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Visual Opsin Diversity in Anurans

Leah Perez
perezlmsc@gmail.com

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VISUAL OPSIN DIVERSITY IN ANURANS

By

LEAH PEREZ, Bachelor of Science

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VISUAL OPSIN DIVERSITY IN ANURANS

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LEAH PEREZ, Bachelor of Science

APPROVED:

_____
Dr. Matthew Kwiatkowski, Thesis Director

_____
Dr. Jennifer Gumm, Committee Member

_____
Dr. Beatrice Clack, Committee Member

_____
Dr. Donald Pratt, Committee Member

_____

Pauline M. Sampson, Ph.D.
Dean of Research and Graduate Studies

ABSTRACT

Among major vertebrate groups, anurans are understudied with regards to their visual systems and how they function. This study sampled North American anurans representing diverse evolutionary and life histories and which likely possess visual systems adapted to meet different ecological needs. Using standard molecular techniques, sequences were obtained for four opsins—the protein component of visual pigments—expressed in anuran retinas. Amino acid sequences of the genes RH1, LWS, SWS1, and SWS2 were compared across taxa to identify variable sites, as such variation can shift the spectral sensitivity of visual pigments and thus alter dim-light and color vision. Some of the amino acid changes observed are known to tune spectral sensitivity in other vertebrates, and tests for positive selection revealed additional candidate tuning sites in LWS. The observed variation cannot fully be explained by evolutionary relationships among species. Taken together, results suggest that other factors may be driving changes to anuran visual systems.

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INTRODUCTION

Vertebrate Visual Systems

Most animals possess some level of photosensitivity (Bowmaker, 2008). One of the most primitive and widespread functions of the visual system is detecting changes in illumination. This provides an organism with basic information about its environment and mediates behaviors such as phototaxis and the entrainment of a circadian rhythm (Lamb et al., 2007; reviewed in Cronin et al., 2014). In addition to this, some invertebrates and nearly all vertebrates also possess image-forming, camera-like eyes, in which light is focused through various media (cornea, pupil, vitreous humor, etc.) onto the light-sensitive retina.

There are two types of photoreceptive cells responsible for image-forming vision in vertebrates: rods and cones (Yokoyama and Yokoyama, 1996). Rods are involved primarily in scotopic (dim-light) vision, while cones function better in brighter conditions and are involved primarily in photopic and color vision (reviewed in Bowmaker, 1998). Rods and cones are composed of three main parts: an outer segment of disc-shaped membranous folds, an inner segment containing most of the cell's membrane-bound organelles, and a synaptic terminal (Lamb et al., 2007). The discs of the outer segment contain transmembrane visual pigments, each consisting of a G-protein-coupled

receptor—an opsin—with seven transmembrane α-helices enclosing a covalently bound chromophore (Palczewski et al., 2000). The chromophore of vertebrate visual pigments is a derivative of vitamin A and can occur in two forms. It can occur as either 11-cis-retinal (vitamin A_1 aldehyde) or 11-cis-3, 4-didehydroretinal (vitamin A_2 aldehyde), which possesses an additional double bond within its β-ionone ring (Yokoyama, 2000; Temple et al., 2006; Porter et al., 2011). Due to the aforementioned structural difference, the wavelength sensitivity of visual pigments expressing 11-cis-3, 4-didehydroretinal are long-wavelength shifted by 20-60 nm with respect to those expressing 11-cis-retinal (Enright et al., 2015). Phototransduction begins when a photon excites the chromophore, causing it to isomerize. This triggers a conformational change in the opsin protein and activates a signal transduction cascade which leads to a neural response (Lamb et al., 2007).

Vertebrate visual pigments are historically divided into five classes based on their wavelength absorption maxima, referred to as their spectral sensitivity (Bowmaker, 2008; Davies et al., 2012). The visual pigment classes include one “rod” pigment class, the protein component of which is rhodopsin, or RH1, having a peak spectral sensitivity (λ_{max}) of approximately 500 nm (reviewed in Cronin et al., 2014). The four “cone” pigment classes include the long-wavelength-sensitive (LWS), mid-wavelength-sensitive (MWS), short-wavelength-sensitive (SWS or SWS2), and very-short- or UV-wavelength-sensitive (UVWS or SWS1) classes

(Yokoyama, 2000; 2008). Historically, vertebrate cone cells have also been divided into four classes synonymous with the aforementioned visual pigment classes, but this is an oversimplification. Other factors, such as the presence of a wavelength-filtering oil droplet in the inner segment, can also influence the spectral sensitivity of a photoreceptor (Wilby and Roberts, 2017). A single photoreceptor cell can also coexpress different opsins and thus different visual pigments, possibly improving its light sensitivity at the cost of spectral discrimination (Dalton et al., 2014). Additional visual pigments can arise due to opsin gene duplication events. In fishes, for example, duplication of the SWS2 opsin has produced SWS2A and SWS2B, and several RH2 duplications have produced RH2Aα, RH2Aβ, and RH2B opsins (reviewed in Rennison et al., 2012). The expressed opsin protein and chromophore of each visual pigment and how those components interact shape its spectral sensitivity and, by extension, affect the spectral sensitivity of individual photoreceptors and the retina as a whole (Bowmaker and Hunt, 2006; reviewed in Cronin et al., 2014).

Spectral Tuning in Response to Photic Environment

Spectral tuning refers to shifts in spectral sensitivity and occurs through two major genetic mechanisms. The first is through changes to an opsin-coding sequence which result in the substitution of amino acid residues lining the chromophore-binding pocket formed by the opsin's seven transmembrane α-

helices. These substitutions can tune spectral sensitivity by altering the interaction between the opsin and the chromophore (Nathans, 1990; Chang et al., 1995; Wilkie et al., 2000; reviewed in Yokoyama, 2000; Hofmann et al., 2009; Davies et al., 2012). One common type of amino acid change that results in spectral tuning is a change that alters polarity and/or charge at a particular site, which can affect the electrostatic environment surrounding the chromophore (reviewed in Wang et al., 2014). In mammals, for example, a change from the polar amino acid serine to nonpolar alanine at site 180 (notated as S180A) results in short-wavelength-shifted spectral sensitivity of the LWS visual pigment in humans, goats, cats, and dogs. This and four other critical amino acid changes (H197Y, Y277F, T285A, and A208S) can predict short-wavelength shifts in spectral sensitivity and control red-green color vision in mammals (Yokoyama and Radlwimmer, 1998). As in mammals, amino acid changes responsible for spectral tuning have been identified in all major vertebrate taxonomic groups (Wilkie et al., 2000; Cowing et al., 2002; Takahashi and Yokoyama, 2005; Takenaka and Yokoyama, 2007; reviewed in Yokoyama, 2008).

The second mechanism of spectral tuning is differential expression of opsin-coding genes. This mechanism tunes visual systems via the expression of distinct subsets of genes and variation in relative opsin expression. In vertebrates, expression of opsin genes is controlled by transcription factors that bind to opsin promoters (reviewed in Nandamuri et al., 2017). Though few in

number, the loci that control opsin expression can produce significant changes in expression profiles (Carleton et al., 2010). This is the case in African rift lake cichlids, which have seven available cone opsin genes, spectrally distinct subsets of which may be expressed in different species (Carleton and Kocher, 2001; Parry et al., 2005; Spady et al., 2006). Alternatively, variation in opsin expression within and among populations occurs via differences in relative opsin expression (Carleton and Kocher, 2001; Fuller et al., 2004; Sandkam et al., 2015; Stieb et al., 2016).

Variation in spectral sensitivity between closely-related species and spectral tuning within species are both associated with photic environment. An example of the former is found in snapper (*Lutjanus*) species residing on the Great Barrier Reef in Australia. Variation in spectral sensitivity among these species corresponds with the spectral characteristics of the clear, chlorophyll-rich, or tannin-stained waters in which they occur (Lythgoe et al., 1994). Ambient light conditions can also drive diverse changes in opsin expression within species (Hofmann et al., 2009; Jokela-Määttä et al., 2009) and influence speciation in the absence of geographical isolation through the process of sensory drive (Seehausen et al., 2008). Spectral tuning can occur via both amino acid changes and differential opsin expression within the same species. Threespine sticklebacks (*Gasterosteus aculeatus*) in natural tannin-stained blackwater environments exhibit amino acid changes on the SWS2 opsin known to cause a

long-wavelength shift in spectral sensitivity. After transplant to a natural clearwater environment, successive generations of sticklebacks exhibit greater frequency of a novel SWS2 haplotype resulting in short-wavelength-shifted spectral sensitivity (Marques et al., 2017). Also in *G. aculeatus*, variation in expression of the SWS1 and RH2 opsin genes tunes spectral sensitivity to longer wavelengths in freshwater populations, relative to marine populations (Rennison et al., 2016). Effects of photic environment on spectral tuning can also be observed in a laboratory setting (Carleton et al., 2008; Ehlman et al., 2015). Bluefin killifish (*Lucania goodei*) raised under different photic conditions in the lab exhibit spectral tuning regardless of the photic environment of the origin population. More specifically, killifish raised in tannin-stained water exhibit greater expression of long-wavelength-sensitive opsins, while those raised in clear water exhibit greater expression of short-wavelength-sensitive opsins (Fuller et al., 2005).

Vertebrates living in photon-limited environments have adapted visual systems to maximize efficiency at low light levels and with fewer available wavelengths. Light attenuates in water, resulting in a narrower range of available wavelengths at greater depth. Lake Victoria cichlids in progressively deeper, long-wavelength-shifted photic environments exhibit long-wavelength-shifted spectral sensitivity and male nuptial color (Seehausen et al., 2008). In cottoid fish of Lake Baikal, the deepest lake in the world, amino acid changes at four spectral

tuning sites result in a step-wise shift in maximum absorbance at different depths. Short-wavelength shifts in spectral sensitivity at greater depths are hypothesized to reduce photoreceptor noise at extremely low light levels (Hunt et al., 1996). Similarly, to accommodate their photon-limited environment, deep-sea fishes often possess rods spectrally shifted to maximum absorbances of approximately 470-480 nm, the range of available wavelengths from down-welling daylight and most common bioluminescence (Hunt et al., 2001).

Photon-limited environments are also found terrestrially, where nocturnal light intensities can vary by as much as eight orders of magnitude due to lunar phase, lunar altitude, weather, foliage density, seasonality, and latitude (reviewed in Veilleux and Cummings, 2012). Lunar light is spectrally similar to sunlight, while starlight is spectrally shifted toward longer wavelengths, above 560 nm (Johnsen et al., 2006; Warrant and Johnsen, 2013). In forests, due to filtering by photosynthetic leaves, nocturnal light conditions are generally dimmer and dominated by wavelengths ranging from 480 to 580 nm under moonlight and 540 to 580 nm on moonless nights (Veilleaux and Cummings, 2012). Variation in the spectral sensitivity of long-wavelength-sensitive visual pigments in nocturnal mammals suggests that they may be tuned to maximize light absorption in photon-limited forest environments at night (Veilleux and Cummings, 2012).

Study System

Anuran Vision

The amphibian eye broadly resembles that of other vertebrates, with photoreceptor cells concentrated on the retina (reviewed in Kardong, 2012). In all studied amphibian retinas, the most abundant photoreceptors are mid-wavelength-sensitive (MWS) rods (Denton and Wyllie, 1955), historically termed "red" rods due to their appearance in freshly-dissected tissue under white light (Boll, 1877). In salamanders (Urodela) and frogs (Anura), these rods possess visual pigments with a peak spectral sensitivity (λ_{max}) of 496-503 nm (Table 1; Liebman and Entine, 1968; Korenyak and Govardovskii, 2013). In caecilians (Gymnophiona), which are limbless and mostly fossorial, MWS rods are the only known photoreceptor type and are short wavelength shifted by 13-19 nm relative to other amphibians (Mohun et al., 2010). Anurans and possibly some salamanders also possess a second rod type that is unique among vertebrates. These historically-termed "green" rods account for approximately 8-9% of rods on the anuran retina (Denton and Wyllie, 1955) and are short-wavelength sensitive (SWS), possessing visual pigments with a λ_{max} of 432-440 nm (Table 1; Reuter, 1966; Dartnall, 1967; Liebman and Entine, 1968; reviewed in Govardovskii and Reuter, 2014). This SWS rod type is understood to be an evolutionarily modified cone with an SWS2 opsin (Hisatomi, 1999; Lamb et al., 2007).

Most amphibians, with the exception of caecilians, possess three cone types. In anurans, the most abundant cone type is long-wavelength sensitive (LWS), with visual pigments of which possess an LWS opsin and typically have a

λ_{max} between 560-575 nm (Table 1; Bowmaker, 2008). LWS cones of anuran retinas have been identified primarily as single cones possessing clear oil droplets, but some may occur as double cones without oil droplets (Liebman and Entine, 1968; Hárosi, 1982). The second-most abundant cone type in anurans is a short-wavelength-sensitive (SWS) single cone with no observed oil droplets, and with visual pigments possessing an SWS1 opsin and λ_{max} between 431-433 nm (Table 1; Hárosi, 1982; Hisatomi, 1998; Takahashi and Yokoyama, 2005). The third cone type observed in anurans is a mid-wavelength-sensitive (MWS) cone, also lacking oil droplets, with λ_{max} measured at 502 nm in two species in the pond frog genus *Lithobates* (Liebman and Entine, 1968; Hárosi, 1982). The opsin component of MWS cone visual pigments in anurans has not yet been identified. The RH2 opsin-coding gene, which is the typical opsin-coding gene associated with MWS cones in fishes and non-mammalian tetrapods, has not been identified in anurans (Bowmaker, 2008). The SWS2 opsin found in the SWS rod cells of anurans may also occur in some anuran cones (Ma et al., 2001; Lamb et al., 2007), but this has not been tied to MWS cones.

Amphibians possess both visual pigments with 11-cis-retinal and 11-cis-3, 4-didehydroretinal chromophores (Dartnall and Lythgoe, 1965). In the MWS rods of salamanders and anurans that have been studied to date, λ_{max} in visual pigments with 11-cis-3, 4-didehydroretinal are long wavelength shifted by 20-30 nm in comparison to those with 11-cis-retinal chromophores (Dartnall and

Lythgoe, 1965; Ala-Laurila et al., 2007; Korenyak and Govardovskii, 2013). The same relationship between visual pigments possessing different chromophores occurs in the cones of several salamander species (Korenyak and Govardovskii, 2013). In adult anurans, the visual pigment composition of photoreceptors on the retina can also change under specific light and temperature conditions (Tsin and Beatty, 1980).

Table 1. Spectral sensitivity (in nm) of five anuran photoreceptors espressing the 11-cis-retinal chromophore; Reported values represent a combination of microspectrophotometric (MSP) and electroretinographic (ERG) methodologies.

Species	SWS rod	MWS rod	SWS cone	MWS cone	LWS cone
Bufo bufo[1] (common toad)	432	502	-	-	562
Agalychnis callidryas[2] (red-eyed treefrog)	-	502	-	-	546
Hyla cinerea[3] (green treefrog)	435	503	-	-	-
Lithobates pipiens[1,4] (Northern leopard frog)	432	502	-	502	575
Lithobates catesbeianus[1,5] (American bullfrog)	433	502	433	502	570
Rana temporaria [1,6] (common frog)	434	503	431	-	562

[1] Govardovskii et al. (2000); [2] Liebau et al. (2015); [3] King et al. (1993); [4] Liebman and Entine (1968); [5] Hárosi (1982); [6] Koskelainen et al. (1994)

Anuran Visual Ecology

A reliance on visual cues, specialization for dim-light vision, and complex visual system metamorphosis make anurans a valuable study system in

vertebrate visual ecology. Anurans have complex life cycles in which larvae and adults inhabit diverse photic environments. Most anuran larvae are fully aquatic and free swimming, while adults may be aquatic, semiaquatic, terrestrial, fossorial, arboreal, semiarboreal, or some combination thereof. During metamorphosis anurans undergo drastic anatomical and physiological changes, including changes to the visual system which can affect spectral sensitivity. Like many freshwater organisms, larval anurans primarily express the chromophore 11-cis-3, 4-didehydroretinal, which likely tunes visual pigment sensitivity to the longer wavelengths that dominate many freshwater photic environments (reviewed in Wald, 1958; Partridge et al., 1992; reviewed in Temple et al., 2006). During metamorphosis, pond frogs (Ranidae) produce less 11-cis-3, 4-didehydroretinal and segregate it within the dorsal retina, a change which correlates with ontogenic changes in habitat and behavior, as adults often rest with eyes only partially submerged in water (Reuter et al., 1971). In the retinas of adult pond frogs, the dominant chromophore becomes 11-cis-retinal (Wald, 1946; Reuter et al., 1971), which is more common in terrestrial organisms (Enright et al., 2015). In *Xenopus laevis*, which is secondarily aquatic, larval retinas exhibit a combination of both chromophores, while fully aquatic adults exhibit mainly 11-cis-3, 4-didehydroretinal (Crescitelli, 1973).

The ability of anurans to detect light levels and the spectral properties of their photic environment affects many aspects of their biology, including

movement patterns, habitat preferences, foraging, reproduction, and possibly thermoregulation (reviewed in Buchanan, 2006). The two spectrally distinct rod types likely allow amphibians to see colors at light intensities too low for cones to detect light (Yovanovich et al., 2017). Nocturnal anurans may use color vision for mate selection in dim light conditions. Nocturnal forests and woodlands have a yellow-green dominant light environment with peak flux at 560 nm (Veilleux and Cummings, 2012). In most frog species that exhibit sexual dichromatism during the mating season, males become yellower or brighter than females (Bell and Zamudio 2012; Rehberg-Besler et al., 2015), which may be associated with the photic environment of forests at night (suggested in Rehberg-Besler et al., 2015). In many anuran species, visual signals are influenced by sexual selection (Abrunhosa and Wogel, 2004; Amézquita and Hödl, 2004; Rosenthal et al., 2004; Giasson and Haddad, 2006; Taylor et al., 2007; Richardson et al., 2010). In the European treefrog (*Hyla arborea*), females exhibit vocal sac coloration preferences under controlled dim light conditions and likely utilize both chromatic and brightness cues for mate assessment at night (Gomez et al., 2009; Gomez et al., 2010).

Objectives

Among major vertebrate groups, anurans are understudied with regards to their visual systems and how they function. Currently, complete opsin sequences have been published for only two species of native North American anurans: the

bullfrog (*Lithobates catesbeianus*) and the cane toad (*Rhinella marina*) (Sayers, 2019). This study includes *L. catesbeianus* and fourteen additional species, representing six families, for which visual opsin sequences have not yet been published. Study species represent diverse evolutionary and life histories and, because of this, likely possess visual systems adapted to meet different ecological needs. The objectives of this study are to (1) Determine which opsin genes are expressed in anuran retinas; (2) Identify variation in opsin sequences among anuran species; and (3) Test for selection at possible spectral tuning sites. I hypothesize that visual opsins of anurans representing diverse evolutionary and life histories exhibit amino acid variation which may contribute to differences in spectral sensitivity.

MATERIALS AND METHODS

Sample Collection

Fourteen of the fifteen anuran species represented in this study are native to eastern Texas. These include two species of “true toad” (*Incilius nebulifer* and *Anaxyrus woodhousii*); one species of cricket frog (*Acris crepitans*); two species of chorus frog (*Pseudacris crucifer* and *P. fouquettei*); three species of treefrog (*Hyla chrysoscelis*, *H. versicolor*, and *H. cinerea*); four species of pond frog (*Lithobates catesbeianus*, *L. clamitans*, *L. palustris*, *and L. sphenocephalus*); one species of narrowmouth toad (*Gastrophryne carolinensis*); and one species of spadefoot toad (*Scaphiopus hurterii*). In addition to the fourteen native species, this study also includes one non-native species, the chirping frog *Eleutherodactylus cystignathoides*. (See the phylogeny in Figure 1 for relationships among study species, as well as common taxonomic groupings.) Up to five individuals per species were collected throughout the study period, from autumn of 2017 through spring of 2019.

Most individuals were collected from ephemeral breeding ponds in the Stephen F. Austin Experimental Forest, which is part of the Angelina National Forest, and the adjacent Alazan Bayou Wildlife Management Area in southwestern Nacogdoches County, TX, USA. The strictly urban *E.*

cystignathoides were collected on or near the Stephen F. Austin State University campus. All study animals were collected under permit and in compliance with the U.S. Forest Service, Texas Parks and Wildlife Department, and Nacogdoches city law enforcement. Following protocol described by the Herpetological Animal Care and Use Committee (2004) of the American Society of Ichthyologists and Herpetologists (ASIH) and approved by the SFASU Institutional Animal Care and Use Committee (Protocol # 2017-007), animals were euthanized via overdose of the anesthetic Tricaine methane sulfonate (MS-222). Euthanasia was confirmed prior to eye dissection by severing and pithing the spinal cord. Upon removal from the eye, each retina was immediately stored at -20°C in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) in preparation for molecular work.

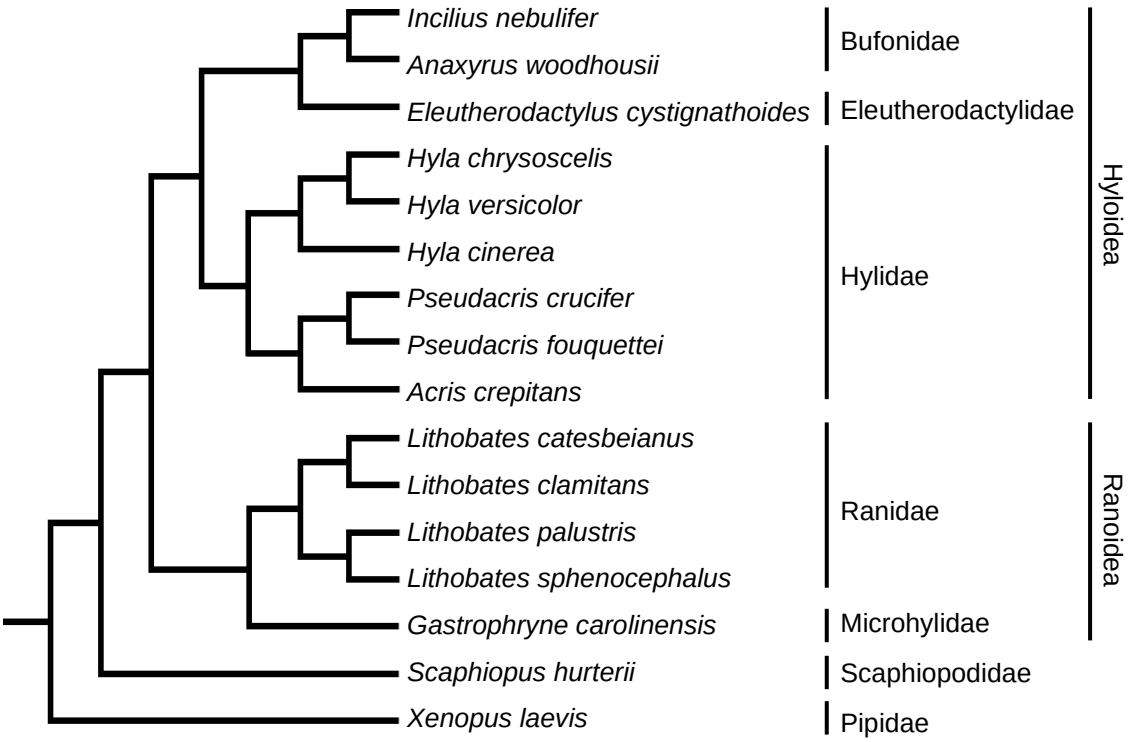


Figure 1. Phylogenetic tree illustrating evolutionary relationships among the fifteen study species and the more ancestral *Xenopus laevis*, simplified from large-scale phylogenies published in Pyron and Wiens (2011); Feng et al. (2017); Jetz and Pyron (2018); and Streicher et al. (2018). Broader taxonomic groupings are shown to the right of the tree.

Expressed Opsin Sequencing

Total retinal mRNA was extracted from one of each study animal's retinas with an RNeasy Mini Kit and QIAshredder (Qiagen, Valencia, CA, USA), quantified with a NanoVue spectrophotometer (GE Healthcare, Chicago, IL, USA), and stored at -80°C; the second retina remained in storage at -20°C. Standardized 0.4 µg mRNA aliquots were reverse transcribed using

SuperScript™ IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with an oligo(dT) primer to synthesize 20 µL aliquots of total cDNA. Fragments of each opsin-coding gene could then be amplified via polymerase chain reactions (PCR) for sequencing. Gene-specific and degenerate primers for anuran RH1, LWS, SWS1, and SWS2(A) (Appendix A) were designed using Primer3 (Rozen and Skaletsky, 2000) from aligned GenBank reference sequences (Appendix B).

Each 25 µL PCR mixture contained 18.4 µL nuclease-free H_2O, 2.0 µL 10X High Fidelity PCR Buffer, 1.0 µL 50 mM $MgSO_4$, 0.5 µL mix of 10 mM-each dNTPs, 1.0 µL 10 µM forward primer, 1.0 µL 10 µM reverse primer, 0.1 µL Platinum™ *Taq* DNA Polymerase High Fidelity (5 U/µL) (Invitrogen, Carlsbad, CA, USA), and 1.0 µL template cDNA. Target gene fragments were amplified in a Mastercycler © ep realplex thermocycler (Eppendorf, Hamburg, Germany) set to the following PCR profile: 95°C for 10 min; 94°C for 120 s; 35-50 cycles at 94°C for 30 s (denaturation), 45-50°C for 60 s (primer annealing), and 72°C for 120 s (polymerization); and 72°C for 120 s. Samples of PCR product were visualized with ethidium bromide in a 1% agarose gel to assess the effectiveness of each primer pair and to select suitable samples for cleanup and sequencing. PCR product was purified with the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA), quantified, and prepared according to specifications set by the DNA Sequencing Facility at the University of Texas at Austin for nucleotide sequencing via the chain-termination method (Sanger et al.,

1977). Returned partial sequences were identified to the gene via nucleotide BLAST (Altschul et al., 1990). Prior to further analysis, partial sequences of the same gene from the same species were cleaned and merged into a consensus sequence in Geneious 10 (Biomatters, Ltd., Aukland, New Zealand; Kearse et al., 2012). Due to the highly conserved nature of opsin-coding genes, although up to five individuals were collected per species, in most cases smaller sample sizes of two or three individuals per species were sufficient for opsin sequencing. In the case of *Lithobates clamitans*, only one of the two individuals collected was sequenced. Among other species, opsins were sequenced from two individuals in *Incilius nebulifer*, *Eleutherodactylus cystignathoides*, *Hyla chrysoscelis*, *H. versicolor*, *Gastrophryne carolinensis*, and *Lithobates clamitans*; three individuals in *Anaxyrus woodhousii*, *H. cinerea*, *Pseudacris fouquettei*, *L. sphenocephalus*, and *L. palustris*; and four individuals in *P. crucifer* and *Scaphiopus hurterii*.

Opsin Alignment and Tree Construction

All sequenced anuran opsins were aligned to complete RH1, LWS, SWS1, and SWS2(A) reference sequences from GenBank (Appendix C). Representative amphibian taxa included one anuran (*Xenopus laevis*) and two salamanders (*Ambystoma tigrinum* and *Cynops pyrrhogaster*) for which complete sequences of all four genes have been published. Also included were the same four opsins of three fish species commonly used in studies of opsin evolution, as well as

bovine rhodopsin. As the first sequenced vertebrate opsin (Nathans and Hogness, 1983), *Bos taurus* RH1 has for decades served as the standard reference by which visual opsin base pairs and amino acid positions are numbered for comparisons across taxa. Additionally, its published crystalline structure (Palczewski et al., 2000) can be used to approximate the locations of features important to spectral tuning, such as the seven transmembrane α-helices which form the chromophore-binding pocket. Alignments were created in Geneious 10 using MUSCLE (Edgar, 2004) with a CLUSTALW sequence weighting scheme (Thompson et al., 1994). Genetic distances were calculated using the Tamura-Nei model (Tamura and Nei, 1993), and reference trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987). The final, consensus opsin gene tree was constructed using the NJ method and node support values calculated via bootstrapping with 5000 replicates (Felsenstein, 1985). An mRNA-sequenced *Xenopus laevis* vertebrate ancient opsin (VAO) from GenBank (Appendix C) was selected as the outgroup. VAOs are non-visual opsins expressed in neurons of the inner retina and brain of many vertebrates (Philp et al., 2000; Porter et al., 2011). They are closely-related but ancestral to the visual opsins (Porter et al., 2011) and are commonly used to root visual opsin gene trees.

Testing for Positive Selection

A well-established method for evaluating selective pressure on protein-coding genes is the comparison of relative nonsynonymous (amino-acid-changing) to synonymous (non-changing) nucleotide substitution rates, respectively termed d_N and d_S (Yang and Bielawski, 2000). When equal to 1, the d_N / d_S ratio (ω) indicates neutral evolution. When nonsynonymous substitution rates are relatively low, ω has a value less than 1, so $\omega < 1$ is usually interpreted as indicative of purifying selection. When nonsynonymous substitution rates are relatively high, $\omega > 1$, indicating positive selection. Site-specific d_N / d_S ratios can be compared phylogenetically among homologous genes to test for positive selection at different codon positions (Goldman and Yang, 1994). Here, the CODEML program from the Phylogenetic Analysis by Maximum Likelihood (PAML) 4 program package was used to calculate site-specific d_N / d_S ratios and identify opsin codon positions likely undergoing positive selection (Yang, 1997; Yang, 2007).

Partial RH1, LWS, SWS1, and SWS2 sequences representing all fifteen study species were used to create four gene-specific alignments to complete *Xenopus laevis* reference sequences. Following recommendations stated in the PAML 4 manual (Yang, 2007), the ends of each gene alignment were trimmed to include only regions that had been sequenced in at least half of all study species. Positive selection at different codon positions within each gene was tested for

using two pairs of nested, site-specific models described in Yang (2007). The first pair, the more conservative of the two, included the null model M1 (“nearly neutral”) and the alternative model M2 (“positive selection”); the second pair included the null model M7 (“beta”) and alternative model M8 (“beta&ω”). In both cases, the null model was a constrained version of the alternative, with the alternative allowing for positive selection and the null excluding it. The null and alternative models were then compared via likelihood ratio tests (LRT) by calculating twice the difference in log-likelihoods ($2\Delta l$). The LRT result obtained in this manner follows a X^2 distribution, with degrees of freedom equaling the difference in free parameters between the two models; a X^2 test was used to determine whether the fit of the alternative model was significantly different from that of the null. If so, Bayes Empirical Bayes (BEB) posterior probabilities (P, with a threshold of 50%) were calculated by CODEML for all codon sites with $\omega > 1$ to identify sites likely undergoing positive selection. Again following the recommendations of Yang (2007), because the selection models used are prone to multiple local optima, all CODEML analyses were run at least twice per gene—at least once with initial $\omega > 1$ and at least once with initial $\omega < 1$.

RESULTS

Opsin Variation

Partial sequences of four opsins—RH1, LWS, SWS1, and SWS2—were recovered from the retinal mRNA of fifteen anuran species, providing a final data set of sixty consensus sequences (one sequence per species per gene). Fifty-three of these sequences included all coding sites within and between all transmembrane regions. With the exception of *Anaxyrus woodhousii* LWS and *Gastrophryne carolinensis* SWS1, obtained sequences covered more than 70% of each gene and averaged 87% coverage across all genes. All but seven of the sixty sequences included all coding sites within and between all seven transmembrane regions as approximated by the structure of bovine rhodopsin (Appendix D). All amino acid positions are numbered in relation to bovine rhodopsin unless otherwise stated (e.g., “anuran LWS-specific position 177,” which corresponds with bovine rhodopsin position 162). Every sequenced anuran opsin possessed the expected lysine residue at amino acid position 296, which forms the protonated Schiff base attachment site for the chromophore, and glutamic acid at position 113, which functions as the Schiff base counterion in vertebrates (Sakmar et al., 1989; reviewed in Porter et al., 2011). The total number of amino acid sites found to vary among anuran opsins ranged from 43

of 366 in LWS, to 68 of 350 in SWS1. Of the four opsins, LWS contained the fewest variable sites within the seven transmembrane regions. All sequences exhibited a similar number of polarity-changing sites, both in total and within the transmembrane regions (Table 2).

Table 2. Summary of opsin variation in the fifteen anuran study species. "TMRs" refer to the seven transmembrane regions which surround the light-sensitive chromophore.

	RH1	LWS	SWS1	SWS2
Total number of amino acids	355	366	350	365
Total number of variable sites	51	42	68	53
Total number of polarity changes	13	12	14	14
Variable sites within TMRs	32	23	33	30
Polarity changes within TMRs	7	7	6	7
Substitutions at known tuning sites[a]	1	1	7	1

[a] refers to gene-specific spectral tuning sites of vertebrate opsins

Variation at Known Tuning Sites

Each of the four opsins possessed at least one amino acid substitution at a gene-specific site known in other vertebrates to tune spectral sensitivity of visual pigments (Table 2; Appendix D). On the RH1 opsin, *Scaphiopus hurterii* and both species of *Pseudacris* exhibited a change from the nonpolar, aliphatic amino acid alanine (A) to the polar, uncharged serine (S) at position 299 (notated as A299S; Appendix D, Table D-1). Additionally, anuran RH1 varied at four

amino acid positions (52, 93, 97, 109) known to affect the spectral sensitivity of other vertebrate visual pigments. At RH1 position 93, a tuning site on SWS1, study species expressed one of two nonpolar, aliphatic amino acids: *Incilius nebulifer*, *Gastrophryne carolinensis*, *S. hurterii*, and *P. crucifer* expressed valine (V), and the remaining eleven species expressed isoleucine (I). Sites 52 and 97 are tuning sites on both RH2 and SWS1. At the former tuning site, four species (*Anaxyrus woodhousii*, *G. carolinensis*, *S. hurterii*, and *P. fouquettei*) expressed the nonpolar, aromatic amino acid phenylalanine (F), and remaining species expressed the nonpolar, aliphatic leucine (L). At the latter tuning site, study species expressed one of two polar, uncharged amino acids: *Eleutherodactylus cystignathoides*, *Acris crepitans*, *G. carolinensis*, and the four species of *Lithobates* expressed threonine (T), and the remaining eight species expressed serine. At amino acid position 109, a known tuning site on both SWS1 and SWS2, all but one study species possessed the nonpolar, aliphatic glycine (G), while *E. cystignathoides* instead expressed polar, uncharged threonine at that position.

On the LWS opsin, an amino acid change occurred at known LWS tuning site 164 (anuran LWS-specific site 179), at which position *I. nebulifer*, *E. cystignathoides*, and *S. hurterii* expressed alanine, and remaining species expressed serine (Appendix D, Table D-2). Anuran LWS also varied at three RH1 tuning sites (96, 124, and 195) and two RH2/SWS1 tuning sites (49 and 52). The

most variable was site 49, at which *P. fouquettei* expressed the polar, uncharged amino acid cysteine (C), and remaining study species expressed one of four nonpolar amino acids. These included glycine in *G. carolinensis*; alanine in *I. nebulifer* and *P. crucifer*; leucine in the three *Hyla* species; and isoleucine in *Acris crepitans*, *E. cystignathoides*, *S. hurterii*, and the four *Lithobates* species. Another polarity change occurred at site 52. Most species expressed valine, though *S. hurterii* instead expressed similarly nonpolar isoleucine, and five species (*G. carolinensis*, *P. crucifer*, and the three *Hyla* species) expressed the polar, uncharged cysteine. Among the RH1 tuning sites that varied on anuran LWS, the most variable was site 96, at which *S. hurterii* expressed alanine; *Acris crepitans*, *G. carolinensis*, and the four species of *Lithobates* expressed isoleucine; and the remaining seven species expressed phenylalanine. At site 124, all but one study species expressed glycine, and *S. hurterii* instead expressed alanine. Finally, at site 195, study species expressed one of two polar, uncharged amino acids: asparagine (N) in *L. palustris* and serine in most remaining species. Due to an ambiguous nucleotide at the second codon position of this site, it could not be determined whether *Acris crepitans*, *L. catesbeianus*, and *L. sphenocephalus* expressed N195 or S195.

SWS1 exhibited the greatest number of amino acid changes at gene-specific tuning sites (Table 2; Appendix D, Table D-3). All seven variable sites (46, 86, 93, 97, 109, 114, and 118) occured within the first three transmembrane

regions, which were not sequenced in *G. carolinensis*. At site 46 (anuran SWS1-specific site 42), the remaining fourteen study species expressed one of four nonpolar, aliphatic amino acids. *Anaxyrus woodhousii*, *I. nebulifer*, and *E. cystignathoides* expressed leucine, methionine (M), and alanine, respectively; the remaining eleven species expressed valine. At site 86, all but one study species expressed isoleucine, and *Anaxyrus woodhousii* instead expressed methionine. A polarity change occurred at site 93. Six species expressed one of two nonpolar amino acids: isoleucine in *L. palustris* and *L. sphenocephalus* and valine in *Acris crepitans*, *L. catesbeianus*, *L. clamitans*, and *S. hurterii*. The remaining eight species expressed the polar, uncharged amino acid threonine. At site 97, *Acris crepitans* and all four species of *Lithobates* expressed asparagine, while remaining species expressed similarly polar, uncharged serine. Site 109 exhibited the greatest variation among the SWS1 tuning sites. *Anaxyrus woodhousii* expressed threonine, while the remaining study species expressed one of three nonpolar amino acids: V109 in *P. fouquettei*; A109 in *P. crucifer*, *I. nebulifer, E. cystignathoides*, and all three species of *Hyla*; and F109 in *Acris crepitans*, *S. hurterii*, and all four species of *Lithobates*. At site 114, all *Hyla* and *Pseudacris* species expressed glycine, and the remaining nine species expressed alanine. Lastly of the SWS1-specific tuning sites, the amino acid change T118S occurred in *P. fouquettei*. Finally, in addition to variation at the aforementioned tuning sites, anuran SWS1 also varied at known RH1 tuning site

96, at which *Acris crepitans*, *S. hurterii*, and all four species of *Lithobates* expressed isoleucine, and the remaining eight species expressed valine.

On the SWS2 opsin, amino acid variation occurred at gene-specific tuning site 122 (anuran SWS2-specific site 131), with *G. carolinensis* expressing isoleucine and remaining species expressing methionine (Appendix D, Table D-4). In addition to that SWS2-specific tuning site, anuran SWS2 varied at five amino acid positions (93, 97, 124, 164, and 207) known in other opsins to affect spectral sensitivity. The same polarity change occurred at SWS2 site 93 as in SWS1, though on this opsin *P. fouquettei* was the only species to express nonpolar valine rather than polar threonine. At amino acid position 97, a known tuning site of both RH2 and SWS1, *G. carolinensis* expressed threonine, and the remaining fourteen species expressed serine. Another polarity change on the SWS2 opsin occurred at known RH1 tuning site 124, at which the two gray treefrog species (*H. chrysoscelis* and *H. versicolor*) expressed glycine, and remaining species expressed serine. Yet another polarity change occurred at site 164, a known tuning site of both LWS and RH2. As in anuran LWS, *I. nebulifer* and *E. cystignathoides*, as well as *Anaxyrus woodhousii*, expressed A164, and all species of *Hyla* and *Pseudacris* expressed S164. Unlike in anuran LWS, *Acris crepitans*, *G. carolinensis*, *S. hurterii*, and the four species of *Lithobates* expressed G164. Lastly, at known RH2 tuning site 207, study species expressed one of three nonpolar, aliphatic amino acids on the SWS2 opsin: leucine in *S.*

hurterii; isoleucine in *Acris crepitans* and the four species of *Lithobates*; and methionine in the remaining nine species.

Variation in the Chromophore-binding Pocket

In all four opsins, amino acid changes also occurred at additional sites forming the chromophore-binding pocket (list of sites provided in Hunt et al., 2001). These included two sites (54 and 119) on RH1, two sites (119 and 160) on LWS, six sites (47, 82, 120, 258, 271, and 307) on SWS1, and two sites (207 and 258) on SWS2. Site 119, which occurs in the third transmembrane α-helix, varied on two different opsins. Variation at site 119 included polarity changes on both RH1, with the amino acid change L119V in *G. carolinensis*, and LWS, with the change V119T in *S. hurterii*. Another polarity change occurred at LWS site 160, at which *I. nebulifer* and *E. cystignathoides* expressed alanine instead of serine. Of the six variable sites lining the chromophore-binding pocket in SWS1, only one inclued a polarity change. *Acris crepitans*, *S. hurterii*, and all four species of *Lithobates* expressed nonpolar aline at SWS1 site 120, while the remaining species expressed the polar amino acid serine.

Site-Specific Positive Selection

Phylogenetic analysis by maximum likelihood (PAML) did not identify statistically significant positive selection on anuran RH1, SWS1, or SWS2 but did

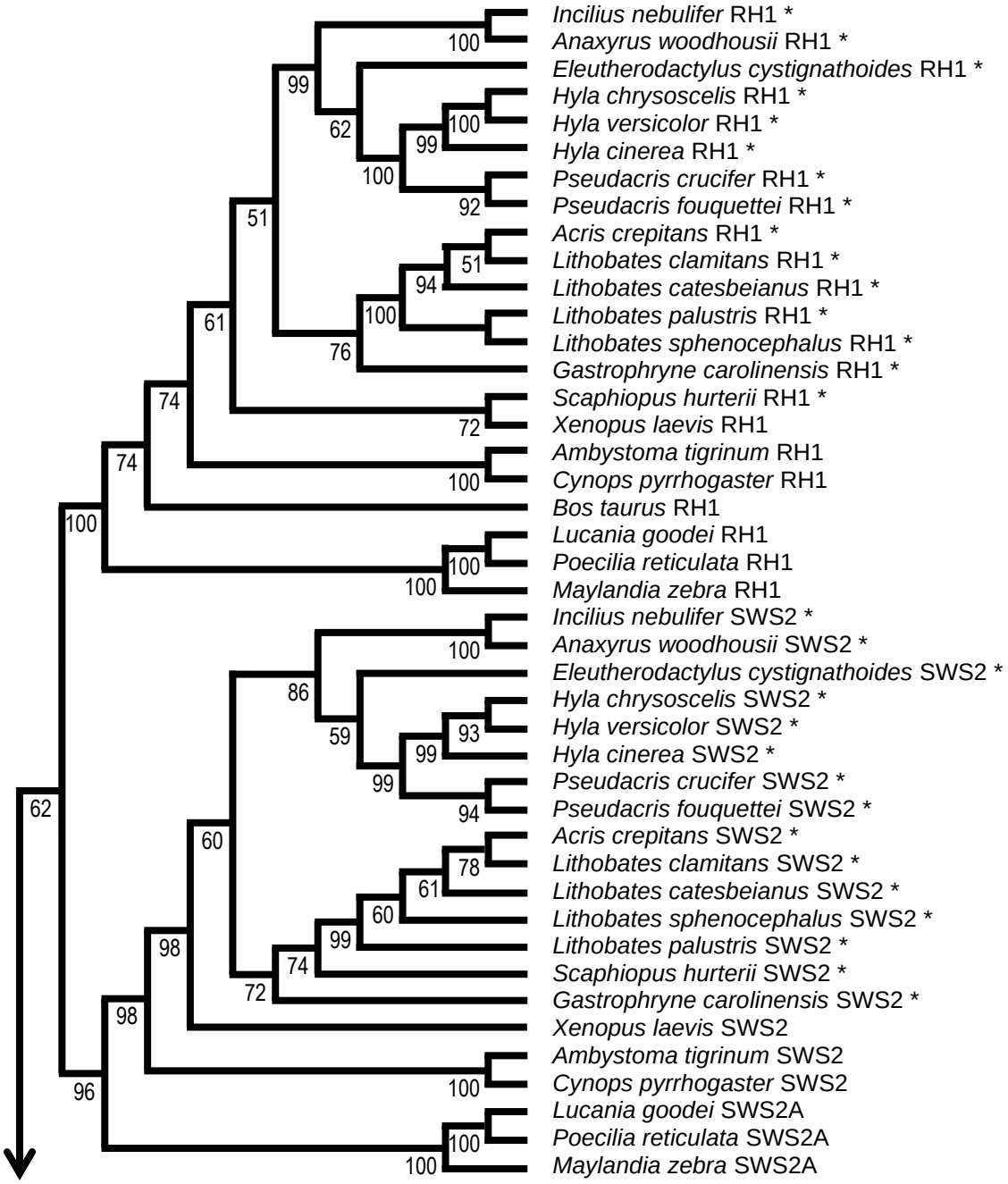
provide strong support for positive selection on anuran LWS (Table 3). In the case of LWS, both evolutionary models allowing for positive selection (M2 and M8) fit the data significantly better than their respective paired null models (M1 and M7; Table 3). The more conservative M2 model identified an LWS codon site class with a d_N / d_S ratio (ω) of 5.78050, indicating positive selection on the five sites falling within that class: 49, 52, 154, 158, and 162 (anuran LWS-specific sites 64, 67, 169, 173, and 177). Site 52 had a Bayes Empirical Bayes (BEB) posterior probability (P) of 93.6%, and for site 49 P > 99%. The less conservative M8 model identified a codon site class with ω = 3.39037 and indicated positive selection on the same five sites: 49 (P > 99%), 52 (P > 95%), 154 (P = 93.8%), 158 (P = 90.3%), and 162 (P > 95%).

Table 3. Likelihood ratio tests (LRT) for positive selection on four anuran opsin genes, based on two pairs of nested evolutionary models (M1 vs M2; M7 vs M8) from the PAML program package (Yang, 2007). The LRT statistic (twice the difference in log likelihoods (2Δl) between paired models) follows a X^2 distribution with degrees of freedom equaling the difference between the number of free parameters. Significant differences between the null (M1 or M7) and alternative (M2 or M8) models are indicated with (*).

Gene	Model	Log-likelihood (l)	2Δl	df	LRT Result
RH1	M1	-3668.843921	0.00000	2	p = 1.00000
	M2	-3668.843921			
	M7	-3661.370679	0.49816	2	p = 0.77950
	M8	-3661.121598			
LWS	M1	-3627.786369	11.17474	2	p = 0.00374 *
	M2	-3622.198999			
	M7	-3626.191376	20.33064	2	p < 0.00005 *
	M8	-3616.026055			
SWS1	M1	-3734.550801	0.00000	2	p = 1.00000
	M2	-3734.550801			
	M7	-3727.513193	0.75811	2	p = 0.68451
	M8	-3727.134136			
SWS2	M1	-3715.999925	0.00000	2	p = 1.00000
	M2	-3715.999925			
	M7	-3710.190555	1.88432	2	p = 0.38979
	M8	-3709.248393			

Comparison of Opsin and Species Tree Topologies

Relationships shown among the four opsin genes on the consensus tree (Figure 2) agree with current understanding of opsin evolution, with LWS forming the most ancestral clade, and RH1 and SWS2 forming a more derived monophyletic group (Bowmaker, 2008; Porter et al., 2011). Within each gene-specific clade, most relationships among species reflected patterns of species evolution evidenced in recent amphibian phylogenetics research (see Figure 1 for species tree). However, several of the gene-specific clusters formed from anuran opsins cannot be explained fully by evolutionary relationships among the study species. Most notably, all opsins of the hylid species *Acris crepitans* grouped more closely with those of the pond frogs (*Lithobates*) than those of other hylids (*Hyla* and *Pseudacris*). Some of the nodes formed within the *Lithobates+Acris* gene clusters, such as the node formed by *Acris crepitans* RH1 and *Lithobates clamitans* RH1, have relatively low support values. However, in all four opsins the common node connecting *Acris crepitans* to all *Lithobates*—or, in the case of the SWS1 opsin, the common node of those five species with *Gastrophryne carolinensis* and *Scaphiopus hurterii*—has a high support value. Again using RH1 as an example, the group formed by *Acris crepitans* and all *Lithobates* species has a support value of 100 and so is very well supported.


Incilius nebulifer RH1 *
Anaxyrus woodhousii RH1 *
Eleutherodactylus cystignathoides RH1 *
Hyla chrysoscelis RH1 *
Hyla versicolor RH1 *
Hyla cinerea RH1 *
Pseudacris crucifer RH1 *
Pseudacris fouquettei RH1 *
Acris crepitans RH1 *
Lithobates clamitans RH1 *
Lithobates catesbeianus RH1 *
Lithobates palustris RH1 *
Lithobates sphenocephalus RH1 *
Gastrophryne carolinensis RH1 *
Scaphiopus hurterii RH1 *
Xenopus laevis RH1
Ambystoma tigrinum RH1
Cynops pyrrhogaster RH1
Bos taurus RH1
Lucania goodei RH1
Poecilia reticulata RH1
Maylandia zebra RH1
Incilius nebulifer SWS2 *
Anaxyrus woodhousii SWS2 *
Eleutherodactylus cystignathoides SWS2 *
Hyla chrysoscelis SWS2 *
Hyla versicolor SWS2 *
Hyla cinerea SWS2 *
Pseudacris crucifer SWS2 *
Pseudacris fouquettei SWS2 *
Acris crepitans SWS2 *
Lithobates clamitans SWS2 *
Lithobates catesbeianus SWS2 *
Lithobates sphenocephalus SWS2 *
Lithobates palustris SWS2 *
Scaphiopus hurterii SWS2 *
Gastrophryne carolinensis SWS2 *
Xenopus laevis SWS2
Ambystoma tigrinum SWS2
Cynops pyrrhogaster SWS2
Lucania goodei SWS2A
Poecilia reticulata SWS2A
Maylandia zebra SWS2A

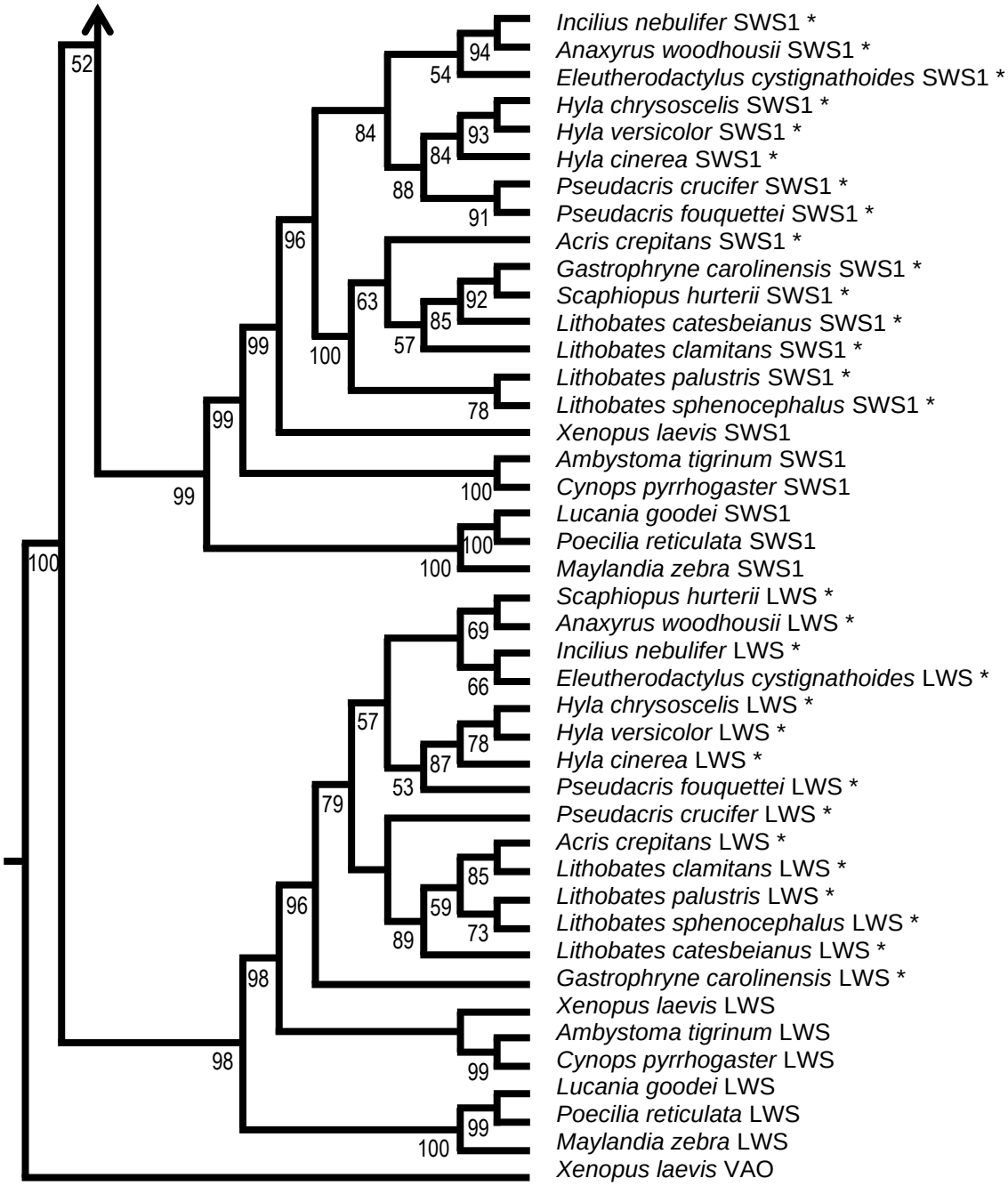


Figure 2. Gene tree generated from anuran, salamander, and fish RH1, LWS, SWS1, and SWS2(A), as well as bovine RH1, using the neighbor-joining (NJ) method (Saitou and Nei, 1987). Study species are indicated with (*). The tree is rooted with a *Xenopus laevis* vertebrate ancient opsin (VAO) as the outgroup. Bootstrap support values (5000 replicates) > 50 are provided at each node.

DISCUSSION

Phylogenetic analyses and amino acid variation at known spectral tuning sites suggest factors other than evolutionary species relationships may be driving changes to anuran visual systems. Some of the most striking evidence comes from opsins of the cricket frog *Acris crepitans*. All four cricket frog opsins (RH1, LWS, SWS1, and SWS2) sequenced in this study grouped more closely with those of the pond frogs (*Lithobates*) than with those of more closely-related species such as *Hyla* and *Pseudacris* (Figure 2). Similarities between the cricket frog and pond frog opsins occurred across many variable sites on each gene, including sites which are known to affect spectral sensitivity in vertebrate visual pigments (Appendix D). Among study species, spectral sensitivity in *Lithobates catesbeianus* is the most well-documented, as it is the only one in which all photoreceptors have been measured (Table 1; Hárosi, 1982; Govardovskii et al., 2000). For this reason, possible variation in spectral sensitivity among study species will be described relative to *L. catesbeianus*. While most study species exhibited variation on the SWS1 opsin indicative of either short- or long-wavelength-shifted spectral sensitivity relative to the pond frogs, *Acris crepitans* did not differ from members of *Lithobates* at any of the seven known SWS1 tuning sites (Appendix D, Table D-3). Such consistent

similarities across all four genes and at key amino acid sites indicate that spectral sensitivity in cricket frogs might resemble that of pond frogs more closely than that of other hylids—possibly as a result of similarities in ecology and life history. For example, daily activity patterns in cricket frogs generally resemble those of pond frogs. While most hylids form strictly nocturnal breeding aggregations, with peak calling activity in *Hyla* occurring between sunset and midnight, in cricket frogs and pond frogs, calling activity peaks between midnight and sunrise and often continues throughout the day (Bridges and Dorcas, 2000). Terrestrial photic conditions vary drastically throughout the day, twilight, and night, and temporal niche partitioning may contribute to similarities in the opsins of some anurans despite more distant species relationships. Also of note in cricket frogs is their considerable color polymorphism as adults—particularly in dorsal stripe coloration, which ranges from green to red to gray. There is some evidence for substrate-matching color change within individuals (see Hoffman and Blouin, 2000), which could necessitate the ability to obtain and process certain visual information (reviewed in Duarte et al., 2017). However, the potential drivers of cricket frog coloration in natural populations are not fully understood (Gray, 1983; Gorman, 1986; reviewed in Hoffman and Blouin, 2000).

The treefrogs (*Hyla*) show variation on the SWS1 opsin that may contribute to differences in spectral sensitivity of SWS cones compared to other taxa. For example, the amino acid substitution A114G on the third

transmembrane α-helix of SWS1, as observed in *Hyla*, can act in synergy with substitutions at other variable sites to tune ultraviolet-violet vision via long- (i.e., more violet) wavelength sensitivity shifts (Yokoyama and Shi, 2000; Yokoyama, 2002; Shi and Yokoyama, 2003). Somewhat conversely, the expression of T93 in *Hyla* could contribute to short-wavelength sensitivity shifts of anuran SWS1 visual pigments (Shi and Yokoyama, 2003; Takahashi and Yokoyama, 2005). Such variation on SWS1 suggests spectral tuning in *Hyla* SWS cones, but the possible direction of those shifts is unclear and interactions among tuning sites unknown. The visual systems of this group may be of particular interest, as previous studies have documented intraspecific visual signaling in several species of *Hyla*. In mate choice trials, female green treefrogs (*H. cinerea*) prefer paired visual and audio cues to audio cues alone (Laird et al., 2016). The squirrel treefrog (*H. squirella*), in which females exhibit preferences for males with larger lateral stripes (Taylor et al., 2007), shares much of its geographic range and many ecological and life history characteristics with the study species *H. cinerea*. It has been suggested that conspicuous lateral stripes in *H. cinerea* may play a similar role in sexual selection (Laird et al., 2016). Furthermore, intraspecific signaling in some hylids utilizes chromatic cues. In the European treefrog (*H. arborea*), for example, females prefer males with darker and more chromatic vocal sacs to those with pale vocal sacs (Gomez et al., 2009; Gomez et al., 2010).

The chorus frogs (*Pseudacris*), like *Hyla*, express T93 and the amino acid change A114G on the SWS1 opsin. In addition, *Pseudacris fouquettei* was the only study species with the amino acid changes A109V and T118S, which can contribute to short-wavelength sensitivity shifts in anuran SWS1 visual pigments (Shi and Yokoyama, 2003; Takahashi and Yokoyama, 2005). As in *Hyla*, possible interactions between these and amino acids at other tuning sites, and thus effects on spectral sensitivity in *Pseudacris* SWS cones, are unclear. More clear, however, are the potential effects of variation at a key RH1 tuning site on spectral sensitivity in *Pseudacris* MWS rods. RH1 amino acid site 299 lies on the seventh transmembrane α-helix, in close proximity to the chromophore and, more specifically, the Schiff base attachment site (Hunt et al., 2001; Bowmaker and Hunt, 2006). In dolphins and teleost fishes, the amino acid change A299S contributes to a long-wavelength shift in spectral sensitivity (Fasick and Robinson, 1998; Hunt et al., 2001; Varela and Ritchie, 2015). While most of the study species expressed A299, *P. crucifer* and *P. fouquettei* expressed S299 (Appendix D); it is possible that this variation on the RH1 opsin contributes to long-wavelength-shifted spectral sensitivity in the MWS rods of these species.

The two bufonids (*Incilius nebulifer* and *Anaxyrus woodhousii*) and the chirping frog (*Eleutherodactylus cystignathoides*) exhibited variation at tuning sites on two cone opsins, LWS and SWS1. While T93 on bufonid and chirping frog SWS1 could contribute to short-wavelength shifts in spectral sensitivity (Shi

and Yokoyama, 2003; Takahashi and Yokoyama, 2005), as in *Hyla* and *Pseudacris*, possible tuning site interactions and their implications are unclear. Amino acid variation at site 86 can also shift spectral sensitivity of anuran SWS1 visual pigments, but the specific change (F86M) published in Takahashi and Yokoyama (2005) was not found in this study. While *Anaxyrus woodhousii* did express M86, the remaining thirteen study species expressed I86 rather than F86. On the LWS opsin, the specific amino acid change S164A has been shown in several vertebrates to cause a 7 nm short-wavelength shift in spectral sensitivity of LWS visual pigments (Asenjo et al., 1994; Bowmaker and Hunt, 2006; Yokoyama, 2008). It is possible that spectral sensitivity in the LWS cones of anurans which express S164 on the LWS opsin, such as *I. nebulifer* and *E. cystignathoides*, may be long-wavelength shifted with respect to those of the other study species, which expressed A164. Interestingly, these species commonly occur in urban areas, where anthropogenic light sources tend to long-wavelength shift available spectra (Johnsen et al., 2006) and have the potential to affect foraging, movement, and calling behavior in anurans (Ferguson, 1960; Buchanan, 1993; Baker and Richardson, 2006; Hall, 2016).

The narrowmouth toad (*Gastrophryne carolinensis*) was the only study species to exhibit an amino acid change at a known SWS2 tuning site—more specifically, at site 122 (anuran SWS2-specific position 131), which occurs on the third transmembrane α-helix and near the β-ionone ring of the chromophore.

Site-directed mutagenesis of amphibian SWS2 has revealed that the amino acid substitution M122I results in a long-wavelength shift of 6 nm and contributes to spectral tuning of SWS2 visual pigments in salamander cones (Takahashi and Ebrey, 2003). In *G. carolinensis*, this amino acid substitution predicts a shift in the peak absorption of SWS2 visual pigments towards 450 nm. Such a shift could be of potential ecological significance, as daily calling activity in *G. carolinensis* peaks just before twilight (Bridges and Dorcas, 2000), a period characterized by drastic changes in illumination and a ~450 nm peak of available spectra (Johnsen et al., 2006). It is also worth noting that in anurans, SWS2 is expressed both in SWS rods and some cones, so variation on the SWS2 opsin may affect both scotopic and photopic color vision.

Finally, the spadefoot toad (*Scaphiopus hurterii*) exhibited variation at tuning sites on two opsins which predict long-wavelength shifts in spectral sensitivity. *Scaphiopus hurterii* was the only species outside of *Pseudacris* to exhibit the amino acid change A299S on the RH1 opsin (Appendix D, Table D-1), which may contribute to long-wavelength shifts in spectral sensitivity in MWS rods (Fasick and Robinson, 1998; Hunt et al., 2001; Varela and Ritchie, 2015). Similarly, the amino acid change A164S on the LWS opsin of *S. hurterii*, as in *I. nebulifer* and *E. cystignathoides* (Appendix D, Table D-2), could shift the spectral sensitivity of LWS cones toward longer wavelengths (Asenjo et al., 1994; Bowmaker and Hunt, 2006; Yokoyama, 2008). The ecological significance of

these predicted long-wavelength shifts in *S. hurterii* spectral sensitivity are uncertain, as the visual ecology of this species is relatively unexplored. However, mate choice trials in the congener *S. couchii* have shown that, given identical audio cues, females prefer males with brighter dorsal coloration, which is a predictor of male size and body condition in that species (Vasquez and Pfennig, 2007). Both *S. couchii* and *S. hurterii* are considered “explosive breeders,” forming large breeding aggregations at ephemeral ponds immediately after heavy rainfall. These breeding aggregations often host multiple species, and where the ranges of these two species overlap, hybrids can occur (Wasserman and Bogart, 1968). Females of both species collected from active breeding sites and presented with conspecific and heterospecific calls do not show preference for conspecific calls (Awbrey, 1968). Whether in addition to or in absence of sufficient auditory cues, color-based female preference in *S. couchii* suggests the importance of visual cues to intraspecific signaling and raises the possibility that visual signals may also play an important role in mixed-species choruses. To fully explore visual signaling in these and other species, however, requires more thorough understanding of the molecular mechanisms underlying their visual systems.

Additional Tuning Sites

While no additional variation on anuran LWS was observed at known LWS tuning sites, PAML indicated positive selection on sites 49, 52, 154, 158, and 162 (anuran LWS-specific positions 64, 67, 169, 173, and 177, respectively). Two of the sites with the strongest support for positive selection (49 and 52) lie on the first transmembrane α-helix and are known spectral tuning sites of vertebrate RH2 and SWS1 (Bowmaker and Hunt, 2006; Yokoyama, 2008). In chameleons, amino acid changes on RH2 at sites 49 and 52 together shift spectral sensitivity in RH2 visual pigments (Takenaka and Yokoyama, 2007). On SWS1, changes at these sites contribute to ultraviolet-violet spectral in mammals and birds (Shi and Yokoyama, 2003; Yokoyama et al., 2006). Among study species, the amino acid variation observed at these sites included changes in polarity (Appendix D, Table D-2). Due to their location within the first transmembrane region, indications of positive selection by PAML, and the contribution to amino acid changes at these sites to spectral tuning in other vertebrate opsins, it is likely that sites 49 and 52 contribute to spectral tuning of anuran LWS. In addition, PAML identified possible positive selection on three sites (154, 158, and 162) in the fourth transmembrane α-helix of the LWS opsin. Site 158 also varied on anuran RH1, and sites 154 and 162 varied on all four anuran opsins and were among the most variable of all sites on RH1 and SWS1, respectively. In LWS, amino acid changes at the latter two sites included changes in polarity. As evidenced by their location within the

fourth transmembrane region, relatively high variability in other opsins, and indications of positive selection by PAML, amino acid changes at sites 154, 158, and 162 may also contribute to spectral tuning of anuran LWS.

Of all four visual opsins sequenced in this study, anuran SWS1 possessed the greatest number of amino acid changes in total (68), at known tuning sites (8; gene-specific and general to all opsins), and at other potential tuning sites, such as those lining the chromophore-binding pocket (6). Although LWS possessed the fewest variable sites overall (42), the percentage of known (6) and candidate tuning sites (5; additional sites lining the chromophore-binding pocket and PAML-identified sites) among all variable sites was highest on this opsin. This reflects trends observed in other vertebrate taxa, where most opsin variation responsible for spectral tuning occurs at photoreceptors sensitive to the extremes of visible spectra (Kawamura and Yokoyama, 1998; Hart and Hunt, 2007; Hofmann et al., 2009; Hofmann et al., 2012).

Conclusions and Future Studies

The purpose of this study was to expand upon our understanding of anuran visual systems in several ways. First, near-complete sequences of RH1, LWS, SWS1, and SWS2 expressed in anuran retinas were obtained from fourteen previously unexamined species representing six families. Sequencing opsins from an increasingly broader and more diverse range of taxa improves the

statistical power of evolutionary analyses such as tests for site-specific positive selection and ancestral state reconstruction, and could reveal novel sources of spectral tuning in vertebrates. In the present study, amino acid variation among study species at known vertebrate tuning sites predict changes in spectral tuning of potential ecological significance. With an increasing body of evidence that visual signaling plays a role in sexual selection in some anuran species, and that anurans may be capable of color vision in conditions that are too dim for cone-mediated color vision, it is becoming increasingly important to understand how selective pressures may be driving changes to anuran visual systems. Finally, with the addition of tests for site-specific positive selection, this study revealed amino acid variation suggesting a several of novel spectral tuning sites in anurans. Taken together, the results of this study provide a foundation for investigating additional outstanding questions of anuran visual ecology.

Of particular concern to amphibian conservation through changes in visual systems and resulting visually-mediated behaviors is an emergent source of environmental pollution: anthropogenically-sourced light. This light pollution, frequently termed “artificial light at night” (ALAN), is increasing at a rate of approximately 6% per year (Hölker et al., 2010) and is capable of disrupting natural light cycles and ambient light conditions (Chalkias et al., 2006; reviewed in Longcore and Rich, 2004; reviewed in Gaston et al., 2013; Power et al., 2017). Artificial light sources contribute multiple high intensity peaks throughout the

spectrum, with especially high intensities at wavelengths greater than 550 nm. At wavelengths below ~530 nm, ALAN can occur at intensities near that of nautical twilight, and can surpass it at wavelengths above ~530 nm (Johnsen et al., 2006; reviewed in Cronin et al., 2014). In addition to altering ambient light conditions, the high-intensity long-wavelength shifts induced by ALAN can significantly alter achromatic and/or chromatic contrast of an object against its background, potentially affecting its perception (Johnsen et al., 2006). There is evidence that ALAN-induced shifts in the spectral characteristics of night lighting and extended photoperiods can cause direct changes to vertebrate visual systems (Kopperud and Grace, 2017). Given that the greatest spectral variation in terrestrial environments occurs during twilight and at night, with ALAN contributing additional but highly dissimilar variation (Johnsen et al., 2006; reviewed in Cronin et al., 2014), the visual systems of crepuscular and nocturnal animals such as most anurans may be particularly sensitive to its effects.

Even in natural light conditions, due to their complex life histories, anurans may be exposed to vastly different photic environments pre- and post-metamorphosis. Most anurans undergo early development as aquatic larvae. Variation in depth and the abundance of phytoplankton, inorganic particulates such as sediment, and dissolved organic matter (reviewed in Cronin et al., 2014), both within and among aquatic microhabitats, can exert selective pressures and drive spectral tuning in vertebrates (Carleton and Kocher, 2001; Fuller et al.,

2005; Spady et al., 2006; Stieb et al., 2016; Marques et al., 2017). For terrestrial or arboreal adults, photic environments can vary greatly as a result of differing light sources throughout the day, and by the transmission and reflectance of light through and by substrate and especially vegetation (reviewed in Cronin et al., 2014). As anurans transition through life stages and seasons, they may utilize macro- and microhabitats with different spectral characteristics, forage on different prey items, and be preyed upon by species with different foraging tactics, all of which may affect how they must perceive and respond to visual stimuli. Previous studies have documented ontogenic changes to anuran visual pigments via differences in chromophore type (Wald, 1946; Reuter et al., 1971). However, potential changes to the opsin component of visual pigments is thus far entirely unexplored. In particular, spectral tuning in response to changing photic environments may occur in anurans through the mechanism of differential gene expression.

While the focus of the present study was of the first major mechanism of spectral tuning—amino acid changes in opsin sequences—this work also supports further investigation into the second major mechanism: differential expression of opsin-coding genes. This second mechanism has so far not been studied in any amphibian. Work in other systems, however, demonstrates the potential for gene expression studies to address several aforementioned, unexplored aspects of anuran visual ecology. In fishes, for example, opsin gene

expression can vary in response to changes in photic environment at different life stages via developmental plasticity (Spady et al., 2006; Carleton et al., 2008; Shand et al., 2008; Hofmann et al., 2010). It is possible that anuran visual systems undergo similar spectral tuning during transition from larval to adult stages, especially in cases of substantial habitat change.

Anurans form a largely understudied but intriguing group of organisms for studies of visual system evolution, in part due to their reliance on visual cues and specialization for dim-light vision, including the unique use of two spectrally-distinct rod classes. Additionally, while most molecular vision studies have focused on organisms living in either aquatic or terrestrial light environments, anurans present an opportunity to study complex visual system metamorphosis between them. How potential tuning mechanisms such as amino acid variation at both known and potential tuning sites actually affects spectral sensitivity at the visual pigment, photoreceptor, and organism level requires further investigation. By contributing to our understanding of spectral tuning mechanisms in anuran visual systems, this study supports future investigative work into the fundamental questions of anuran visual ecology.

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APPENDICES

Appendix A. Anuran opsin primers. The letters “F” and “R” at the end of each primer code indicate forward and reverse primers, respectively. Start and stop positions are based on complete *Xenopus laevis* reference sequences for each gene.

Primer Code	Sequence (5’ to 3’)	Start Position	Stop Position
RH1P1F	TCACCATCCAGCACAAGAAG	182	202
RH1P1R	GGGTGGTGATCATGCAGTTA	942	962
RH1P2F	CCCTTTGGTCCCTGGTG	371	388
RH1P2R	TGCAGCCTCTTTCACAGT	685	703
RH1P3F	AGACTGGGGTNGTNCGAAGCC	47	68
RH1P3R	TTGTGCTGGATGGTGACGTA	178	198
RH1P4F	CCATGTCNAACAAGACTGGGGT	35	57
RH1P4R	TGCTGGATGGTGACGTANAN	175	195
LWSP1F	GTCCAGATGTGTTCAGTGGTAG	608	630
LWSP1R	CTGTCCGGGATGTAGAAGAAAG	1036	1058
LWSP2F	GCACCAAGATGGGTCTACAA	139	159
LWSP2R	TGCCCAAGTCCAAGAGAATAC	517	538
LWSP3F	GAGGAAGCCTGATGATGAAGAA	39	61
LWSP4F	TCTTGGTCAACCTGGCTATTG	269	290
LWSP4R	TGCTGGGCTACCTTTCTAATG	732	753
LWSP5F	GGCTGCAACATGGAATGAAG	3	23
LWSP5R	CTGCTGGGCTACCTTTCTAAT	733	764
LWSP6R	GGATACTTCTGTCCGGGATGTA	1044	1066

Appendix A, continued.

LWSP7F	GGAGGAAGCCTGATGATGAA	38	58
LWSP7R	GTTGTAAATGGTGGCACTCTTG	930	952
SWS1P1F	GTTGGGTGACGAAGACTTCTA	3	24
SWS1P1R	CCATAGCTGCATAGGGAACA	780	800
SWS1P2F	GTTATGGTTGGATCCTTCTGTCT	751	774
SWS1P2R	ACCTGGCTGTGGGATACT	1014	1032
SWS2AP1F	CTGCTTCTGCTTTGGGTTTC	651	671
SWS2AP1R	CATCGCCAAGTGGATTCTTTC	998	1019
SWS2AP2F	CCACCTTAACTACATCCTGGTAAA	237	261
SWS2AP2R	ACCCAAAGCAGAAGCAGAA	649	668
SWS2AP3F	CCCCATTCCTCTGGAAACCA	54	73
SWS2AP3R	ACAGCTGACCATTCCTCCG	384	403

Appendix B. Complete anuran opsin reference sequences obtained from GenBank for primer design.

Gene	Species	Accession Number
RH1	*Xenopus tropicalis*	NM_001097334.2
	Xenopus laevis (v1)	L04692
	Xenopus laevis (v2)	L07770
	Bufo bufo	U59921
	Rhinella marina (*Bufo marinus*)	U59922
	Nanorana parkeri (predicted)	XM_018555227
	Rana temporaria	U59920
LWS	*Xenopus tropicalis*	NM_001102861
	Xenopus laevis	U90895
	Nanorana parkeri (predicted)	XM_018560714
SWS1	*Xenopus tropicalis*	NM_001126076
	Xenopus laevis	U23463
	Nanorana parkeri (predicted)	XM_018560743
	Lithobates catesbeianus (*Rana catesbeiana*)	AB001983
SWS2	*Xenopus laevis*	BC080123
	Mantella baroni	LC180362
	Lithobates catesbeianus (*Rana catesbeiana*)	AB010085

Appendix C. Complete opsin sequences obtained from GenBank for sample alignment and gene tree construction with an anuran vertebrate ancient opsin (VAO) outgroup.

Gene	Clade	Species	Accession Number
VAO	Amphibia	*Xenopus laevis*	NM_001171892
RH1	Mammalia	*Bos taurus*	NM_001014890.2
	Actinopterygii	*Lucania goodei*	AY296738
		Poecilia reticulata	LC127191
		Maylandia zebra	XM_004546085.3
	Amphibia	*Cynops pyrrhogaster*	AB043890
		Ambystoma tigrinum	U36574
		Xenopus laevis	L04692
LWS	Actinopterygii	*Lucania goodei*	AY296740
		Poecilia reticulata	HQ260677
		Maylandia zebra	XM_004571915.2
	Amphibia	*Cynops pyrrhogaster*	AB043891
		Ambystoma tigrinum	AF038947
		Xenopus laevis	U90895
SWS1	Actinopterygii	*Lucania goodei*	AY296735
		Poecilia reticulata	NM_001297490
		Maylandia zebra	NM_001310074
	Amphibia	*Cynops pyrrhogaster*	AB052889
		Ambystoma tigrinum	AF038948
		Xenopus laevis	U23463
SWS2	Actinopterygii	*Lucania goodei*	AY296737.2
		Poecilia reticulata	NM_001297456
		Maylandia zebra	XM_004571913.3

Appendix C, continued.

Amphibia	*Cynops pyrrhogaster*	AB040148
	Ambystoma tigrinum	LC180360
	Xenopus laevis	BC080123

Appendix D. Alignments of consensus opsin sequences for each study species (labeled according to the first two letters of the genus followed by specific epithet, such that *Incilius nebulifer* becomes INNE, for example). The top of each alignment includes an opsin-specific consensus sequence of all fifteen study species, indicated with (Cons.). Amino acid positions and locations of transmembrane regions (TMRs), provided above each alignment, are based on alignment to bovine rhodopsin. Amino acid positions at which no variation was found among study species are indicated with (.); unsequenced positions with (-); and ambiguous amino acids with (X). Variable gene-specific tuning sites are highlighted in orange. RH1, LWS, SWS1, and SWS2 alignments are provided in **Tables D-1**, **D-2**, **D-3**, and **D-4**, respectively.

Table D-1. Variable amino acid sites on anuran RH1, aligned to *Bos taurus* (BOTA) rhodopsin.

```
Anuran RH1
     001                                                TMR 1          060
BOTA    MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLY
Cons.   -----------SMSNKTGVVRSPFEYPQYYLAEPWXYSXLXAYMFLLILLGXPXNFXTLY
INNE    -----------.......................Q..A.S.........L.I..M...
ANWO    -----------.......................Q..V.C.........F.I..M...
ELCY    -----------.......................K..V.A.........L.I..M...
ACCR    -----------.......................K..V.A.........L.I..M...
HYCH    -----------.......................K..I.A.........L.I..M...
HYVE    -----------.......................K..I.A.........L.I..M...
HYCI    -----------.......................K..V.A.........L.I..M...
PSCR    -----------.......................K..I.A.........L.I..M...
PSFO    -----------.......................K..I.A.........F.I..M...
GACA    -----------.......................K..V.A.........F.I..M...
LICA    -----------.......................K..V.A.........L.I..M...
LICL    -----------.......................K..V.A.........L.I..M...
LIPA    -----------.......................K..V.A.........L.I..M...
LISP    ------------------------------------------------------------
SCHU    -----------.......................K..I.A.........F.V..L...
```

Table D-1, continued.

```
Anuran RH1
     061                TMR 2                          TMR 3  120
BOTA    VTVQHKKLRTPLNYILLNLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLG
Cons.   VTIQHKKLRTPLNYILLNLXXANXFMVLXGFTXTXYXSXXGYFXXGXTXCXXEGFFATXG
INNE    ...................AF..H....C...V.M.S.MN...VF.Q.G.YV......L.
ANWO    ...................AF..H....C...I.M.S.MN...VF.Q.G.YV......L.
ELCY    ...................AF..H....C...I.M.T.MH...VL.W.T.QI......L.
ACCR    ...................AF..H....C...I.M.T.LH...VF.Q.G.YF......L.
HYCH    ...................AF..H....G...I.M.S.MH...VF.H.G.YI......L.
HYVE    ...................AF..H....G...I.M.S.MH...VF.H.G.YI......L.
HYCI    ...................AF..H....G...I.M.S.MH...VF.H.G.YI......L.
PSCR    ...................AF..H....C...V.M.S.MH...VF.Q.G.YV......L.
PSFO    ...................VF..H....C...I.M.S.MH...VF.Q.G.YI......L.
GACA    ...................AI..L....C...V.L.T.LH...IF.S.G.YI......T.
LICA    ...................AF..H....C...I.M.T.LH...VF.Q.G.YF......L.
LICL    ...................AF..H....C...I.M.T.LH...VF.Q.G.YF......L.
LIPA    ...................AF..H....C...I.M.T.LH...VF.Q.G.YF......L.
LISP    ----................AF..H....C...I.M.T.LH...VF.Q.G.YF......L.
SCHU    ...................AF..H....C...V.M.S.IN...VF.Q.G.YV......L.

Anuran RH1
     121   TMR 3                          TMR 4              180
BOTA    GEIALWSLVVLAIERYVVVCKPMSNFRFGENHAIMGVAFTWVMALACAAPPLVGWSRYIP
Cons.   GEXALWSLVVLAIERYXVVCKPMSNFRFXENHAXMGVXFTWXMAXACXXPPLXGWSRYIP
INNE    ..I.............V...........S....I...V...V..L..AA...F.......
ANWO    ..I.............V...........S....F...A...I..L..AV...F.......
ELCY    ..I.............I...........G....F...A...I..L..AV...A.......
ACCR    ..I.............I...........G....M...A...I..L..AV...F.......
HYCH    ..I.............V...........G....I...A...I..M..AA...F.......
HYVE    ..I.............V...........G....I...A...I..M..AA...F.......
HYCI    ..I.............V...........G....I...A...I..M..AA...F.......
PSCR    ..I.............V...........G....I...A...L..L..AA...F.......
PSFO    ..I.............V...........G....I...A...I..L..AV...F.......
GACA    ..M.............V...........T....F...A...I..L..SA...V.......
LICA    ..I.............I...........G....M...A...I..L..AV...F.......
LICL    ..I.............I...........G....M...A...I..L..AV...F.......
LIPA    ..I.............I...........G....M...A...I..L..AV...F.......
LISP    ..I.............I...........G....M...A...I..L..AV...F.......
SCHU    ..I.............V...........G....I...A...I..L..AA...M.......
```

Table D-1, continued.

```
Anuran RH1
     181                              TMR 5                          240
BOTA     EGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGQLVFTVKEAAAQQQES
Cons.    EGMQCSCGVDYYTLKPEVNNESFVIYMFXVHFXIPLXXIXFCYGRLVCTVKEAAAQQQES
INNE     ............................I...L...VI.F.....................
ANWO     ............................V...L...VI.F.....................
ELCY     ............................L...T...IV.F.....................
ACCR     ............................V...L...II.S.....................
HYCH     ............................V...L...II.F.....................
HYVE     ............................V...L...II.F.....................
HYCI     ............................I...L...VI.F.....................
PSCR     ............................I...L...II.F.....................
PSFO     ............................I...L...II.F.....................
GACA     ............................I...C...AI.F.....................
LICA     ............................V...L...II.S.....................
LICL     ............................V...L...II.S.....................
LIPA     ............................V...L...II.S.....................
LISP     ............................V...L...II.S.....................
SCHU     ............................I...L...VV.F.....................

Anuran RH1
     241                TMR 6                    TMR 7          300
BOTA     ATTQKAEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTHQGSDFGPIFMTIPAFFAKTSAV
Cons.    ATTQKAEKEVTRMVXIMVXXFXICWXPYAXVAFXIFXXQGXEFGPXFMTXPAFFAKSSXI
INNE     ..............V...VF.L...V...T...F..TH..S....V...L.........A.
ANWO     ..............I...VF.L...V...Y...Y..TH..S....V...V.........A.
ELCY     ..............I...VF.L...V...T...F..TH..S....V...V.........A.
ACCR     ..............V...IF.L...V...Y...Y..TH..S....I...V.........A.
HYCH     ..............I...VF.L...V...T...F..CN..S....V...I.........A.
HYVE     ..............I...VF.L...V...T...F..CN..S....V...I.........A.
HYCI     ..............I...VF.L...V...T...F..TH..S....V...I.........A.
PSCR     ..............I...VF.L...V...T...F..MN..S....V...I.........S.
PSFO     ..............I...VF.L...V...T...F..MN..S....V...V.........S.
GACA     ..............I...IF.C...F...Y...Y..TH..S....I...V.........A.
LICA     ..............V...IF.L...V...Y...Y..TH..S....I...V.........A.
LICL     ..............V...IF.L...V...Y...Y..TH..S....I...V.........A.
LIPA     ..............V...IF.L...V...Y...Y..TH..S....I...V.........A.
LISP     ..............I...IF.L...V...Y...Y..TH..S....I...V.........A.
SCHU     ..............I...VG.L...V...T...F..TH..T....V...V.........S.
```

Table D-1, continued.

```
Anuran RH1
     301 TMR 7                                                  355
BOTA     YNPVIYIMMNKQFRNCMVTTLCCGKNPLGDDEASTTVSKTETSQVAPA*
Cons.    YNPVIYIXLNKQFRNCMIT-----------------------------------
INNE     .......M........-------------------------------------
ANWO     .......M........-------------------------------------
ELCY     .......M.......-------------------------------------
ACCR     .......M........-------------------------------------
HYCH     .......M........-------------------------------------
HYVE     .......M........-------------------------------------
HYCI     .......M...........-----------------------------------
PSCR     .......M...........-----------------------------------
PSFO     .......M........-------------------------------------
GACA     .......M........-------------------------------------
LICA     .......M........-------------------------------------
LICL     .......M........-------------------------------------
LIPA     .......M........-------------------------------------
LISP     .......M........-------------------------------------
SCHU     .......V........-------------------------------------
```

Table D-2. Variable amino acid sites on anuran LWS. Position 001 on bovine rhodopsin (at which this alignment begins) aligns with anuran LWS position 016. Potential sites of positive selection, as identified by PAML, are highlighted in purple.

```
Anuran LWS
     001                                                  TMR 1          060
Cons.   XDXEXTTRSXXFXYTNXNNTRGPFEGPNYHIAPRWXYNXTTXWMXFVVXASXXTNGXVLX
INNE    -.D.D....SI.T...S.................V..I..L..V...A..VF...L..V
ANWO    ------------------------------------------------------------
ELCY    P.D.D....TI.T...S.................V..I..L..V...I..VF...L..V
ACCR    ------...SV.T...S.................A..V..L..I...I..VL...I..V
HYCH    --------.SV.T...S.................V..V..V..I...L..CF...L..A
HYVE    --------.SV.X...S.................V..V..V..I...L..CF...L..A
HYCI    H.E.E....SV.T...S.................V..V..V..I...L..CF...L..A
PSCR    ---------------------------------------------...A..CF...L..A
PSFO    ----------------------.............V..V..V..I...C..VF...L..A
GACA    ------------------------------------V..I..V..I...G..CF...L..V
LICA    ------------------------------------V..V..L..I...I..VL...I..V
LICL    ------------------------------------V..V..L..I...I..VL...I..V
LIPA    -------------------------------------..V..L..I...I..VL...I..V
LISP    ------------------------------------V..V..L..I...I..VL...I..V
SCHU    D.D.E....SV.V...F.................V..I..L..V...I..IF...L..V

Anuran LWS
     061              TMR 2                                TMR 3   120
Cons.   ATXKFKKLRHPLNWILXNXAIADLGETXIASXISVXNQXFGYFXLGHPXCVXEGYTVSXC
INNE    ..F.............V..........V...T...F..I....V.......I......V.
ANWO    ------------------------------------------------------------
ELCY    ..F.............V..........I...T...F..I....V.......I......V.
ACCR    ..F.............M..........I...T...I..I....V.......I......V.
HYCH    ..F.............V..........I...T...F..I....I.......L......V.
HYVE    ..F.............V..........I...T...F..I....I.......L......V.
HYCI    ..L.............V..........I...T...F..I....I.......L......V.
PSCR    ..F.............V..........I...T...F..I....I.......L......V.
PSFO    ..F.............V..........I...T...F..I....I.......L......V.
GACA    ..F.............M..........I...T...I..L....V.......I......V.
LICA    ..F.............M..........L...S...I..I....V.......I......V.
LICL    ..F.............M..........I...T...I..I....V.......I......V.
LIPA    ..F.............M..........I...T...I..I....V.......I......V.
LISP    -.F.............M..........I...T...I..I....V.......I......V.
SCHU    ..Y.............V..........V...T...A..C....I.......I......T.
```

Table D-2, continued.

```
Anuran LWS
     121    TMR 3                                  TMR 4                  180
Cons.    GITXLWSLTVIAWERWFVVCKPFGNIKFDGKLAXXGIXFXWXWXAXWCXPPXFGWSRYWP
INNE     ...G.............................AG..I.A.V.A.C..A..I........
ANWO     ---------------------------------------------------------------
ELCY     ...G.............................AG..I.A.T.A.C..A..I........
ACCR     ...G.............................AG..A.S.L.S.G..A..I........
HYCH     ...G.............................AG..I.S.V.S.V..A..M........
HYVE     ...G.............................AG..I.S.V.S.V..A..M........
HYCI     ...G.............................AG..I.S.V.S.V..A..I........
PSCR     ...G.............................TG..V.S.V.S.V..S..I........
PSFO     ...G.............................TG..I.S.T.S.V..A..I........
GACA     ...G.............................IA..A.S.I.S.A..A..I........
LICA     ...G.............................AG..A.S.L.S.G..A..I........
LICL     ...G.............................AG..A.S.L.S.G..A..I........
LIPA     ...G.............................AG..A.S.L.S.G..A..I........
LISP     ...G.............................AG..A.S.L.S.G..A..I........
SCHU     ...A.............................CA..L.S.I.A.S..A..I........

Anuran LWS
     181                          TMR 5                          240
Cons.    HGLKTSCGPDVFSGXSDPGIQSYMMVLMITCCIIPLXIIXLCYXXVWWAIRKVAQQQKES
INNE     ..............S.....................T..V...IH...............
ANWO     --------......S.....................T..V...IH...............
ELCY     ..............S.....................T..V...IH...............
ACCR     ..............X.....................T..V...IH...............
HYCH     ..............S.....................T..V...IH...............
HYVE     ..............S.....................T..V...IH...............
HYCI     ..............S.....................T..V...IH...............
PSCR     ..............S.....................X..X...IA...............
PSFO     ..............S.....................T..V...IH...............
GACA     ..............S.....................T..I...LQ...............
LICA     ..............S.....................A..V...IH...............
LICL     ..............X.....................T..V...IH...............
LIPA     ..............N.....................T..V...IH...............
LISP     ..............X.....................T..V...IH...............
SCHU     ..............S.....................T..V...IH...............
```

Table D-2, continued.

```
Anuran LWS
     241                TMR 6                TMR 7       300
Cons.   ESTQKAEREVSRMVVVMIMAYCFCWGPYTXFACFAAANPGYSFGPLAASLPAYFAKSATI
INNE    ............................V.............................
ANWO    ............................V.............................
ELCY    ............................V.............................
ACCR    ............................I.............................
HYCH    ............................V.............................
HYVE    ............................V.............................
HYCI    ............................X.............................
PSCR    ............................F.............................
PSFO    ............................V.............................
GACA    ............................V.............................
LICA    ............................X.............................
LICL    ............................X.............................
LIPA    ............................V.............................
LISP    ............................V.............................
SCHU    ............................F.............................

Anuran LWS
     301 TMR 7                                              351
Cons.   YNPVIYVFMNRQFRNCIYQLLGKKVEDXSELSSTSRT--------------
INNE    ........................A.........--------------
ANWO    ........................A.........--------------
ELCY    ........................A.........--------------
ACCR    ........................G.........--------------
HYCH    ........................A.........--------------
HYVE    ........................A.........--------------
HYCI    ........................A.........--------------
PSCR    ........................A.........--------------
PSFO    ........................A.........--------------
GACA    .....-----------------------------------------
LICA    ..................-----------------------------
LICL    ........................-------------------------
LIPA    ........................A.........--------------
LISP    ........................A.........--------------
SCHU    ........................A.........--------------
```

Table D-3. Variable amino acid sites on anuran SWS1. Anuran SWS1 position 001 aligns with bovine rhodopsin position 006, so the first five positions on this alignment are blank.

```
Anuran SWS1
     001                                                  TMR 1          060
Cons.        --GDEDFYXFXNXXXXXPWDGPQYHIAPXWAFXLQAIFMGXXFIXGTPLNXXVLX
INNE         ----....I.K.ITDVS..........K...T.......LV..I.....SV..L
ANWO         --......I.R.ISDVG..........K...T.......MV..I.....SX..L
ELCY         ----------------G..........K...T.......AV..I.....AV..V
ACCR         ----....L.K.VSDIR..........R...T.......VI..I.....AV..L
HYCH         ----....L.K.ISHVS..........K...H.......VV..F.....AV..V
HYVE         -----...L.K.ISHVS..........K...H.......VV..F.....AV..V
HYCI         ------..L.K.ISHVS..........K...H.......VV..F.....SI..V
PSCR         ----------.K.VSHIS..........K...H.......VV..F.....AV..V
PSFO         ----....L.K.VSHIG..........K...H.......VV..F.....AV..V
GACA         ----------------------------------------------------------
LICA         ----....L.K.VSDIR..........R...T.......VV..I.....AV..L
LICL         ----....L.K.VSDIR..........R...T.......VI..I.....AV..L
LIPA         ----....L.K.VSDIR..........R...T.......VV..I.....AV..V
LISP         ----....L.K.VSDIR..........R...T.......VV..I.....AV..V
SCHU         ----------------------.....R...T.......VV..I.....AV..L

Anuran SWS1
     061                TMR 2                                  TMR 3  120
Cons.    VTXKYKKLRQPLNYILVNISVXGLXXCXFSIFXVFXXSXXGYFXFGKXXCXIEXFVGXLX
INNE     ..V..................A..VI.I....T..VS.CH...V...IA.G..A...T.S
ANWO     ..V..................A..VM.I....T..VS.CQ...V...TT.G..A...T.S
ELCY     ..A..................A..VI.I....T..VS.CQ...V...TA.G..A...T.S
ACCR     ..V..................G..LI.L....V..IN.WQ...F...AF.A..A...T.A
HYCH     ..V..................A..LI.I....Y..VS.CY...V...IA.Y..G...T.S
HYVE     ..V..................A..LI.I....Y..VS.CY...V...IA.T..G...T.S
HYCI     ..V..................A..LI.I....Y..VS.CN...I...IA.A..G...T.S
PSCR     ..V..................A..LI.V....Y..VS.CN...V...IA.A..G...T.S
PSFO     ..V..................A..LI.V....Y..VS.CN...V...TV.A..G...S.S
GACA     ------------------------------------------------------------
LICA     ..V..................G..LI.I....V..IN.WQ...F...AF.A..A...T.A
LICL     ..V..................G..LI.L....V..IN.WQ...F...AF.A..A...T.A
LIPA     ..V..................G..LI.I....I..IN.WQ...I...AF.A..A...T.A
LISP     ..V..................G..LI.I....I..IN.WQ...I...AF.A..A...T.A
SCHU     ..V..................G..LI.I....V..IN.WQ...F...AF.A..A...T.A
```

Table D-3, continued.

```
Anuran SWS1
     121    TMR 3                                  TMR 4                 180
Cons.   GLVTGWSLAFLAFERYIVICKPMGXFXFXSKHAXXVVLXTWXIGXXVSXPPFXGWSRXXP
INNE    ........................N.C.S....MA...C..F..IG..V...F....YI.
ANWO    ........................N.C.S....MA...C..L..FG..I...F....YI.
ELCY    ........................N.C.S....MS...V..L..FG..V...F....FL.
ACCR    ........................T.T.T....LA...T..M..VG..V...F....YI.
HYCH    ........................N.C.S....LA...C..L..IA..V...L....YI.
HYVE    ........................N.C.S....LA...C..L..IA..V...L....YI.
HYCI    ........................N.C.S....LA...C..L..IA..V...F....YI.
PSCR    ........................N.C.S....LA...I..I..IA..V...L....YI.
PSFO    ........................N.C.S....LT...I..M..LS..V...F....YI.
GACA    ------------------------------------------------------------
LICA    ........................T.T.T....LA...T..M..VG..V...F....YI.
LICL    ........................T.T.T....LA...T..M..VG..V...F....YI.
LIPA    ........................T.T.T....LA...S..L..VG..V...L....YI.
LISP    ........................T.T.T....LA...S..L..IG..V...L....YI.
SCHU    ........................T.T.T....LA...T..M..VG..V...F....YI.

Anuran SWS1
     181                                TMR 5                        240
Cons.   EGLQCSCGPDWYTVGTKYXSEYYTWFIFXFCFXXPLXLICXXYAXLLGALRAVAAQQQES
INNE    ...................R.........I...VI..T...F...Q...............
ANWO    ...................R.........I...VI..T...F...Q...............
ELCY    ...................R.........I...IV..T...F...Q...............
ACCR    ...................H.........V...II..T...Y...R...............
HYCH    ...................R.........I...VI..T...F...Q...............
HYVE    ...................R.........I...VI..T...F...Q...............
HYCI    ...................R.........I...VI..T...F...Q...............
PSCR    ...................R.........I...VI..T...F...Q...............
PSFO    ...................R.........I...VI..T...F...Q...............
GACA    ------------------------------------------------------------
LICA    ...................H.........V...II..T...Y...R...............
LICL    ...................H.........V...II..T...Y...R...............
LIPA    ...................H.........V...II..S...Y...R...............
LISP    ...................H.........V...LI..S...Y...R...............
SCHU    ...................H.....-------------------------------------
```

Table D-3, continued.

```
Anuran SWS1
     241                TMR 6                TMR 7       300
Cons.   ASTQKAEKEVSRMVXVMXGSFCLCYVPYAAXAXXMXTNRNHGXDLRXVTXPAFFSKSACV
INNE    ..............I..I............M.MY.V......M...L..I..........
ANWO    ..............I..I............M.MY.V......M...L..I..........
ELCY    ..............I..I............L.MY.V......M...F..I..........
ACCR    ..............V..V............M.MY.I......L...F..I..........
HYCH    ..............I..I............M.LY.V......M...L..L..........
HYVE    ..............I..I............M.LY.V......M...L..L..........
HYCI    ..............I..I............M.MY.V......M...L..L..........
PSCR    ..............I..I............M.LF.V......M...L..I..........
PSFO    ..............I..I............M.LY.V......M...L..I..........
GACA    ---------------..V............M.MY.I......L...F..I..........
LICA    ..............V..V............M.MY.I......L...F..I..........
LICL    ..............V..V............M.MY.I......L...F..I..........
LIPA    ..............V..V............M.MY.I......L...F..I..........
LISP    ..............V..V............M.MY.I......L...F..I..........
SCHU    ---------------..V............M.MY.I......L...F..I..........

Anuran SWS1
     301 TMR 7                                                  355
Cons.   YNPIIYXFMNKQFRGCIXETVCGRPXTDDSXXSXXSQK-----------------
INNE    ......S..........M.......I...---------------------------
ANWO    ......S..........M.......I....SL.TS...-----------------
ELCY    ......S..........L.......M....SV.TS...-----------------
ACCR    ......T..........M......-------------------------------
HYCH    ......S..........M.......I....-------------------------
HYVE    ......S..........M.......I...---------------------------
HYCI    ......S..........M.......I....SV-----------------------
PSCR    ......S..........M.......I...---------------------------
PSFO    ......S..........M.......I....SV.----------------------
GACA    ......T..........M.......M....TL.ST...-----------------
LICA    ......T..........M.......M....T-----------------------
LICL    ......T..........M.......M...---------------------------
LIPA    ......T..........M.......M....TL.ST...-----------------
LISP    ......T..........M.......M....TL.ST...-----------------
SCHU    ......T..........M.......M....TL.ST...-----------------
```

Table D-4. Variable amino acid sites on anuran SWS2. Position 001 on bovine rhodopsin (at which this alignment begins) aligns with anuran LWS position 010.

```
Anuran SWS2
     001                                                TMR 1
060
Cons.    ---------PIPXETTNXXXXSPFLVPQXHLGXPGXFMXMSAFMLFTIIFGFPLNXLTXX
INNE     --------------...ISSV.......T...S..I..S.................L..II
ANWO     --------------...ISSL.......T...S..I..S.................V..II
ELCY     -------------....ISSV.......T...T..I..S.................M..VI
ACCR     --------------...ITAL.......S...T..M..S.................V..VI
HYCH     --------------...VTAL.......T...T..I..S.................L..VI
HYVE     --------------...VTAL.......T...T..I..S.................L..VI
HYCI     ---------...S....VTAL.......T...T..I..S.................L..VI
PSCR     ---------------..VTAL.......T...T..V..S.................L..VI
PSFO     --------------...VTAL.......T...T..I..S.................L..VI
GACA     -------------....VTAL.......T...T..I..S.................L..IF
LICA     --------------...ITAL.......S...T..M..S.................V..VI
LICL     -------------....ITAL.......S...T..M..S.................V..VI
LIPA     ----------------------.......T...T..M..S.................V..VI
LISP     ---------...L....ITAL.......T...T..M..S.................V..VI
SCHU     ---------...L....ITSL.......T...T..V..G.................L..VI

Anuran SWS2
     061                TMR 2                                TMR 3  120
Cons.   CTXKYKKLRSHLNYILVNLAXANLXVIXXGSTXAFYXFSQMYFXXGXLACKXEGFTATLG
INNE    ..A.................V...V..CF...T...S......SL.T....I........
ANWO    ..A.................V...V..CF...T...S......SL.T....I........
ELCY    ..A.................V...V..CF...T...S......AL.T....L........
ACCR    ..I.................V...I..CF...T...S......AL.T....I........
HYCH    ..I.................V...L..SI...T...S......AM.P....I........
HYVE    ..I.................V...L..SI...T...S......AM.P....I........
HYCI    ..I.................V...L..CI...T...S......AM.P....I........
PSCR    ..I.................V...V..CI...T...S......AM.P....I........
PSFO    ..I.................V...V..CI...V...S......SL.P....I........
GACA    ..I.................I...I..CF...T...T......SL.T....I........
LICA    ..I.................V...I..CF...T...S......AL.T....I........
LICL    ..I.................V...I..CF...T...S......AL.T....I........
LIPA    ..I.................V...I..CF...T...S......AL.T....I........
LISP    ..I.................V...I..CF...T...S......AL.T....I........
SCHU    ..V.................V...I..CF...T...S......AL.T....I........
```

Table D-4, continued.

```
Anuran SWS2
     121    TMR 3                                  TMR 4                 180
Cons.   GXXXLWSLAVVAFERFXVICKPMGSFTFREXHAXXGCIXTWXXXXXAXXPPLXGWSRYIP
INNE    .MVS...........L.............S..VM...F..VIALV.AA...L.......
ANWO    .MVS...........I.............S..VI...F..VIAFM.AV...L.......
ELCY    .MVS...........L.............S..VM...F..VIALV.AS...L.......
ACCR    .MVS...........L.............N..IL...F..VIGLV.AS...L.......
HYCH    .MVG...........I.............S..VM...F..VISLV.SV...L.......
HYVE    .MVG...........I.............S..VM...F..VISLV.SV...L.......
HYCI    .MVS...........I.............S..VM...F..VISLV.SV...L.......
PSCR    .MVS...........I.............S..VL...F..VISLV.SV...L.......
PSFO    .MVS...........I.............S..VL...F..VISLV.SV...L.......
GACA    .IIS...........L.............N..IM...L..LMGIV.AT...V.......
LICA    .MVS...........L.............N..IL...F..VIGLV.AS...L.......
LICL    .MVS...........L.............N..IL...F..VIGLV.AS...L.......
LIPA    .MVS...........L.............N..IL...F..VIGLV.AS...L.......
LISP    .MVS...........L.............N..IL...F..VIGLV.AS...L.......
SCHU    .MVS...........L.............N..IL...F..VIGLV.AS...L.......

Anuran SWS2
     181                              TMR 5                          240
Cons.   EGLQCSCGPDWYTVNNKWNNESYVXFXFCFCFGFPLXVIXFSYGRLLLTLGAVAKQQEQS
INNE    ........................M.M.........A..V.....................
ANWO    ........................L.M.........T..V.....................
ELCY    ........................L.M.........A..I.....................
ACCR    ........................I.I.........A..V.....................
HYCH    ........................L.M.........A..V.....................
HYVE    ........................L.M.........A..V.....................
HYCI    ........................L.M.........A..V.....................
PSCR    ........................L.M.........A..V.....................
PSFO    ........................L.M.........A..V.....................
GACA    ........................M.M......----------.....................
LICA    ........................I.I.........A..V.....................
LICL    ........................I.I.........A..V.....................
LIPA    ........................I.I.........A..V.....................
LISP    ........................I.I.........A..V.....................
SCHU    ........................L.L.........A..V.....................
```

Table D-4, continued.

```
Anuran SWS2
     241                TMR 6                TMR 7       300
Cons.   AXTQKAEREVTRMVIXHXXGFLVCWLPYASFALWAVTHRGEXFDLRMASXPSVFSKASTV
INNE    .T.............M.VF.....................E.......I..........
ANWO    .T.............V.VF.....................E.......I..........
ELCY    .T.............M.VV.....................T.......I..........
ACCR    .S.............M.IA.....................T.......I..........
HYCH    .S.............V.VA.....................V.......I..........
HYVE    .S.............V.VA.....................V.......I..........
HYCI    .T.............V.VA.....................V.......I..........
PSCR    .T.............F.VA.....................T.......I..........
PSFO    .T.............F.VA.....................T.......V..........
GACA    .T.............F.VA.....................T.......I..........
LICA    .S.............M.IA.....................T.......I..........
LICL    .S.............M.IA.....................T.......I..........
LIPA    .S.............M.IA.....................T.......I..........
LISP    .S.............M.IA.....................T.......I..........
SCHU    .T.............F.VA.....................T.......I..........

Anuran SWS2
     301 TMR 7                                                356
Cons.   YNPFIYIXMNRQFRXCXXKLIFCGKNPLGDD-------------------------
INNE    .......F......S.MM............-------------------------
ANWO    .......F......A.IM............-------------------------
ELCY    .......L......S.IM............-------------------------
ACCR    .......F......S.MM.......-----------------------------
HYCH    .......F......S.MM........-----------------------------
HYVE    .......F......S.MM........-----------------------------
HYCI    .......F......S.MM........-----------------------------
PSCR    .......L......S.IL............-------------------------
PSFO    .......F......S.IM............-------------------------
GACA    .......L......S.IL............-------------------------
LICA    .......F......S.MM............-------------------------
LICL    .......F......S.MM........-----------------------------
LIPA    .......F......S.MM............-------------------------
LISP    .......F.....-----------------------------------------
SCHU    .......L......S.IL............-------------------------
```

VITA

Leah Perez graduated from Klein High School in 2012 and immediately began higher education through the Lone Star College System. In the fall of 2013, she transferred to Stephen F. Austin State University, declaring a major in Biology and minor in Environmental Science. She completed additional coursework at Lone Star College again in the summer of 2014. In December of 2016, she graduated with honors from Stephen F. Austin State University as a University Scholar and received the degree of Bachelor of Science.

Permanent Address: 9415 Wallingham Dr.
Spring, TX, 77379

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