for the positive selection pressure for specific amino acid substitutions at key functional amino acid sites. For example, RH1 opsin in the ancestral snake is believed to have N83 and A292 with λ_{max} values of about 491-496 nm (Hunt et al., 1996; Yokoyama et al., 2008b; Davies et al., 2009;

Simões et al., 2015). Various living snakes (especially more basal snakes such as Henophidians) maintain these amino acids whereas some of the more advanced snakes such as *B. atrox* carry a N83D substitution, shifting its λ_{max} to 503-505 nm. Although all Henophidians studied so far possess a N83, the opposite (i.e. the invariable presence of D83) is not true for all Caenophidians (Simões et al., 2016b). This is not surprising as Caenophidia is a very diverse clade, and includes snakes from very different habitats and photic lifestyles (diurnal vs. nocturnal). Snakes carrying this amino acid substitution on RH1 seem to be mostly nocturnal or crepuscular and can be found in more tropical or subtropical areas (Conant and Collins, 1998).

As mentioned above, nocturnal habitats can be extremely photon-limited and exert strong selective pressures to nocturnal animals. Even within the different nocturnal light environments, great differences may exist in terms of opened/closed canopy, foliage density, lunar phases, cloud cover, etc. These differences could greatly modify the amount of light available (Lythgoe, 1972; Munz and McFarland, 1973; Pariente, 1980). Both, the African viper *Echis ocellatus* and *B. atrox* inhabit rainforest grounds; where light can be extremely limited because of the dense foliage. These probably makes the selective pressures different from other animals, which inhabit more open environments with less foliage like grass lands and savannas like the two other African vipers (*Causus rhombeatus* and *Bitis nasicornis*) we compared it with. Rainforests are also richer in longer wavelengths (red-shifted) than dry forest and grasslands (Veilleux and Cumming, 2012), which could be another reason why *B. atrox* and *E. ocellatus* RH1 opsin has a D83 substitution resulting in a predicted λ_{max} a bit shifted to the red (503-505 nm) in comparison to other snakes (Simões et al., 2016b).

In general, LWS opsin has a larger wavelength range than RH1 and its λ_{max} can vary between 502 and 562 nm in nocturnal vertebrates, whereas the λ_{max} of RH1 varies within a smaller range (498-507nm). Furthermore, LWS λ_{max} in known not to vary according to the environment in which animals live (Veilleux and Cumming, 2012). This is not surprising since there is little light available during the night, and cones are therefore mostly inactive (something that may change somewhat depending on the lunar phase or time of the year in seasonal environments). In snakes specifically (nocturnal or diurnal), the λ_{max} of this protein seems to be much less variable; where blue-shifted varies in between 536-538 nm, whereas the more red-shifted ones vary between 550-560 nm (Simões et al., 2016b). The most common substitution in snakes

and the only one present in *B. atrox* and the previously studied African vipers (Simões et al., 2016b), is the S164A (SHYTA) substitution which causes a small blue shift from the 560 nm value, but is within the range for LWS opsins which absorb at longer wavelengths (550-560 nm, see above). Another mutation has been observed in snakes (T269A), but not in *B. atrox* or other vipers, and in combination with S164A causes a greater blue shift to LWS opsin to reach its blue-shifted form. Given that *B. atrox* only has the S164A substitution we predict the λ_{max} of LWS from *B. atrox* to be around 553 nm which is well within the absorbance range for both snakes and vertebrates in general as described in other studies (Davies et al., 2007; Davies et al., 2009; Yokoyama et al., 2008b; Simões et al., 2016b, amongst others).

Although we did not manage to clone the full length sequence, probably because of the relatively low expression of the transcript (generally animals possess far less blue cones than rods or red/green cones and therefore the protein is also expressed in far smaller amounts), we managed to predict the absorbance of SWS1 opsin based on the identity of amino acid 86 (compared to bovine rhodopsin). The *B. atrox* SWS1 opsin contained the F86 substitution and we predict it to be UV light sensitive, similarly to its previously studied homologue opsins (Sillman et al., 1999; Cowing et al., 2002; Fasick, Applebury and Oprian, 2002; Parry et al., 2004; Davies et al., 2009; Simões et al., 2016b). This seems particularly interesting, given that when SWS1 opsins were examined in nocturnal vertebrates, most of them were UV-sensitive albeit the sampling was not extensive (Veilleux and Cumming, 2012). It would be of interest to determine whether SWS1 sensitivity to UV wavelengths is correlated with a nocturnal life style within snakes.

To get a more complete picture on how our findings compare to those of others, taking in consideration the biology of the animals (habitats, activity patterns), we also decided to compare the λ_{max} of the three opsins from *B. atrox* with the λ_{max} of their homologues from one diurnal and one nocturnal snake mentioned in the study of Simões et al. (2016b). *Oxyrhopus melanogeny* is a nocturnal colubrid which occupies the same type of habitat as *B. atrox* and possesses opsins with λ_{max} values similar to the ones predicted here for *B. atrox*. *Spalerosophis diadema* on the other hand is a diurnal colubrid which inhabits more open locations without a canopy (such as grasslands and deserts) and the λ_{max} for its RH1 and LWS opsins is about 20 nm blue-shifted from the *B. atrox* homologues. These values conform to the view that the habitat of an animal,

as well as its activity patterns, can play a crucial role in the evolution of its visual system. *S. diadema* is a diurnal snake which has different visual needs than nocturnal snakes; it is therefore advantageous for it to be able to absorb shorter wavelengths which are more abundant in sunlight (Veilleux and Cumming, 2012). These results also agree with our comparisons with the three African vipers, where *E. ocellatus,* that inhabits close areas like forests as *B.atrox,* presents the same substitutions in RH1 opsin. Not surprisingly, the visual system, known for its plasticity, has evolved to satisfy the specific visual necessities of the organism.

Predicting the λ_{max} values for opsins is a practice which has been widely used as these proteins have been studied in detail and are well understood. However, the λ_{max} predicted from sequences may be slightly different from its actual value as measured by microspectrophotometry (MSP), a technique where an opsin can be expressed in a heterologous system and its absorbance can be directly measured (Liebman and Entine, 1964; Mollon, Bowmaker and Jacobs, 1984; Bowmaker, Astell, Hunt and Mollon, 1991). In one such case, Davies et al. (2009) reported MSP-determined λ_{max} values which were slightly different than the λ_{max} values predicted based on the opsin sequence. Although the results can be slightly different, considering that MSP is a technique which requires highly specialized equipment and expertise, using the sequence to predict the λ_{max} can be an easier alternative which can provide similar results. Furthermore, as more studies are done we can discover additional tuning sites, which are presently unknown, and this detailed knowledge could make this technique as accurate as MSP.

Opsins, although different at crucial sites for their spectral tuning, are highly conserved. On the other hand, photoreceptors (cones and rods) have experimented evolutionary changes, and have in fact switched from one to the other, as originally proposed by Wall's Transmutation Theory in 1934 and recently experimentally confirmed in snakes and lizards (Walls, 1934; Walls, 1942; Underwood, 1967; Schott et al., 2015; Simões et al., 2015). These changes have generated great diversification in photoreceptors, such as the acquisition of double cones, and the loss or gain of both cones and rods (Walls, 1934; Walls, 1942; Underwood, 1967; Simões et al., 2015). Recent studies in various snakes have shown that these animals have transmuted cones (Simões et al., 2015; Schott et al., 2015). However, the animals thus far studied are mostly colubrids and do not include vipers. It would be interesting to examine the *B.*

atrox retina with electron microscopy, in order to determine whether it has transmuted rods or cones and therefore if it supports (or possibly rejects) Wall´s transmutation theory as other reptiles do.

5. REFERENCES

Asenjo, A. B., Rim, J., & Oprian, D. D. (1994). Molecular determinants of human red/green color discrimination. *Neuron*, *12*(5), 1131-1138.

Atick, J.J. (1992). Could information theory provide an ecological theory of sensory processing? *Network 3*, 213 – 251.

Bowmaker, J. K., Astell, S., Hunt, D. M., & Mollon, J. D. (1991). Photosensitive and photostable pigments in the retinae of Old World monkeys. *Journal of Experimental Biology*, *156*(1), 1-19.

Bowmaker, J. K., & Hunt, D. M. (1999). Molecular biology of photoreceptor spectral sensitivity. In *Adaptive mechanisms in the ecology of vision* (pp. 439-462). Springer Netherlands.

Bowmaker, J. K. (2008). Evolution of vertebrate visual pigments. *Vision research*, *48*(20), 2022-2041.

Caprette, C. L., Lee, M. S., Shine, R., Mokany, A., & Downhower, J. F. (2004). The origin of snakes (Serpentes) as seen through eye anatomy. *Biological Journal of the Linnean* Society, 81(4), 469-482. ISO 690

Carter-Dawson, L. D., & Lavail, M. M. (1979). Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. *Journal of Comparative Neurology*, *188*(2), 245-262.

Cermakian, N., & Sassone-Corsi, P. (2002). Environmental stimulus perception and control of circadian clocks. *Current opinion in neurobiology*, *12*(4), 359-365.

Conant, R., & Collins, J. T. (1998). *A field guide to reptiles & amphibians: eastern and central North America* (Vol. 12). Houghton Mifflin Harcourt.

Crescitelli, F. (1972). The visual cells and visual pigments of the vertebrate eye. In *Photochemistry of vision* (pp. 245-363). Springer Berlin Heidelberg.

Cowing, J. A., Poopalasundaram, S., Wilkie, S. E., Robinson, P. R., & Bowmaker, J. K. (2002). The molecular mechanism for the spectral shifts between vertebrate ultraviolet-and violet-sensitive cone visual pigments. *Biochemical Journal*, *367*(1), 129-135.

Cummings, M., & Partridge, J. (2001). Visual pigments and optical habitats of surfperch (Embiotocidae) in the California kelp forest. *Journal of Comparative Physiology A*, *187*(11), 875-889.

Davies, W. L., Cowing, J. A., Carvalho, L. S., Potter, I. C., Trezise, A. E., Hunt, D. M., & Collin, S. P. (2007). Functional characterization, tuning, and regulation of visual pigment gene expression in an anadromous lamprey. *The FASEB Journal*, *21*(11), 2713-2724.

Davies, W. L., Cowing, J. A., Bowmaker, J. K., Carvalho, L. S., Gower, D. J., & Hunt, D. M. (2009). Shedding light on serpent sight: the visual pigments of henophidian snakes. *The Journal of Neuroscience*, *29*(23), 7519-7525.

Davies, W. L., Hankins, M. W., & Foster, R. G. (2010). Vertebrate ancient opsin and melanopsin: divergent irradiance detectors. *Photochemical & Photobiological Sciences*, *9*(11), 1444-1457.

Doyle, S., & Menaker, M. (2007). Circadian photoreception in vertebrates. In *Cold Spring Harbor symposia on quantitative biology* (Vol. 72, pp. 499-508). Cold Spring Harbor Laboratory Press.

Fasick, J. I., Applebury, M. L., & Oprian, D. D. (2002). Spectral tuning in the mammalian short-wavelength sensitive cone pigments. *Biochemistry*, *41*(21), 6860-6865.

Hart, N. S., Coimbra, J. P., Collin, S. P., & Westhoff, G. (2012). Photoreceptor types, visual pigments, and topographic specializations in the retinas of hydrophiid sea snakes. *Journal of Comparative Neurology*, *520*(6), 1246-1261.

Hauzman, E., Bonci, D. M., Grotzner, S. R., Mel a, M., Liber, A. M., Martins, S. L., & Ventura, D. F. (2014). Comparative Study of Photoreceptor and Retinal Ganglion Cell Topography and Spatial Resolving Power in Dipsadidae Snakes. *Brain, behavior and evolution*, *84*(3), 197-213.

Hecht, S., & Mintz, E. U. (1939). The visibility of single lines at various illuminations and the retinal basis of visual resolution. *The Journal of general physiology*, *22*(5), 593-612.

Hill, R. W., Wyse, G. A., Anderson, M., & Anderson, M. (2004). *Animal physiology* (Vol. 2). Massachusetts: Sinauer Associates.

Hsiang, A. Y., Field, D. J., Webster, T. H., Behlke, A. D., Davis, M. B., Racicot, R. A., & Gauthier, J. A. (2015). The origin of snakes: revealing the ecology, behavior, and evolutionary history of early snakes using genomics, phenomics, and the fossil record. *BMC evolutionary biology*, *15*(1), 87.

Hunt, D. M., Fitzgibbon, J., Slobodyanyuk, S. J., & Bowmakers, J. K. (1996). Spectral tuning and molecular evolution of rod visual pigments in the species flock of cottoid fish in Lake Baikal. *Vision research*, *36*(9), 1217-1224.

Hunt, D. M., & Collin, S. P. (2014). The Evolution of Photoreceptors and Visual Photopigments in Vertebrates. In *Evolution of Visual and Non-visual Pigments* (pp. 163-217). Springer US.

Hunt, D. M., & Peichl, L. (2014). S cones: evolution, retinal distribution, development, and spectral sensitivity. *Visual neuroscience*, *31*(02), 115-138.

Janke, A., & Arnason, U. (1997). The complete mitochondrial genome of Alligator mississippiensis and the separation between recent archosauria (birds and crocodiles). *Molecular Biology and Evolution*, *14*(12), 1266-1272

Kazmin, R., Rose, A., Szczepek, M., Elgeti, M., Ritter, E., Piechnick, R., ... & Bartl, F. J. (2015). The activation pathway of human rodopsin in comparison to bovine rhodopsin. *Journal of Biological Chemistry*, *290*(33), 20117-20127.

Liebman, P. A., & Entine, G. (1964). Sensitive low-light-level microspectrophotometer: detection of photosensitive pigments of retinal cones. *JOSA*, *54*(12), 1451-1458.

Loew, E. R., & Govardovskii, V. I. (2001). Photoreceptors and visual pigments in the red-eared turtle, Trachemys scripta elegans. *Visual neuroscience*, *18*(05), 753-757.

Lythgoe, J. N. (1972). The adaptation of visual pigments to the photic environment. In *Photochemistry of vision* (pp. 566-603). Springer Berlin Heidelberg.

Lythgoe, J. N. (1984). Visual pigments and environmental light. *Vision research*, *24*(11), 1539-1550.

Miller WH, ed (1981). Molecular Mechanisms of Photoreceptor Transduction, Current Topics in Membranes and Transport (*Academic, New York)*

Mollon, J. D., Bowmaker, J. K., & Jacobs, G. H. (1984). Variations of colour vision in a New World primate can be explained by polymorphism of retinal photopigments. *Proceedings of the Royal Society of London B: Biological Sciences*, *222*(1228), 373-399.

Mustafi, D., Engel, A. H., & Palczewski, K. (2009). Structure of Cone Photoreceptors. *Progress in Retinal and Eye Research*, *28*(4), 289–302. http://doi.org/10.1016/j.preteyeres.2009.05.003

Munz, F. W., & McFarland, W. N. (1973). The significance of spectral position in the rhodopsins of tropical marine fishes. *Vision research*, *13*(10), 1829IN1-1874.

Nilsonn, D.-E. (2013). Eye evolution and its functional basis. *Visual Neuroscience*, *30*(1-2), 5–20. http://doi.org/10.1017/S0952523813000035

Oliveira, M. E., & Martins, M. (2002). When and where to find a pitviper: activity patterns and habitat use of the lancehead, Bothrops atrox, in central Amazonia, Brazil. Herpetological Natural History, 8(2), 101-110. ISO 690

Osorio, D., & Vorobyev, M. (2005). Photoreceptor sectral sensitivities in terrestrial animals: adaptations for luminance and colour vision. *Proceedings of the Royal Society B: Biological Sciences*, *272*(1574), 1745–1752. http://doi.org/10.1098/rspb.2005.3156ç

Pariente, G. F. (1980). Quantitative and qualitative study of the light available in the natural biotope of Malagasy prosimians. *Nocturnal Malagasy primates: ecology, physiology and behavior. Academic*, *New York*, 117-134.

Parry, J. W., Poopalasundaram, S., Bowmaker, J. K., & Hunt, D. M. (2004). A novel amino acid substitution is responsible for spectral tuning in a rodent violet-sensitive visual pigment. *Biochemistry*, *43*(25), 8014-8020.

Partridge, J. C., & Cummings, M. E. (1999). Adaptation of visual pigments to the aquatic environment. In *Adaptive mechanisms in the ecology of vision* (pp. 251-283). Springer Netherlands.

Röll, B. (2000). Gecko vision—visual cells, evolution, and ecological constraints. *Journal of neurocytology*, *29*(7), 471-484.

Scanlon, J. D., & Lee, M. S. (2011). The major clades of living snakes: morphological evolution, molecular phylogeny, and divergence dates. *Reproductive biology and phylogeny of snakes*, *9*, 55-95.

Schott, R. K., Müller, J., Yang, C. G. Y., Bhattacharyya, N., Chan, N., Xu, M., … Chang, B. S. W. (2016). Evolutionary transformation of rod photoreceptors in the all-cone retina of a diurnal garter snake. *Proceedings of the National*

Schramm, G., Bruchhaus, I., & Roeder, T. (2000). A simple and reliable 5′-RACE approach. *Nucleic acids research*, *28*(22), e96-e96.

Shichida, Y., & Matsuyama, T. (2009). Evolution of opsins and phototransduction. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1531), 2881–2895. http://doi.org/10.1098/rstb.2009.0051

Sillman, A. J., Carver, J. K., & Loew, E. R. (1999). The photoreceptors and visual pigments in the retina of a boid snake, the ball python (Python regius). *Journal of Experimental Biology*, *202*(14), 1931-1938.

Sillman, A. J., Johnson, J. L., & Loew, E. R. (2001). Retinal photoreceptors and visual pigments in Boa constrictor imperator. *Journal of Experimental Zoology*, *290*(4), 359-365.

SIMMONS, J. (2005). Historia de las colecciones biológicas: 17-30 (en) SIMMONS, JE & MUÑOZ-SABA, Y. (eds.) Cuidado, manejo y conservación de colecciones biológicas.

Simões, B. F., Sampaio, F. L., Jared, C., Antoniazzi, M. M., Loew, E. R., Bowmaker, J. K., ... & Gower, D. J. (2015). Visual system evolution and the nature of the ancestral snake. *Journal of evolutionary biology*, *28*(7), 1309-1320.

(a) Simões, B. F., Sampaio, F. L., Loew, E. R., Sanders, K. L., Fisher, R. N., Hart, N. S., ... & Gower, D. J. (2016, January). Multiple rod–cone and cone–rod photoreceptor transmutations in snakes: evidence from visual opsin gene expression. In *Proc. R. Soc. B* (Vol. 283, No. 1823, p. 20152624). *The Royal Society*.

(b) Simões, B. F., Sampaio, F. L., Douglas, R. H., Kodandaramaiah, U., Casewell, N. R., Harrison, R. A., ... & Gower, D. J. (2016). Visual pigments, ocular filters and the evolution of snake vision. *Molecular biology and evolution*, *33*(10), 2483-2495.

Stocker, K., & Barlow, G. H. (1976). [18] The coagulant enzyme from Bothrops atrox venom (batroxobin). *Methods in enzymology*, *45*, 214-223.

Underwood, G. (1967). A contribution to the classification of snakes (No. 653). *Trustees of the British Museum* (Natural History).

Van Hazel, I., Santini, F., Müller, J., & Chang, B. S. (2006). Short-wavelength sensitive opsin (SWS1) as a new marker for vertebrate phylogenetics. *BMC Evolutionary Biology*, *6*, 97. http://doi.org/10.1186/1471-2148-6-97

Veilleux, C. C., & Cummings, M. E. (2012). Nocturnal light environments and species ecology: implications for nocturnal color vision in forests. *Journal of Experimental Biology*, *215*(23), 4085-4096.

Walls, G. L. (1934). The Reptilian Retina: I. A new concept of visual-cell evolution. *American journal of ophthalmology*, *17*(10), 892-915.

Walls, G. L. (1942). The vertebrate eye and its adaptive radiation.

Yau, K. W., & Hardie, R. C. (2009). Phototransduction motifs and variations.*Cell*, *139*(2), 246-264.

Yokoyama, S. (2000). Molecular evolution of vertebrate visual pigments. *Progress in retinal and eye research*, *19*(4), 385-419.

Yokoyama, S., Takenaka, N., Agnew, D. W., & Shoshani, J. (2005). Elephants and human color-blind deuteranopes have identical sets of visual pigments. *Genetics*, *170*(1), 335-344.

(a) Yokoyama, S., Yang, H., & Starmer, W. T. (2008). Molecular Basis of Spectral Tuning in the Red- and Green-Sensitive (M/LWS) Pigments in Vertebrates.*Genetics*, *179*(4),2037–2043. http://doi.org/10.1534/genetics.108.090449

(b) Yokoyama, S., Tada, T., Zhang, H., & Britt, L. (2008). Elucidation of phenotypic adaptations: Molecular analyses of dim-light vision proteins in vertebrates. *Proceedings of the National Academy of Sciences*, *105*(36), 13480-13485.

Figures

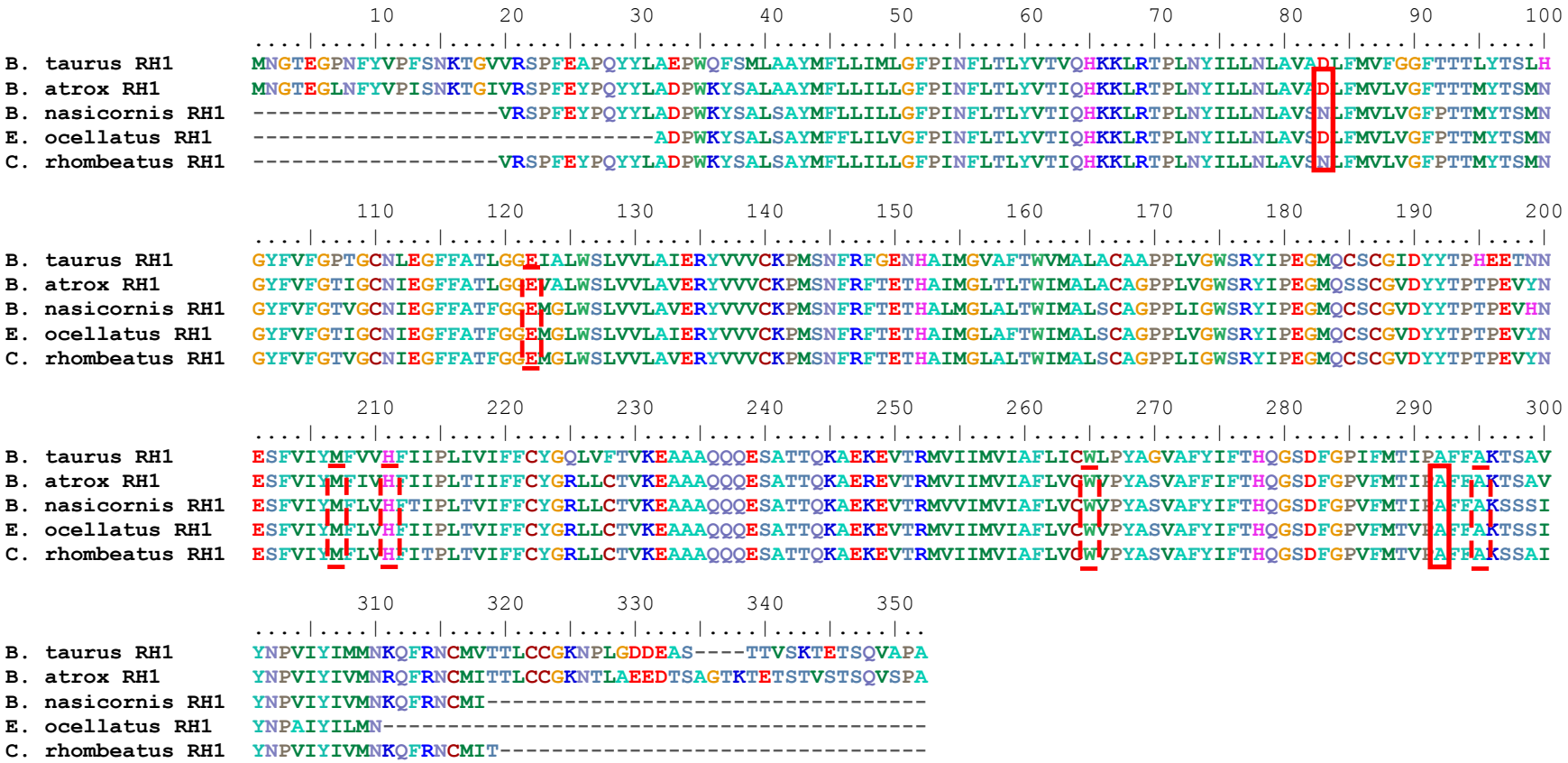

Figure 1: Alignment of the complete amino acid sequence of RH1 expressed in the retina of *Bothrops atrox*, with *Bos taurus* rod opsin (RH1) and partial RH1 sequences of *Bitis nasicornis*, E*chis ocellatus* and *Causus rhombeatus*. Amino acid numbering is based on bovine rod opsin (*Bos taurus*). All amino acids located at spectral tuning sites are shown in boxes. Boxes with solid lines indicate amino acids D83 and A292 which have been shown to change in snakes.

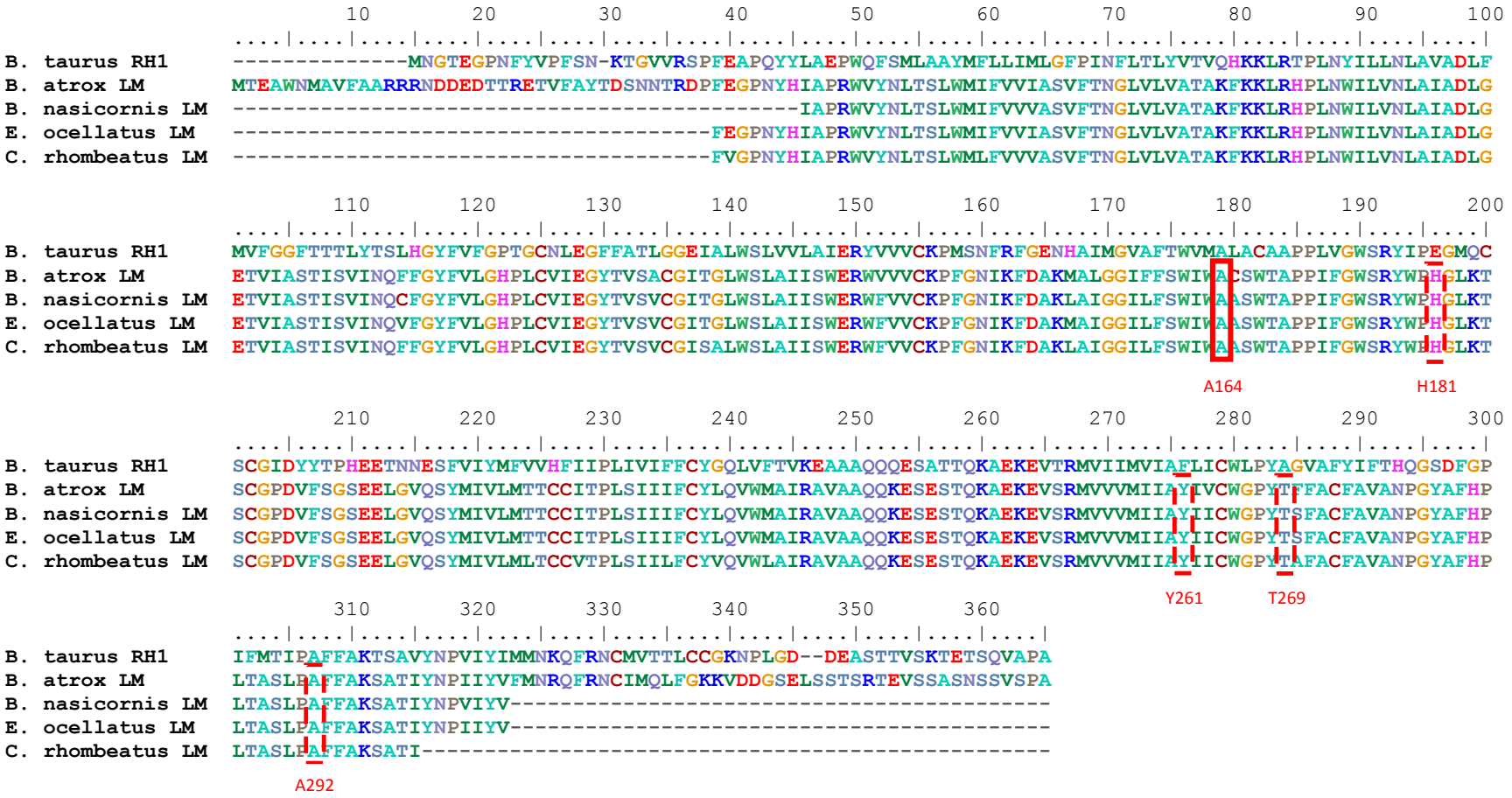

Figure 2: Alignment of the complete amino acid sequence of LWS expressed in the retina of *Bothrops atrox*, with Bos taurus rod opsin (RH1) and partial LWS sequences of *Bitis nasicornis*, *Echis ocellatus* and *Causus rhombeatus*. Amino acid numbering (boxes) is based on the bovine (*Bos taurus*) RH1 opsin. All amino acids located at spectral tuning sites are shown in boxes. The box with solid lines indicates amino acid A164 which has been shown to change in some snakes.

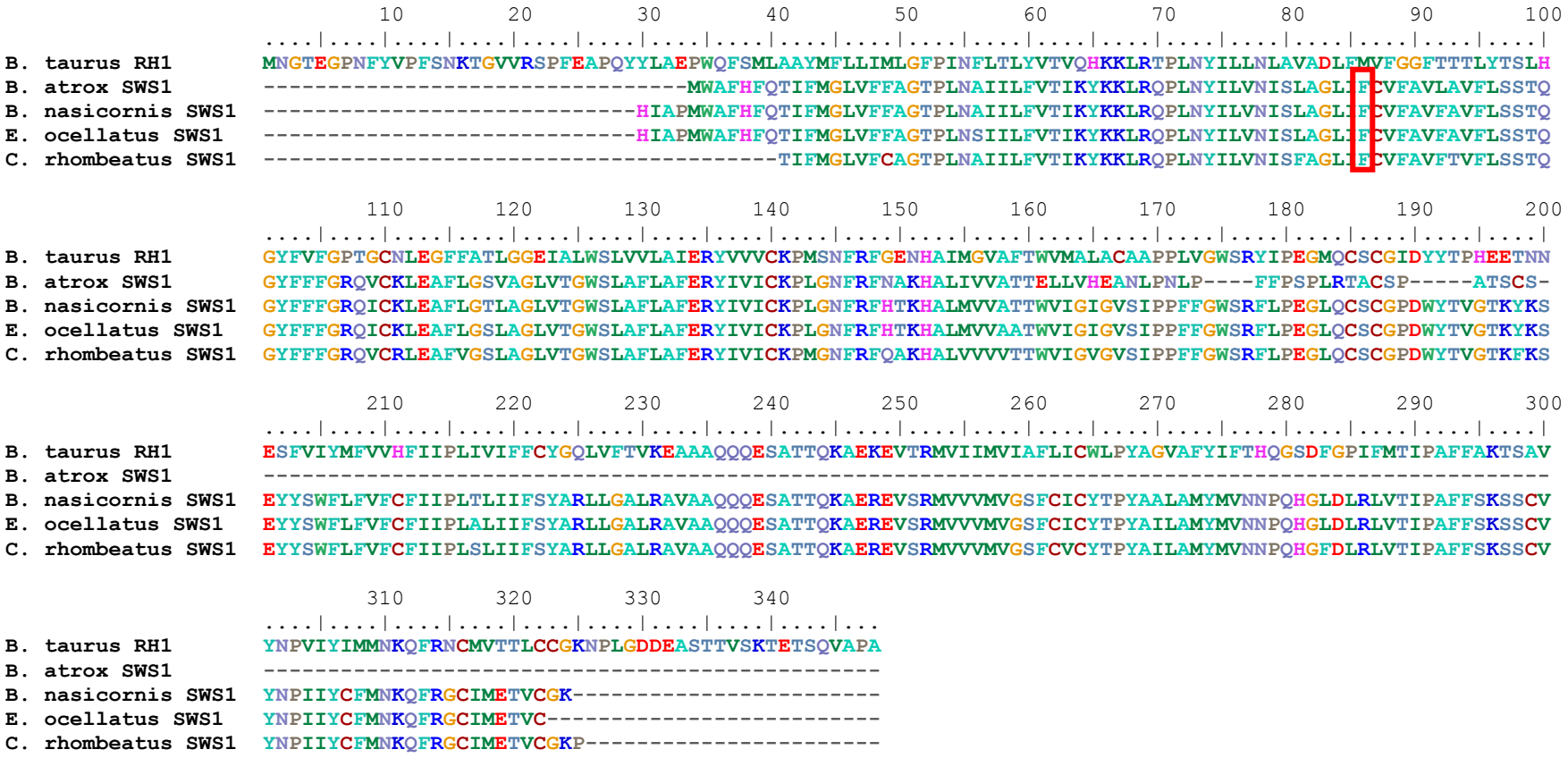

Figure 3: Alignment of the partial amino acid sequence of SWS1 expressed in the retina of *Bothrops atrox*, with Bos taurus rod opsin (RH1) and the partial SWS1 sequences of *Bitis nasicornis*, *Echis ocellatus* and *Causus rhombeatu*s. Amino acid numbering is based on the bovine (*Bos taurus*) RH1 opsin. Amino acid 86 (boxed) is known to determine the spectral sensitivity (UV- or violet-sensitive) of SWS1. In *B. atrox* a phenylalanine is present making this protein UV-sensitive.

Tables

Primer	Sequence	Gene	Reference
DIAPLMF1	5’-AAGCGTATTYAYTTAYACCRACASCAACAA-3’	LWS	Davies et al. 2009
DIAPLMF2	5’-AGTGTCATCAACCAGWTCTYBGGSTAYTTC-3’	LWS	Davies et al. 2009
DIAPLMR1	5’-CATCCTBGACACYTCCYTCTCVGCCTTCTG-3’	LWS	Davies et al. 2009
DIAPLMR2	5’-CATCATCCACTTTYTTSCCRAASAGCTGCA-3’	LWS	Davies et al. 2009
DIAPR1F1	5’-GTCAAAATTTCTAYRTBCCCWTKTCCAACA-3’	RH1	Davies et al. 2009
DIAPR1F2	5’-AATAGGATGCWRCWTYGARGGCTTCTTTGC-3’	RH1	Davies et al. 2009
DIAPR1R1	5’-ACAGTGCAGACAAGRYKYCCRTAGCAGAAG-3’	RH1	Davies et al. 2009
DIAPR1R2	5’-ATTCTTTCCACARCARAGRGTBRTGATCAT-3’	RH1	Davies et al. 2009
DIAPS1F1	5’-TCCCATGTCCGGAGAVGAVGABTTYTACCT-3’	SWS1	Davies et al. 2009
DIAPS1F2	5’-GGCCTTCGARCGHTACATYGTYATCTGCAA-3’	SWS1	Davies et al. 2009
DIAPS1R1	5’-CACCACSACCATSCGVGASACCTCCCGCTC-3’	SWS1	Davies et al. 2009
DIAPS1R2	5’-TTAGCTGGGGCYGACYTGRCTGGAGGACAC-3’	SWS1	Davies et al. 2009
All_DIAP_S1_F3	5’-GGGGCCBTTSGAYGGBCCCCARTAYCACAT-3’	SWS1	Davies et al. 2009
All_DIAP_S1_R3	5’-TGCTTCATGAACAARCAGTTCCGNGSHTGC-3’	SWS1	Davies et al. 2009
5’RACE_ANCHOR_F	5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG- 3'	RACE	Davies et al. 2009
5’RACE_SHORT_F	5'-GGCCACGCGTCGACTAGTAC-3'	RACE	Davies et al. 2009
3’RACE_INNER_R	5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'	RACE	Davies et al. 2009
3’RACE_OUTER_R	5'-GCGAGCACAGAATTAATACGACT-3'	RACE	Davies et al. 2009
LM_FA_REP	5'-GGAAGTRTCRAGRATGGTMGTGG-3'	LWS	This work
LM_FB_REP	5’-GGGGACCMTATRCRDYYTTTGCC-3'	LWS	This work
LM_FB_SN	5’-GGGGACCATATRCAKYTTTTGCC-3'	LWS	This work

(Continue)

LM_RA_REP	5’-ATCRGCKAYHGCCAAGTTSACC-3'	LWS	This work
LM_RB_REP	5’-CVACAAAGAYCATCCAGAGDGAAG-3'	LWS	This work
LM_atxden_R	5’-ACTGGTTGATGACACTGATRGTGCTAGC-3'	LWS	This work
LM_REP_Rc	5’-CDGTGGCCACCAATACCAARCCATTGG-3'	LWS	This work
RH1_FA_atx	5’-TGGGTCCCTTATGCCTCTGTGGC-3'	RH1	This work
RH1_FB_REP	5’-CYGTCTTTATGACCATCCCRGCC-3'	RH1	This work
RH1_RBB_ATX	5’-GAAACATATACGCAGCCAAGGC-3'	RH1	This work
RH1_sn_F	5’-CCCCAGAAGTCYATAAYGAGTCC-3'	RH1	This work
RH1_sn_R	5’-GGGCTTACAAACTACTAMRTATC-3'	RH1	This work
RH1_atx_R	5’-GACCATGAAAAGATCGGCTACTGCC-3'	RH1	This work
RH1_atx_Rc	5’-CAAGGCAGAATATTTCCATGGATCTGCC-3'	RH1	This work
SWS1_atr_R	5’-TGACCAAAATGTAGTTGAGCGGCTGCC-3'	SWS1	This work
SWS1_atr_FF	5’-GCGGTCTTCTTGTCCAGCACCCAAGGG-3'	SWS1	This work
S1_atr_R2	5’-CGAAGAACACCAGGCCCATGAAGATGG-3'	SWS1	This work
S1_atrden_F1	5’-GCWCGGTRGCAGGTCTGGTCACYGGC-3'	SWS1	This work
S1_atr_F2	5’-GCATGCTTTGATTGTGGTGGCAACC-3'	SWS1	This work
S1_atrden_R1	5’-CGAAGAGGATGATGGYGTTGAGSGG-3'	SWS1	This work
S1_atr_F3	5’-CCTCCGAACTGCTTGTTCCCCCGC-3'	SWS1	This work
S1_atr_F2	5’-GCATGCTTTGATTGTGGTGGCAACC-3'	SWS1	This work

Table 1: Primers used in nested-PCR and RACE reactions to generate sequences for Rhodopsin-like 1 (RH1), Short-wavelength sensitive 1 (SWS1) and Long-wavelength sensitive (LWS) opsins from *Bothrops atrox*

DECLARACIÓN Y AUTORIZACIÓN

Yo, Micaela Stacey Solís, con CC. 1713069969, autora del trabajo de graduación titulado "Cloning of the opsin genes present in *Bothrops atrox* (Viperidae) retina.", previa la obtención del grado académico de **LICENCIADA EN CIENCIAS BIOLÓGICAS** en la Facultad de Ciencias Exactas y Naturales.

1.- Declaro tener pleno conocimiento de la obligación que tiene la Pontificia Universidad Católica del Ecuador, de conformidad con el artículo 144 de la Ley Orgánica de Educación Superior, de entregar a la SENECYT en formato digital una copia del referido trabajo de graduación para que sea integrado al Sistema Nacional de Información de la Educación Superior del Ecuador para su difusión pública respetando los derechos de autor.

2.- autorizo a la Pontificia Universidad Católica del Ecuador a difundir a través del sitio web de la Biblioteca de la PUCE el referido trabajo de graduación, respetando las políticas de propiedad intelectual de la Universidad.

Quito, 10 de febrero de 2017

f)_____

CC#__1713069969_____