



City Research Online

City St George's, University of London

Citation: Simoes, B. F., Sampaio, F. L., Douglas, R. H., Kodandaramaiah, U., Casewell, N. R., Harrison, R., Hart, N. S., Partridge, J. C., Hunt, D. M. & Gower, D. J. (2016). Visual pigments, ocular filters and the evolution of snake vision. *Molecular Biology and Evolution*, 33(10), pp. 2483-2495. doi: 10.1093/molbev/msw148

This is the accepted version of the paper.

This version of the publication may differ from the final published version. To cite this item please consult the publisher's version.

Permanent repository link: <https://city-ref-test.eprints-hosting.org/id/eprint/15201/>

Link to published version: <https://doi.org/10.1093/molbev/msw148>

Copyright and Reuse: Copyright and Moral Rights remain with the author(s) and/or copyright holders. Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge, unless otherwise indicated, provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way. For full details of reuse please refer to [City Research Online policy](#).

Visual pigments, ocular filters and the evolution of snake vision

Bruno F. Simões^{1,*}, Filipa L. Sampaio¹, Ronald H. Douglas², Ullasa Kodandaramaiah³, Nicholas R. Casewell⁴, Robert A. Harrison⁴, Nathan S. Hart⁵, Julian C. Partridge⁶, David M. Hunt^{6,7} & David J. Gower^{1,*}.

¹Department of Life Sciences, The Natural History Museum, London, SW7 5BD, UK; ²Department of Optometry and Visual Science, City University London, London EC1V 0HB, UK; ³School of Biology, Indian Institute of Science Education and Research Thiruvananthapuram, Thiruvananthapuram 695 016, India; ⁴Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK; ⁵Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia; ⁶School of Animal Biology and The Oceans Institute, The University of Western Australia, Perth, WA 6009, Australia; ⁷Lions Eye Institute, University of Western Australia, Perth, 6009, Australia

*Email: bruno.simoes@me.com; d.gower@nhm.ac.uk

Abstract

Much of what is known about the molecular evolution of vertebrate vision comes from studies of mammals, birds and fish. Reptiles (especially snakes) have barely been sampled in previous studies despite their exceptional diversity of retinal photoreceptor complements. Here we analyse opsin gene sequences and ocular media transmission for up to 69 species to investigate snake visual evolution. Most snakes express three visual opsin genes (*rh1*, *sws1*, *lws*). These opsin genes (especially *rh1* and *sws1*) have undergone much evolutionary change, including modifications of amino acid residues at sites of known importance for spectral tuning, with several tuning site combinations unknown elsewhere among vertebrates. These changes are particularly common among dipsadine and colubrine ‘higher’ snakes. All three opsin genes are under purifying selection, though dN/dS varies with respect to some lineages, ecologies, and retinal anatomy. Positive selection was detected at multiple sites in all three opsins, these being concentrated in transmembrane domains and thus likely to have a substantial effect on spectral tuning and other aspects of opsin function. Snake lenses vary substantially in their spectral transmission. Snakes active at night and some of those active by day have very transmissive lenses, while some primarily diurnal species cut out shorter wavelengths (including UVA). In terms of retinal anatomy, lens transmission,

visual pigment spectral tuning and opsin gene evolution the visual system of snakes is more diverse than in any other tetrapod order.

Key words: ocular media, sensory evolution, photoreception, Serpentes, spectral tuning, vision

Introduction

Animal vision has become one of the best examples of the power of integrative biology. A great deal is known about the anatomy of eyes at many levels, but much is also known about how eyes function and have evolved, including aspects of the physiology underlying photon capture, spectral sensitivity, signal transduction and propagation, and the identity of several key genes and proteins. Indeed, vision is one of the best characterized of all biological sensory systems. In addition, selective pressures can often be determined from physical first principles, allowing the identification and quantification of many aspects of the evolution of eyes (Land 1981; Nilsson 1996). In general, vision in vertebrates is especially well studied, and studies of the evolution of their visual pigments have been able to both identify evolutionary changes, and to ascribe such changes to adaptive evolutionary processes (e.g. Hughes 2008).

The fundamentals of vertebrate vision have been particularly well studied in terms of the molecular basis of photoreception and phototransduction. A cornerstone of this is knowledge of the photosensitivity of visual pigments, members of the large family of G-protein-coupled-receptor (GPCR) proteins, which share a common arrangement of an opsin protein linked to a chromophore derived from vitamin A (Wald 1968). Visual pigments play a core role in photon detection and colour vision and they are a leading example of how gene duplications (Dulai et al. 1999) and changes in amino acid sequences (Yokoyama 2008), type of chromophore (vitamin A1 or A2: Enright et al. 2015) and gene expression (Hofmann and Carleton 2009; Carleton et al. 2010) underlie adaptations to differing ecological and behavioural selection pressures. Visual opsins in some vertebrates have been intensely studied over the past 20 years, to the extent that changes in specific amino acid ('spectral tuning') sites are known to change the peak absorbance wavelength ($\lambda_{\max}$) of the visual pigments (Yokoyama 2008; Yokoyama et al. 2014). However, there is no universal consensus about the tuning impacts of all such mutations (Hauser et al. 2014), with some data suggesting that additional mechanisms to change spectral sensitivity may exist (Davies et al. 2009; Martin et al. 2015).

Much of our knowledge about the function and evolution of vertebrate vision, including its molecular basis, comes from empirical studies on a relatively small proportion of living vertebrates, predominantly some groups of mammals, birds and fish (Nickle and Robinson 2007; Davies et al. 2012). Investigation of vision in other

vertebrates is needed to test inferred generalities, especially in those taxa having visual systems with very different anatomical arrangements of the eye, and/or great phenotypic diversity. Snakes are one such lineage that shows substantial diversity of ocular anatomy, especially retinal photoreceptor complement. Indeed, Walls (1942) and Underwood (1967; 1970) argued that, by virtue of their great diversity of photoreceptor complements, there must have been more evolutionary changes within snakes than in all the other vertebrates combined. The eyes of snakes are also remarkable for being highly divergent in gross morphology from that of non-snake squamates ('lizards'), in lacking photoreceptor oil droplets, in mostly being covered by a transparent head scale (spectacle or Brille), and in presenting evidence for evolutionary transitions ('transmutation' sensu Walls 1934) between rods and cones (Walls 1942).

The approximately 3,500 species of living snakes are distributed across all continents except Antarctica (Van Wallach et al. 2014). They are very diverse ecologically (e.g., Greene 1997) and include burrowing, arboreal, gliding, fully aquatic, nocturnal and diurnal species. Some have small eyes lying under typical head scales, while others are visual hunters with well developed binocular vision, some of which have horizontal pupils and a fovea (Walls 1942). Since Walls' and Underwood's pioneering anatomical surveys, we have learned that the ancestral snake likely had three of the five visual opsin genes present in the ancestral vertebrate (Davies et al. 2009; Simões et al. 2015), but not much more is known.

In order for light to be absorbed by the visual pigments it first has to pass through the ocular media. In vertebrates these comprise the cornea, lens, and aqueous and vitreous humour. Snakes additionally have a covering over the cornea (brille or spectacle). Lens transmission characteristics of most major vertebrate groups have been widely studied (e.g. Douglas and Marshall 1999; Douglas and Jeffery 2014 for reviews), but there are few reports of the spectral transmission of snake lenses. Walls (1931) noted yellow (blue-absorbing) lenses in a number of diurnal snakes and uncoloured lenses in nocturnal species. However, these observations were qualitative, using the UV-insensitive human visual system, such that the spectral characteristics of both coloured and transparent lenses in the UV are unknown, with the exception of two species of sea snake whose lenses transmit significant amounts of UV (Hart et al. 2012). The spectral characteristics of the reptilian spectacle have been reported only twice (Hart et al. 2012; van Doorn and Sivak 2015).

Given the anatomical diversity of snake retinal photoreceptors and the relative lack of previous studies, we address the following major questions: (1) What are the major patterns in the diversity and molecular evolution of snake visual opsins? (2) Is the diversity in retinal photoreceptor anatomy, visual opsin and ocular media transmission

linked in a predictable way? (3) To what extent is visual opsin spectral tuning and/or opsin molecular evolution explained by major shifts in ecology and/or retinal anatomy? (4) Do snakes present diversity in visual opsins beyond that known for other major groups of vertebrates, mirroring the diversity of their ocular morphology?

Here we report the largest dataset of visual opsin genes in reptiles to date, covering the major types of snake retinal anatomy and taxonomic and ecological diversity. We also report data on the spectral transmission of important components of the ocular media (lens and spectacle) of a subset of these snakes. We find that although the vast majority of snakes retain three of the visual opsin genes likely present in the ancestral snake, these have undergone considerable diversification through functionally important amino acid substitutions. Notably, many of these substitutions are unreported in other vertebrate groups. There are also changes in the transmission of the lens, particularly with respect to the filtering of short wavelengths that will significantly affect overall spectral sensitivities. Snakes are an important system for understanding of the evolution of the vertebrate visual system.

Material & Methods

Taxon sampling and sample storage

Snakes were acquired through fieldwork, the Liverpool school of Tropical Medicine, from hobbyists and the commercial trade. Our sampling (SI Table S1) aimed to maximise taxonomic (phylogenetic), ecological and ocular anatomical diversity. One specimen each of 48 species was newly sampled. The use of animals in this research was conducted using standard protocols approved by the Liverpool school of Tropical Medicine Animal Welfare and Ethical Review Board and the UK Home Office. Following euthanasia, spectacle scales (brilles) were removed and the eyes extracted. After removing the lens, each eye was coarsely macerated and stored in RNAlater (Ambion) at -80°C until the RNA extraction. Where possible, undamaged lenses and spectacles were stored dry at -20°C until measurement of spectral transmission was performed.

RNA extraction and cDNA synthesis

Total RNA was extracted from eyes using TRIzol® (Life Technologies/Ambion) followed by purification with PureLink™ RNA Mini Kit (Life Technologies/Ambion) using the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche) with 500ng of total RNA according to manufacturer's instructions. RNA complementary to the cDNA was removed using 2

units of *E. coli* RNase H (Ambion) and incubated at 37°C for 20 minutes. For the following species freshly synthesized cDNA was dehydrated, stored at ambient temperature for 24 hours, and returned to -20°C and rehydrated after a further 24 hours before subsequent amplification: *Melanophidium* sp., *Uropeltis* cf. *macrolepis*, *Gongylophis conicus*, *Pareas monticola*, *Amphiesma stolata*, *Xenochrophis piscator*, *Xylophis captaini*, *Boiga forsteni*, *Boiga ceylonensis*. All other cDNA samples were kept hydrated and stored at -20°C prior to amplification.

Visual opsin gene amplification and cloning

Here we denote opsin genes in lower case italics and opsin proteins in upper case (e.g. *rh1* and RH1, respectively). We amplified the coding regions of *sws1*, *lws* and *rh1* visual opsin genes using universal primers designed to amplify visual opsin genes across snakes and squamates (Simões et al. 2015). All fragments were amplified in 25 µl Polymerase Chain Reactions (PCR): 1x PCR buffer (Invitrogen), 1.5 mmol (mM) of MgCl₂ (Invitrogen), 50 µmol/L of deoxynucleotides (Bioline), 0.4 µmol/L of each primer and 1 unit Platinum Taq Polymerase (Invitrogen) and 100ng of cDNA. PCR products were amplified by touchdown PCR with the following cycling parameters: initial denaturation at 95°C for 5 minutes; 20 cycles of 1 minute at 95°C (denaturation), 30 seconds at 60°C (annealing), and 1 minute at 72°C (extension) with a decrease of 0.5°C per cycle; 15 cycles of 1 minute at 95°C (denaturation), 30 seconds at 50°C (annealing), and 1 minute at 72°C (extension) followed by a final extension at 72°C for 5 minutes. PCR products were run on a 1% agarose gel, excised in a Blue Light Transilluminator (Safe Imager, Invitrogen) and purified with a PureLink Quick Gel Extraction Kit (Invitrogen). PCR fragments were cloned with a StrataClone PCR Cloning Kit (Agilent) and corresponding chemically competent cells following the manufacturer's protocol. Transformed cells were grown overnight on agar medium treated with 100 mg/ml of Ampicilin (Bioline) and 1ml of 2% X-GAL at 37°C. Sixteen white colonies were picked and used as DNA template in 25µl PCR reactions: 1x PCR buffer (Bioline), 1 mmol (mM) of MgCl₂ (Bioline), 80 µmol/L of deoxynucleotides (Bioline), 0.2 µmol/L of M13F and M13R vector primers and 1 unit of BioTAQ Polymerase (Bioline) and 2µl of DNA (1 colony twirled in 50µl of ultra-pure water). The PCR had the following cycling parameters: initial denaturation at 95°C for 10 minutes; 30 cycles of 15 seconds at 95°C (denaturation), 30 seconds at 58°C (annealing), and 1 minute and 30 seconds at 72°C (extension) and a final extension at 72°C for 1.5 minutes. Between four and eight positive clones were sequenced in both directions with M13 universal primers in an automated DNA sequencer. Sequences were assembled in Geneious R8 (Kearse et al. 2012) and are deposited in GenBank, accession numbers XXXX-XXXX (SI Table S1).

183

184 *Barcoding*

185 Genomic DNA (gDNA) was extracted from each eye tissue sample using the DNA layer in
186 Trizol of the RNA extraction, following the Trizol manufacturer's instructions and/or
187 from muscle tissue stored in ethanol using the Qiagen blood and tissue kit. We
188 generated mitochondrial *16s rRNA* 'barcodes' for most specimens (SI Table S1) using
189 universal primers (Palumbi, 1996) in 25 µl PCR reactions: 1x PCR buffer (Invitrogen), 1
190 mmol (mM) of MgCl₂ (Invitrogen), 50 µmol/L of deoxynucleotides (Bioline), 0.4 µmol/L
191 of each primer and 1 unit Platinum Taq Polymerase (Invitrogen) and 100ng of gDNA. The
192 PCR cycling parameters were: initial denaturation at 95°C for 10 minutes; 30 cycles of 15
193 seconds at 95°C (denaturation), 30 seconds at 55°C (annealing), and 1 minute at 72°C
194 (extension) and a final extension at 72°C for 1 minute. All successfully amplified
195 products were sequenced in both directions using the same primers used for PCR, in an
196 automated DNA sequencer. The barcodes were assembled in Geneious R8.

197

198 *Phylogenetic analysis*

199 Visual opsin gene cDNA sequences were aligned with published sequences from other
200 reptiles including other snakes (SI, Table S1) with MAFFT (Katoh et al. 2002) (settings:
201 algorithm; auto; gap penalty: 3; off-set value: 0.1) implemented in Geneious R8, Muscle
202 (Edgar, 2004) (default settings: 15 interactions), and PRANK (Löytynoja and Goldman,
203 2005) (HKY model with empirical base frequencies and kappa=2). These alignments
204 were inspected by eye for both nucleotides and amino acids and were adjusted
205 manually to ensure nucleotides were in-frame and that indels did not include partial
206 codons. The final alignments based on the results of all three programs were identical.
207 jModelTest 2 (Darriba et al. 2012) was used to ascertain the best-fit model of sequence
208 evolution for each alignment according to their AIC and BIC scores. GTR+G+I was the
209 best-fitting model for the three visual opsin genes amplified. Given concerns about
210 incorrectly estimating G when including I in the model (Yang, 2006), we also ran
211 analyses under GTR+G. Phylogenetic analyses were conducted using Maximum (ML)
212 Likelihood and Bayesian Inference (BI) approaches. ML analyses were run with RAxML
213 v8 (Stamatakis 2014) using majority rule bootstopping criteria (Pattengale et al. 2009);
214 randomized MP starting trees, and a fast hill-climbing algorithm. BI analyses were run
215 with Mr. Bayes v3.1.2 (Huelsenbeck and Ronquist 2001) for 1,000,000 generations with
216 chains sampled every 100 generations (after 25% of trees were discarded as burn-in),
217 random starting trees, 4 chains (3 hot and 1 cold), and convergence was assumed when
218 the standard deviation of split frequencies fell below 0.01. Gekkota was used as the

outgroup to root the *sws1* and *lws* trees, and other non-snake squamate visual opsin gene sequences were used to root the *rh1* tree (SI Table S1).

Analyses of molecular evolution

We used selection test analyses to identify patterns in visual opsin gene evolution across the snake evolutionary tree (using branch models) and within the individual visual opsin genes (site models). Codeml implemented in the PAML 4.7 package (Yang 2007) was used to estimate non-synonymous (dN) and synonymous (dS) substitution rates and the respective ratio (dN/dS, or ω) for the *sws1*, *lws* and *rh1* genes in snakes. Sequence alignment indels were removed if present in only one taxon or recoded as missing data if present in more.

Branch models (Yang 1998) allow the ω ratio to vary across branches in the tree and can be used to infer positive selection ($\omega > 1$) acting in particular lineages. The simplest branch model (one-ratio) allows only one ω ratio value across the tree, whereas the more complex free-ratio model assumes independent ω ratios for each branch. Branch models were also used to estimate ω for two branch categories based on ecotypes (primarily fossorial or not, aquatic/semiaquatic or not, primarily arboreal or not, primarily diurnal or not). The ecological classification applied to each species is reported in Fig. 1 and Table S2. Given the substantial diversity of retinal morphology, ecology and density of our sampling within the family, we also estimated two-ratio branches within Colubridae alone. All branch models were compared using the Likelihood Ratio Test (LRT) and the simpler model (one-ratio) was rejected where $p < 0.05$. Branch models were also carried out for a subset of taxa for which the photoreceptor cell complement is known (SI Table S2) to test for possible links between molecular evolution and the presence/absence of double cones or transmuted (sensu Walls 1934) rod-like cones. Retinal anatomy is not known for all species sampled so we removed such taxa from the data set for corresponding molecular evolution analyses and pruned them from the phylogeny in investigations of the relationship between opsin gene evolution and retinal morphology.

Site models (M1a nearly-neutral and M2a positive selection; M7 β and M8 β & ω) allow ω to vary among sites (amino-acids or codons) (Yang et al. 2000). Site models M2a and M8 were compared (using LRT) with the simpler site models M1a and M7, respectively and the simpler models rejected where $P > 0.05$. Bayes empirical Bayes (BEB) (Yang et al. 2005) implemented in models M2a and M8 β & ω was used to identify sites inferred to be under positive selection for each visual opsin gene.

Under branch-site models, ω can vary across both sites and lineages (Zhang 2005) and this was used to infer positive selection at sites among major lineages of snakes (Colubridae, snakes with transmutated, rod-like cones and snakes that are primarily fossorial, arboreal, aquatic/semiaquatic and diurnal). Branch-site models were compared with the simplest model M1a using LRT. Ancestral visual opsin gene sequences were estimated by marginal and joint reconstruction using Codeml.

We used PRIME analysis executed on the Datamonkey server (Delpont et al. 2010) to estimate amino acid exchangeability (as in BEB) but also radical substitutions that result in amino acids with very different biochemical properties. We used both sets of five amino-acid properties available in PRIME: Conant-Stadler (Conant et al. 2007) and Atchley et al. (Atchley et al. 2005). CMS (Delpont et al. 2010) was used to identify the most appropriate codon model for PRIME analysis.

For analyses of molecular evolution and ancestral state reconstruction we used a phylogenetic tree congruent with those published by (Wiens et al. 2012; Pyron et al. 2013; Reeder et al. 2015) (Species Tree, Figure 1). Although the monophyly of the colubrid clades Colubrinae, Natricinae and Dipsadinae are well supported (e.g., Wiens et al. 2012; Pyron et al. 2013), there is currently no compelling resolution of the relationships among them. Thus, as well as following the weakly supported resolution in the trees of (Pyron et al. 2013) (Colubrinae lying outside Natricinae+Dipsadinae), we accounted for phylogenetic uncertainty and repeated the branch and site model analyses for the two alternative phylogenetic resolutions: ((Dipsadinae, Colubrinae), Natricinae) and ((Natricinae, Colubrinae), Dipsadinae). The Indian snake *Xylophis captaini* or any congeners have not yet been included in molecular phylogenetic analyses. Although some workers have reported similarities between *Xylophis* and xenodermatids (e.g., McDowell 1987), we consider the similarity to the Sri Lankan *Aspidura* suggestive of phylogenetic affinity (e.g., Gans and Fetcho 1982; Gower and Winkler 2007) and so we include it as a correspondingly resolved natricine here.

Chi-squared tests of null hypotheses that sites inferred to be under positive selection do not occur unevenly among functional bipartitions (trans-membrane domains; extra- and intracellular loops) of opsins were conducted online at graphpad.com. These tests used one degree of freedom and expected values were calculated under the assumption that sites inferred to be under positive selection are distributed randomly between the functional bipartitions (i.e., in proportion to the total number of sites in each partition). A significance level of $p = 0.05$ was applied.

Estimating visual pigment $\lambda_{\max}$

It is possible, to some extent, to predict peak absorbance ($\lambda_{\max}$) of visual pigments from amino acid sequences of their constituent opsins. Such predictions are possible because correlations exist between amino acid sequences of opsins and $\lambda_{\max}$ of corresponding pigments where this has been measured directly in photoreceptors or where opsin genes have been cloned and pigments regenerated *in vitro*. We made predictions of $\lambda_{\max}$ by assuming a vitamin A1 chromophore (A2 chromophores have not been reported in snakes (Davies et al. 2009, Hart et al. 2012, Schott et al. 20152016, Sillman et al. 1997, Simões et al. 2015, 2016) and assessing combinations of amino acids at ‘spectral tuning’ sites known to be especially important in determining $\lambda_{\max}$ in other vertebrates (see Yokoyama 2008 and references cited therein). Predicting $\lambda_{\max}$ based on selected (spectral tuning) amino acid sites is somewhat controversial because additional tuning sites and different tuning mechanisms might remain undiscovered (Hauser et al. 2014). The limited MSP data published thus far for snake visual pigments generally match predictions based on known tuning sites in other vertebrates (e.g. Davies et al. 2009, Simões et al. 2015, 2016). However, we were unable to make confident $\lambda_{\max}$ predictions in cases in which we found spectral tuning amino acids (or combinations thereof) not reported in other vertebrates, or where they occur in other vertebrates but in pigments for which $\lambda_{\max}$ has not been measured.

Ocular media spectral transmission

We examined spectral transmission of lenses and spectacles. Corneas and humours were not scanned because, with the exception of some fish corneas (Kondrasiv et al. 1986; Douglas and McGuigan 1989; Siebeck and Marshall 2000), the vertebrate lens always removes more shortwave radiation than either the cornea or humours (Douglas and Marshall 1999; Douglas and Jeffery 2014). Lenses, and some spectacle samples were thawed and briefly rinsed in phosphate-buffered saline (PBS) and mounted in purpose-built holders in air in front of a Shimadzu ISR 260 integrating sphere within a Shimadzu UV-2101PC spectrophotometer. Transmission at 700 nm was set to 100% and ocular media scanned at 1 nm intervals from 300 to 700 nm. We averaged the measurements of both eyes unless we had only one usable spectacle scale or lens. The lenses were small, 1–3 mm diameter (SI Table S22), limiting the amount of light transmitted through the measuring system, and the use on an integrating sphere reduced sensitivity further, thus the raw data are noisy at short wavelengths where lamp output is low. Data from scans were therefore smoothed using a cubic Savitzky-Golay filter (data frame length 51nm) using Matlab R2011a (The MathWorks Inc, MA, USA). The 50% cut-off wavelength ($\lambda_{50\%}$), the wavelength at which transmission is 50%, was determined for each sample and rounded to the nearest integer. The proportion of UVA (315–400 nm) transmission was calculated for each lens and spectacle following (Douglas and Jeffery 2014). $\lambda_{50\%}$ and %UVA values were plotted for primarily diurnal and

nocturnal species using the package ggplots2 (Wickham 2010) implemented in R (Team 2014) (R Core Team, 2014).

Results

We sequenced approximately 1100bp of cDNA for each of the three visual opsin genes, *sws1*, *lws* and *rh1* found in 48 snake species. Almost the entire coding region for *sws1*, *lws* and *rh1* was amplified and sequenced in the vast majority of species newly sampled. We amplified *rh1* in *Boiga ceylonensis* and *Macroprotodon brevis* (based on single gel bands of approximate expected fragment size) and perhaps *Phyllorhynchus decurtatus* (occasional multiple gel bands in 10 PCRs with various primer and annealing temperature combinations) but sequencing failed. We failed to amplify *rh1* in *Malpolon monspessulanus*, *sws1* in *Pareas monticola*, *Boiga ceylonensis* and *B. forsteni*, and *lws* in *Melanophidium khairei* and *Pareas monticola*. In each case between four and fourteen PCRs were repeated using various combinations of primers and annealing temperatures. With one exception (*M. monspessulanus*), the lack of amplification in the latter cases occurred in samples in which the cDNA was temporarily dehydrated, so the failed PCRs may be an artefact. With the addition of the visual opsin gene cDNA sequences previously published for other snakes, the dataset includes 69 snake species covering most major lineages, and representing a broad range of ecologies and retinal anatomies. Spectral transmission was determined for lenses and spectacles of 18 and 15 snake species, respectively.

Functionality and spectral sensitivity

The residues present at amino acid sites of known functional importance for spectral tuning of the visual pigments are reported in SI Tables S3–5. Predictions of visual pigment $\lambda_{\max}$ values are based on the assumed presence of a vitamin A1-derived chromophore; an A2 chromophore is not known in snakes but has been reported for some lizards (e.g. Martin et al. 2015). For *rh1* sequences, substitutions N83D and A292S are widespread across snake evolutionary history with multiple independent origins and reversals in Colubridae, Elapidae and Lamprophiidae (SI Table S3). The ancestral snake is reconstructed as having an RH1-based pigment with predicted $\lambda_{\max}$ of 493 nm. However, the ancestral colubrine and colubrid *rh1* sequences encode a combination of N83 and S292 (seen in several extant colubroids: Fig. 1), For *lws* in *Heterodon nasicus*, an A308S substitution is observed; this substitution is also found in combination with A180 in the mouse and rat LWS sequences (Davies et al. 2012) and in a number of aquatic mammals

(Newman and Robinson, 2006). The introduction of a S308A substitution by site directed mutagenesis in mouse LWS produces a 20 nm long-wave shift (Davies et al. 2012), so the presence of S308 in *H. nasicus* produce a short-wave shift. Ancestors of most major snake lineages are reconstructed as having an LWS pigment $\lambda_{\max}$ of 555 nm, with shifts to shorter wavelength $\lambda_{\max}$ occurring independently on multiple occasions within Colubridae (Fig. 1, SI Table S5).

Phylogenetics

The inferred *rh1* and *sws1* trees (SI Figs. S6–S11) are broadly consistent with recently published snake phylogenies estimated using more neutral markers, irrespective of whether analyses were run using ML or BI or under GTR+G or GTR+G+I. Notable exceptions to relationships found in recent molecular phylogenies of snakes are the nesting of *Lampropeltis* within dipsadine rather than colubrine colubrids (*rh1*), monophyly of the Scolecophia (*rh1*), and non-monophyly of Anomalepididae (*rh1*) and Lamprophiidae (*rh1*, *sws1*). The *lws* tree is less well supported and lacks some monophyletic higher taxa (e.g., Dipsadinae, Natricinae, Colubrinae, Colubroidea) present in the *rh1* and *sws1* trees. *Xylophis* (not sampled in molecular snake phylogenies) is recovered variably as closely related to some natricines (*lws*) or lying outside most colubroids (*rh1*, *sws1*).

Adaptive molecular evolution

Values for dN/dS (ω) (SI Table S12) suggest that all three visual opsin genes are under purifying selection (ω_{Rh1} = 0.237; ω_{SWS1} = 0.107; ω_{LWS} = 0.312), indicative of strong functional constraint (Li et al. 1985). Additional tests performed with alternative phylogenetic relationships among dipsadine, colubrine and natricine colubrids yield ω estimates that are not significantly different (data not shown), indicating that these results are robust with respect to this phylogenetic uncertainty.

For the *sws1* opsin gene, branch models (SI Table S12) suggest that non-fossorial (0.112), non-arboreal (0.08), non-aquatic (0.308) and diurnal (0.149) snake lineages have higher ω values than their counterparts (0.062, 0.097, 0.092 and 0.099, respectively). Colubrids have higher ω values (0.119) than non-colubrids (0.088). In the dataset pruned to species for which retinal anatomy is known, ω values are similar between taxa with (0.117) and without (0.101) transmuted cones, and lower in species with doubles cones (0.097 vs 0.110). The free-ratio model ω values vary between 0.001 and 0.409.

For the *lws* opsin gene ω values are higher in non-fossorial (0.340), arboreal (0.398), non-aquatic (0.308) and diurnal snakes (0.342) than their counterparts (0.016, 0.284, 0.268 and 0.246, respectively). Colubrids have significantly higher ω values (0.422) than non-colubrids (0.193). Higher ω occur in species with transmuted cones (0.543 vs. 0.273) and in species with double cones (0.373 vs 0.245). The free-ratio ω ranges from 0.001–7.1 (SI Table S12).

The *rh1* ω ratios among non-fossorial (0.252), non-arboreal (0.240), aquatic/semiaquatic (0.253) and nocturnal (0.240) snake lineages are higher than for their counterparts (0.161, 0.212, 0.0.229 and 0.141, respectively). Colubrids have higher (0.252) ω ratios than non-colubrids (0.212) whereas ω ratios are lower for the thoroughly fossorial Scolecophidia (0.141) than their sister group Alethinophidia (0.244). The *rh1* opsin gene is inferred to be under less functional constraint in snakes with transmuted, rod-like cones (0.388 vs. 0.212) and in snakes with double cones (0.283 vs 0.190) (SI Table S12).

The free-ratio ω ranges from 0.001–1.73, suggesting positive selection ($\omega = 1.44$) in the Colubridae stem. With branch models, for all opsin genes, separate values for each of the contrasted ecologies and retinal types are a significantly better fit than a single ω value for all snakes when compared by LRT (SI Table S12).

Site models results infer several instances of positive selection at the codon level across the three visual opsin genes present in snakes (SI Table S13). Models 2a and M8 ($\beta&\omega$) are significant better fit when compared with the simpler models M1a and M7, respectively (SI Table S13)

According to Bayes Empirical Bayes (BEB) implemented in site models M2a and M8 ($\beta&\omega$) there are two and seven *sws1* amino acid sites that can be inferred to be under positive selection, respectively (SI Table S14). With M8 ($\beta&\omega$), two of these seven sites (86 and 93) are known to have a substantial impact on SWS1 spectral tuning, and five of the sites are located in trans-membrane (TM) domains (Fig. 2). In *lws*, BEB results infer 12 and 18 amino acid sites under positive selection under models M2a and M8 ($\beta&\omega$), respectively. Among the 18, two are involved in LWS spectral tuning and two others are located within the retinal pocket (Fig. 2). A total of 15 of the 18 inferred positively selected sites are located in TM domains, particularly TM 3, 4 and 5 (11 sites). In *rh1*, positive selection is inferred in 11 and 16 amino-acid sites according to M2a and M8 ($\beta&\omega$) models, respectively. Under model M8 ($\beta&\omega$), BEB results infer positive selection in spectral sites 83 and 292 and in two sites within the retinal pocket (Fig. 2, SI Table S14). The majority of the *rh1* amino acids inferred to be under positive selection are located in TM domains, especially TM 3, 4, 5 and 7 (Fig. 2). For the results of both M2a and M8 ($\beta&\omega$) models, chi-squared tests rejected the null that inferred positively

selected sites are not located within TMs versus loops more than expected for RH1 and LWS but not for SWS1. Pooling all visual opsins, chi squared-tests also rejected the null hypothesis that inferred positively selected sites are not located within extracellular versus intracellular loops more than expected.

Using PRIME (SI Table S16-S21), positive selection is inferred at amino acid sites at which substitutions with changes in biochemical properties occurred. Among these sites are spectral tuning sites 86 in *sws1* and 180 in *lws*, and amino acid sites situated within the retinal pocket in *lws* and *rh1* (Fig 2).

Ocular media transmission

The sampled snakes have lenses with a broad range of transmission properties at short wavelengths, ranging from those that filter out all of the UV and even some of blue (the lenses thus appearing yellow) to those that transmit most of the UVA (Fig 3A: SI Table S22). All spectacles transmitted the UVA well (Fig 3B), corroborating recent work on 42 snake species by van Doorn and Sivak (2015). All nocturnal species have very UVA transmissive lenses, while all species with lenses that cut out shorter wavelengths to varying degrees are diurnal (Fig 3C). However, not all snakes with some diurnal activity have UV-blocking lenses.

Discussion

Walls (1934; 1942) and Underwood (1967) documented extensive diversity in retinal anatomy among snakes. Our results demonstrate that snakes also display remarkable diversity in spectral transmission of the lens and variability in visual opsin gene sequences and visual pigment spectral sensitivity that together point to an evolutionarily complex system.

It has been argued that snakes passed through a nocturnal and/or fossorial stage early in their evolutionary history, with some associated diminution of their visual systems (see Simões et al. 2015) followed by possible re-elaboration in ‘higher’ snakes (Alethinophidia), including substantial diversification in retinal photoreceptor complements, at least at a morphological level (Walls 1942; Underwood 1967). The results presented here indicate that the complement of visual pigments, in contrast, has remained largely stable through notable evolutionary events such as the acquisition of double cones, the loss of classes of single cone (and perhaps rods), and the transmutations of both rods and cones.

The vast majority of species surveyed express the same three (*rh1*, *sws1*, *lws*) visual opsin genes that were likely to have been present in the ancestral snake (Davies et al. 2009; Simões et al. 2015). A striking feature is however the absence of *rh1* in the *Malpolon monspessulanus*. Given the good quality of the template cDNA available for this species, this is unlikely to be a PCR artefact. *Malpolon monspessulanus*, a highly diurnal species, is reported to have only cones (Underwood 1967; Underwood 1970) and a previous microspectrophotometric (MSP) study (Govardovskii and Chkheidze 1989) failed to find any visual pigments with a $\lambda_{\max}$ close to the c. 500 nm expected for RH1 pigments (typically occurring in rods). The presence of *rh1* in two other colubrids, *Phyllorhynchus decurtatus* and *Macroprotodon brevis* remains unconfirmed. *Phyllorhynchus decurtatus* is nocturnal but its ‘rods’ have been argued to be transmuted (rod-like) cones (Walls 1934), consistent therefore with the lack of RH1. Among vertebrates, absence of an expressed *rh1* has previously been reported only in another group of squamate reptiles, geckos (e.g. Loew et al. 1996; Yokoyama and Blow 2001). More work examining the physiology of visual pigments and gene expression will be required to test this further for snakes. Among those snakes with three functional visual opsin genes is *Uropeltis* cf. *macrolepis*. Like all uropeltids, this is a mostly fossorial species, though it is more likely to be seen above ground during daylight (D.J.G., pers. obs.) and has a larger eye than the distantly related, but also burrowing, scolecophidians and *Anilius scytale*, for which Simões et al. (2015) failed to amplify either *sws1* or *lws*. The presence of *sws1* and *lws* in *U. cf. macrolepis* adds support to Simões et al. (2015) conclusion that loss of all visual opsins except *rh1* has occurred in snakes in only the most dedicated of burrowers, and that if the ancestral snake was a burrower it was likely not as fossorial as living scolecophidians.

We predict that in snakes where the ocular media filter out most of the UVA (Fig 3A&B, SI Table S22), the SWS1 pigment $\lambda_{\max}$ is long-wave shifted. However, the *sws1* sequences of these species include previously unreported amino acid residues at some key tuning sites and direct measurements of visual pigment absorbance (e.g. by MSP) are currently lacking. Nevertheless, evidence from other studies (Carvalho et al. 2012; Cowing et al. 2002; Hunt and Peichl, 2014; Parry et al. 2004; Yokoyama et al. 2005) suggests that the replacement of is sufficient to shift the $\lambda_{\max}$ from UV to violet. only six of the 60 snakes listed in Table S4 may have lost a UVS SWS1 pigment. Removal of UV has been linked to increased acuity rather than an adaptation underpinning a particular form of colour vision or protection from harmful UV light (Douglas and Jeffery 2014). This hypothesis receives support here because the snakes with the least transparent lenses are highly visual hunters. These include a gliding species (*Chrysopelea ornata*) known to track distant objects (Socha and Sidor 2005) and a taxon (*Ahaetulla*) with horizontal pupils, binocular vision and a fovea (Walls 1942). The latter structure is

known from very few snakes (Rasmussen 1990) and is indicative of high visual acuity in a specialised area of the retina.

Based on ancestral state reconstruction for the *sws1* gene (and predictions of $\lambda_{\max}$), the most recent common ancestor of living snakes was UV sensitive, and UV vision is also predicted to be present in many nocturnal caenophidians, matching the situation in other vertebrate groups (e.g., Veilleux and Cummings 2012) in which nocturnality is associated with UV sensitivity. Although there is evidence of a substantial amount of evolutionary change in snake *sws1* sequences, it is not possible to predict the $\lambda_{\max}$ of the SWS1-based visual pigments in 42 of the 63 species for which sequences are available (many of these species are not primarily nocturnal) because of tuning site amino acid substitutions (or combinations of substitutions) not known in other vertebrates. It is very likely (based on lens transmission) that at least some of these species have substantially long-wave shifted SWS1-based visual pigments. Hart et al. (Hart et al. 2012) found (using MSP) that the probable SWS1-based pigments in two sea snakes is not maximally sensitive in the UV, with $\lambda_{\max}$ of c. 429nm. Although many snakes have previously unknown *sws1* tuning site substitutions, there is evidence that some sequences discovered here produce substantial changes in SWS1 $\lambda_{\max}$. Mutations at site 86 are known to cause major shifts in SWS1 $\lambda_{\max}$, with F86Y (Fasick and Robinson 1998; Cowing et al. 2002) and F86S (Shi et al. 2001) short-wave shifting $\lambda_{\max}$ by 66 and 51 nm (Yokoyama 2005) respectively, and the latter mutation is observed in the snakes *Malpolon monspessulanus* and *Pantherophis guttatus*. In *Ahaetulla nasuta*, *Chrysopelea ornata*, *Helicops angulatus* and *Chironius* spp. F86V is observed. The guinea pig has a 86V substitution and an SWS1 $\lambda_{\max}$ of 420nm and, furthermore, the V86F substitution produces one of the most substantial shifts towards the UV with a decrease of 53nm in the SWS1 pigment $\lambda_{\max}$ (Parry et al. 2004). Given the filtering out of UV light by the lens a short-wave shifted $\lambda_{\max}$ would seem very unlikely, otherwise SWS1 would not function as an efficient visual pigment in these snakes.

In *Helicops angulatus*, cloning the *sws1* gene revealed polymorphism at site 86 with either valine or phenylalanine. The exact change in spectral tuning is not known, but, speculatively, this polymorphism indicates that pigments with spectral peaks in the UV and violet may be present simultaneously and potentially may therefore provide the basis for a form of trichromacy. This would be similar to the form of trichromacy in polymorphic female platyrrhine monkeys (Jacobs et al. 2002), if some random allele inactivation is present that ensures only one allele is expressed per photoreceptor. Alternatively, both alleles in *H. angulatus* may be fully active to give a broader spectrum of sensitivity.

In contrast to *rh1* and *sws1*, the *lws* spectral sites in snakes are identical to those known in other vertebrates, with the exception of the A308S substitution unique to the colubrine *Heterodon nasicus*. Variation in the amino acid residues at LWS spectral sites in snakes suggests multiple LWS $\lambda_{\max}$ shifts between long (555-560nm) and medium wavelengths (536nm) within Caenophidia. Substitutions are particularly common in Dipsadinae and Colubrinae, with most shifts to predicted shorter wavelength $\lambda_{\max}$ values occurring in nocturnal taxa, thereby providing a possible adaptation to maximize photon capture and potentially colour vision in low light conditions. In a study of forest mammals, Veilleux and Cummings (2012) found that SWS spectral tuning appeared to be strongly associated with foraging target and LWS tuning to dominant light field characteristics. Although the shorter wavelength shifted LWS $\lambda_{\max}$ values of nocturnal snakes match this, we are unable to address whether snake SWS1 is more tuned to foraging targets because SWS1 $\lambda_{\max}$ is not known for most snakes (see above), dietary classification is non-trivial, and many snakes are probably primarily using olfaction rather than visual clues to detect prey

The results of our analyses of positive selection in snake visual opsins are notable on two counts. Firstly, unlike some other studies of vertebrate visual opsins (e.g. Yokoyama et al. 2008) we infer multiple sites as under positive selection in all three visual opsins and some of these occur in sites of known functional importance, including known spectral tuning sites. This is consistent with the interpretation that the tuning of snake LWS pigments is influenced by positive selection at sites known to be important in effecting tuning variation in many other vertebrate groups (Hunt and Collin 2014). Secondly, shifts in the molecular evolution (functional constraint) of the visual pigment genes are correlated with many variables, including ecological niche characteristics and retinal anatomy. That the inferred functional constraint is lower in all visual opsin genes in snakes with transmuted, rod-like cones is an important observation indicating that visual pigment adaptation occurs in association with morphological transmutation of photoreceptors — an incompletely understood process with poorly known functional outcomes (Simões et al. 2016). Although we found evidence for less functional constraint in the evolution of *rh1* and *lws* (but not *sws1*) in lineages with double cones, this is difficult to interpret because the function of double cones remains largely unknown (e.g. Pignatelli et al. 2010).

Of the three visual opsins found in snakes, *sws1* has fewer amino acid sites inferred to be under positive selection, consistent with higher purifying selection estimates on branch models (SI Table S12) and possibly indicating possibly greater purifying selection than in *lws* and *rh1*. This is consistent with the relatively few tuning sites identified in SWS1 opsins. Indeed, two of the seven *sws1* sites inferred to be under

positive selection are the spectral tuning sites 86 and 93 known to impart substantial $\lambda_{\max}$ shifts (Fasick and Robinson 1998; Shi et al. 2001; Yokoyama 2005), suggestive of at least some localized positive selection on sites of functional importance. Similarly, *lws*, sites 180 and 285 and *rh1* sites 83 and 292 are inferred to be under positive selection and also mediate important changes in $\lambda_{\max}$ of their respective pigments (see above). Thus, some of the evolution of snake visual opsins inferred here is interpreted as likely adaptive change related to spectral tuning of pigments. Colour vision has yet to be demonstrated behaviourally in snakes, but our results suggest it is almost certainly an important part of their sensory biology, especially for many caenophidians. The visual pigment complement of most snakes, comprising RH1, SWS1 and LWS based pigments, is strongly suggestive of photopic cone dichromacy and scotopic monochromacy as found in most mammals. However, there remains the possibility of trichromacy, either by the involvement of transmuted cone-like rods in the case of diurnal species (Schott et al. 2016), or by the use of transmuted rod-like cones in nocturnal species (as occurs in geckos: Roth & Kelber 2004). As with the observed polymorphism of *sws1* found in *Helicops angulatus*, further studies are required to elucidate these possibilities and the consequences of different visual pigment complements in snake colour vision.

Most of the amino acid sites inferred to be under positive selection in the three visual opsin genes found in snakes are in transmembrane domains (Fig. 3), and most observed changes at these sites are non-conservative in terms of amino acid properties (Fig. 2, SI Table S13). Transmembrane domains impact the tertiary structure, thermal stability (Kobilka 2007) and aspects of the retinal binding pocket (Yokoyama et al. 2006) of the opsin, such that positive selection at these sites is likely to have major influence on opsin function. Change in spectral tuning is only one of the possible functional outcomes of visual opsin amino acid substitutions - there is more to visual pigments than spectral absorption - and these other aspects of visual sensory transduction will need to be part of the future investigations of this system.

Conclusion

Based on surveys of retinal anatomy, the eyes of snakes have been cited as one of the most interesting cases of visual adaptation among vertebrates (Walls 1942; Underwood 1967), but they remained overlooked during the revolution in molecular analyses of visual pigment genes. Our results show that in addition to the substantial anatomical diversity, snakes also have notable diversity in their lens transmission and visual opsin genes, including diversity not known in other vertebrates, and these aspects of snake vision are shown to have undergone considerable evolution. Snake visual opsin

genes contain signals of positive selection in sites of functional importance that are (perhaps causally) associated with shifts in ecology and retinal anatomy. We conclude that the diversity, function and evolution of snake vision are worthy of additional research, and that understanding of vertebrate vision is incomplete without a consideration of snakes.

Acknowledgements

This work was funded by a grant from the Leverhulme Trust (RPG-342 to DJG, NSH, DMH & JCP). Permits for research and export were granted by Direction de l'Environnement de l'Aménagement et du Logement and the Direction des Services Vétérinaires de la Guyane, Cayenne, French Guiana, and by the Ministry of Forestry & Wildlife of Cameroon. For assistance in the field, we thank Gabriela Bittencourt-Silva, Antoine Fouquet, Philippe Gaucher, Jeannot and Odette (Camp Patawa) and Mark Wilkinson. Additional practical assistance with obtaining and processing samples, and with literature and analysis was provided by Christian Cox, Vivek Philip Cyriac, David Donaire, Robert Fisher, Varad Giri, Rachunliu G. Kamei, Panagiotis Kornilios, Marcel Kouete, Dileep Kumar, Michelle Lawing, Mruganka Rahul Lele, Gopal Murali, Jesse Meik, Simon Maddock, Bartosz Nadol, David Richards, Gill Sparrow, Jeff Streicher, Colin Strine, Ed Wade, Chris Williamson, Mark Wilkinson, and the NHM Sequencing Facility, and we thank them all. Chris Hull provided help with the smoothing of the ocular media transmission data.

References

- Atchley WR, Zhao J, Fernandes AD, Drüke T. 2005. Solving the protein sequence metric problem. *Proc Natl Acad Sci. USA*. 102:6395–6400.
- Bowmaker J, Hunt D. 2006. Evolution of vertebrate visual pigments. *Curr Biol*. 16:R484–R489.
- Carleton KL, Hofmann CM, Klisz C, Patel Z, Chircus LM, Simenauer LH, Soodoo N, Albertson RC, Ser JR. 2010. Genetic basis of differential opsin gene expression in cichlid fishes. *J Evol Biol*. 23:840–853.
- Conant GC, Wagner GP, Stadler PF. 2007. Modeling amino acid substitution patterns in orthologous and paralogous genes. *Mol Phyl and Evol*. 42:298–307.
- Cowing JA, Poopalasundaram S, Wilkie SE, Robinson PR, Bowmaker JK, Hunt DM. 2002. The molecular mechanism for the spectral shifts between vertebrate ultraviolet- and violet-sensitive cone visual pigments. *Biochem J*. 367:129.
- Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new

653 heuristics and parallel computing. *Nat Methods*. 9:772.

654 Davies WIL, Collin SP, Hunt DM. 2012. Molecular ecology and adaptation of visual
655 photopigments in craniates. *Mol Ecol*. 21:3121–3158.

656 Davies WL, Cowing JA, Bowmaker JK, Carvalho LS, Gower DJ, Hunt DM. 2009. Shedding
657 Light on Serpent Sight: The Visual Pigments of Henophidian Snakes. *J Neurosci*. 29:7519–
658 7525.

659 Delport W, Poon AFY, Frost SDW, Kosakovsky Pond SL. 2010. Datamonkey 2010: a suite
660 of phylogenetic analysis tools for evolutionary biology. *Bioinformatics*. 26:2455–2457.

661 Delport W, Scheffler K, Botha G, Gravenor MB, Muse SV, Kosakovsky Pond SL. 2010.
662 CodonTest: Modeling Amino Acid Substitution Preferences in Coding Sequences. *PLoS*
663 *Comput Biol*. 6:e1000885.

664 Douglas RH, Jeffery G. 2014. The spectral transmission of ocular media suggests
665 ultraviolet sensitivity is widespread among mammals. *Proc R Soc. B* 281:20132995–
666 20132995.

667 Douglas RH, Marshall NJ. 1999. A review of vertebrate and invertebrate ocular filters. In:
668 Adaptive Mechanisms in the Ecology of Vision. Dordrecht: Springer Netherlands. pp. 95–
669 162.

670 Douglas RH, McGuigan CM. 1989. The spectral transmission of freshwater teleost ocular
671 media – an interspecific comparison and a guide to potential ultraviolet sensitivity.
672 *Vision Res*. 29:871–879.

673 Dulai K, Dornum M, Mollon J, Hunt D. 1999. The Evolution of Trichromatic Color Vision
674 by Opsin Gene Duplication in New World and Old World Primates. *Genome Res*. 9:629–
675 638.

676 Edgar, RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high
677 throughput. *Nucleic Acids Res*. 32: 1792–1797.

678 Enright JM, Toomey MB, Sato S-Y, Temple SE, Allen JR, Fujiwara R, Kramlinger VM, Nagy
679 LD, Johnson KM, Xiao Y, et al. 2015. Cyp27c1 Red-Shifts the Spectral Sensitivity of
680 Photoreceptors by Converting Vitamin A1 into A2. *Curr Biol*. 25: 1-10.

681 Fasick JJ, Robinson PR. 1998. Mechanism of spectral tuning in the dolphin visual
682 pigments. *Biochemistry* 37:433–438.

683 Gans C, Fetcho JR. 1982. The Sri Lankan genus *Aspidura* (Serpentes, reptilia, Colubridae).
684 *Annals of Carnegie Museum* 51(14): 271–316.

685 Govardovskii VI, Chkheidze NI. 1989. Retinal photoreceptors and visual pigments in

686 certain snakes. *Biol Abstr.* 90:1036.
 687 Gower DJ, Winkler JD. 2007. Taxonomy of the Indian snake *Xylophis Beddome*
 688 (Serpentes: Caenophidia), with description of a new species. *Hamadryad* 31:315–329.
 689 Hart NS, Coimbra JP, Collin SP, Westhoff G. 2012. Photoreceptor types, visual pigments,
 690 and topographic specializations in the retinas of hydrophiid sea snakes. *J Comp Neurol.*
 691 520:1246–1261.
 692 Hauser FE, van Hazel I, Chang BSW. 2014. Spectral tuning in vertebrate short
 693 wavelength-sensitive 1 (SWS1) visual pigments: Can wavelength sensitivity be inferred
 694 from sequence data? *J Exp Zool B Mol Dev Evol.* 322:529–539.
 695 Hofmann CM, Carleton KL. 2009. Gene Duplication and Differential Gene Expression Play
 696 an Important Role in the Diversification of Visual Pigments in Fish. *Integr Comp Biol.*
 697 49:630.
 698 Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees.
 699 *Bioinformatics* 17:754–755.
 700 Hunt DM, Collin SP. 2014. Evolution of Visual and Non-visual Pigments. In: Hunt DM,
 701 Hankins MW, Collin SP, Marshall NJ, editors. *Evolution of Visual and Non-visual*
 702 *Pigments.* Boston, MA: Springer. p. 276.
 703 Hunt DM, Dulai KS, Partridge JC, Cottrill P, Bowmaker JK. 2001. The molecular basis for
 704 spectral tuning of rod visual pigments in deep-sea fish. *J Exp Biol.* 204:3333–3344.
 705 Jacobs G, II J, Tan Y, Li W-H. 2002. Opsin gene and photopigment polymorphism in a
 706 prosimian primate. *Vision Res.* 42:11–18.
 707 Janz JM, Farrens DL. 2001. Engineering a Functional Blue-Wavelength-Shifted Rhodopsin
 708 Mutant†. *Biochemistry* 40:7219–7227.
 709 Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple
 710 sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30:3059–3066.
 711 Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A,
 712 Markowitz S, Duran C, et al. 2012. Geneious Basic: an integrated and extendable
 713 desktop software platform for the organization and analysis of sequence data.
 714 *Bioinformatics* 28:1647–1649.
 715 Kobilka BK. 2007. G protein coupled receptor structure and activation. *BBA -*
 716 *Biomembranes* 1768:794–807.
 717 Kondrasiv SL, Gamburzena AG, Gnjbkina VP, Orlov OJ, My PT. 1986. Coloration of
 718 corneas in fish. A list of species. *Vision Res.* 26:287–290.

719 Land MF. 1981. Optics and vision in invertebrates. In: Autrum HJ, editor. Handbook of
720 Sensory Physiology. Vol. VII/6B. Berlin. pp. 471–592.

721 Li WH, Wu CI, Luo CC. 1985. A new method for estimating synonymous and
722 nonsynonymous rates of nucleotide substitution considering the relative likelihood of
723 nucleotide and codon changes. *Mol Biol Evol.* 2:150–174.

724 Loew ER, Govardovskii VI, Rohlich P, Szél A. 1996. Microspectrophotometric and
725 immunocytochemical identification of ultraviolet photoreceptors in geckos. *Vis*
726 *Neurosci.* 13:247–256.

727 Löytynoja A & Goldman N. 2005. An algorithm for progressive multiple alignment of
728 sequences with insertions. *Proc Nat Acad Sci USA.* 102(30): 10557–10562.

729 Martin M, Le Galliard J-F, Meylan S, Loew ER. 2015. The importance of ultraviolet and
730 near-infrared sensitivity for visual discrimination in two species of lacertid lizards. *J Exp*
731 *Biol.* 218:458–465.

732 McDowell SB. 1987. Systematics. In: Seigel RA, Collins JT, Novak SS, editors. Snakes:
733 Ecology and Evolutionary Biology. New York. MacMillan pp. 3–50.

734 Nathans J. 1990. Determinants of Visual Pigment Absorbance: Role of Charged Amino
735 Acids in the Putative Transmembrane Segments. *Biochemistry* 29:937.

736 Nickle B, Robinson PR. 2007. The opsins of the vertebrate retina: insights from
737 structural, biochemical, and evolutionary studies. *Cell Mol Life Sci.* 64:2917–2932.

738 Nilsson DE. 1996. Eye ancestry: old genes for new eyes. *Curr Biol.* 6:39–42.

739 Parry J, Poopalasundaram S, Bowmaker J, Hunt D. 2004. A Novel Amino Acid
740 Substitution Is Responsible for Spectral Tuning in a Rodent Violet-Sensitive Visual
741 Pigment. *Biochemistry* 43:8014–8020.

742 Pattengale ND, Alipour M, Bininda-Emonds ORP, Moret BME, Stamatakis A. 2009. How
743 Many Bootstrap Replicates Are Necessary? In: Springer-Verlag. pp. 184–200.

744 Pignatelli V, Champ C, Marshall J, Vorobyev M. 2010. Double cones are used for colour
745 discrimination in the reef fish, *Rhinecanthus aculeatus*. *Biol Lett.* 6:537–539.

746 Pyron R, Burbrink FT, Wiens JJ. 2013. A phylogeny and revised classification of
747 Squamata, including 4161 species of lizards and snakes. *BMC Evol Biol.* 13:93.

748 Rasmussen JB. 1990. The retina of *Psammodynastes pulverulentus* (Boie, 1827) and
749 *Telescopus fallax* (Fleischmann, 1831) with a discussion of their phylogenetic
750 significance (Colubroidea, Serpentes). *J Zool Syst Evolut.* 28:269–276.

751 Reeder TW, Townsend TM, Mulcahy DG, Noonan BP, Wood PL, Sites JW, Wiens JJ. 2015.
 752 Integrated Analyses Resolve Conflicts over Squamate Reptile Phylogeny and Reveal
 753 Unexpected Placements for Fossil Taxa. *PLoS ONE* 10:e0118199.

754 Schott RK, Müller J, Yang CGY, Bhattacharyya N, Chan N, Xu M, et al. . Evolutionary
 755 transformation of rod photoreceptors in the all-cone retina of a diurnal garter snake.
 756 *Proc Nat Acad Sci USA*. 201513284.

757 Shi Y, Radlwimmer FB, Yokoyama S. 2001. Molecular genetics and the evolution of
 758 ultraviolet vision in vertebrates. *Proc Natl Acad Sci USA*. 98:11731–11736.

759 Siebeck UE, Marshall NJ. 2000. Transmission of ocular media in labrid fishes. *Phil. Trans.*
 760 *Royal Soc B: Biol Sc*. 355:1257–1261.

761 Sillman AJ, Govardovskii VI, Rohlich P, Southard JA, Loew ER. 1997. The photoreceptors
 762 and visual pigments of the garter snake (*Thamnophis sirtalis*): a
 763 microspectrophotometric, scanning electron microscopic and immunocytochemical
 764 study. *J Comp Physiol A* 181:89.

765 Simões BF, Sampaio FL, Jared C, Antoniazzi MM, Loew ER, Bowmaker JK, Rodriguez A,
 766 Hart NS, Hunt DM, Partridge JC, et al. 2015. Visual system evolution and the nature of
 767 the ancestral snake. *J Evol Biol*. 28:1309–1320.

768 Simões, B. F., Sampaio, F. L., Loew, E. R., Sanders, K. L., Fisher, R. N., Hart, N. S., et al.
 769 2016. Multiple rod-cone and cone-rod photoreceptor transmutations in snakes:
 770 evidence from visual opsin gene expression. *Proc R Soc. B* 283: 1823.

771 Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis
 772 of large phylogenies. *Bioinformatics* 30:1312–1313.

773 Team RC. 2014. R: A language and environment for statistical computing. Vienna,
 774 Austria: R Foundation for Statistical Computing

775 Thorpe A. 1991. Spectral transmission and short-wave absorbing pigments in the
 776 lenses of fish and other animals. City University, London.

777 Underwood G. 1967. A Contribution to the Classification of Snakes. London: British
 778 Museum Natural History Publications

779 Underwood G. 1970. The Eye. In: Gans C, Parsons TS, editors. *Biology of the Reptilia:*
 780 *Morphology B*. Vol. 2. New York: Academic Press. pp. 1–97.

781 van Doorn K, Sivak JG. 2015. Spectral transmittance of the spectacle scale of snakes and
 782 geckos. *Contrib Zool*. 8:1–12.

783 Veilleux CC, Cummings ME. 2012. Nocturnal light environments and species ecology:

784 implications for nocturnal color vision in forests. *J Exp Biol.* 215:4085–4096.

785 Wald G. 1968. The molecular basis of visual excitation. *Nature.* 219:800–807.

786 Walls GL. 1931. The Occurrence of Colored Lenses in the Eyes of Snakes and Squirrels,
787 and Their Probable Significance. *Copeia* 1931:125–127.

788 Walls GL. 1934. The reptilian retina. I. A new concept of visual cell evolution. *Am J*
789 *Ophthalmol.* 17:892–915.

790 Walls GL. 1942. The Vertebrate Eye and Its Adaptive Radiation. New York: Fafner
791 Publishing Company

792 Wickham H. 2010. ggplot2. New York, NY: Springer

793 Wiens JJ, Hutter CR, Mulcahy DG, Noonan BP, Townsend TM, Sites JW, Reeder TW.
794 2012. Resolving the phylogeny of lizards and snakes (Squamata) with extensive sampling
795 of genes and species. *Biol Lett.* 8:1043–1046.

796 Yang Z. 1998. Likelihood ratio tests for detecting positive selection and application to
797 primate lysozyme evolution. *Mol Biol Evol.* 15:568–573.

798 Yang, Z. 2006. Computational molecular evolution. Oxford University Press, USA.

799 Yang Z. 2007. PAML 4: Phylogenetic Analysis by Maximum Likelihood. *Mol Biol Evol.*
800 24:1586–1591.

801 Yang ZH, Nielsen R, Goldman N, Pedersen A. 2000. Codon-substitution models for
802 heterogeneous selection pressure at amino acid sites. *Genetics* 155:431–449.

803 Yang ZH, Wong W, Nielsen R. 2005. Bayes Empirical Bayes Inference of Amino Acid Sites
804 Under Positive Selection. *Mol Biol Evol.* 22:1107.

805 Yokoyama S, Blow NS. 2001. Molecular evolution of the cone visual pigments in the pure
806 rod-retina of the nocturnal gecko, *Gekko gekko*. *Gene* 276:117–125.

807 Yokoyama S, Starmer WT, Takahashi Y, Tada T. 2006. Tertiary structure and spectral
808 tuning of UV and violet pigments in vertebrates. *Gene* 365:95–103.

809 Yokoyama S, Tada T, Zhang H, Britt L. 2008. Elucidation of phenotypic adaptations:
810 Molecular analyses of dim-light vision proteins in vertebrates. *Proc Natl Acad Sci USA.*
811 105:13480–13485.

812 Yokoyama S, Xing J, Liu Y, Faggionato D, Altun A, Starmer WT. 2014. Epistatic Adaptive
813 Evolution of Human Color Vision. *PLoS Genet.* 10:e1004884.

814 Yokoyama S. 2005. Elephants and Human Color-Blind Deuteranopes Have Identical Sets

815 of Visual Pigments. *Genetics* 170:335–344.

816 Yokoyama S. 2008. Evolution of Dim-Light and Color Vision Pigments. *Annu Rev*
817 *Genomics Hum Genet.* 9:259–282.

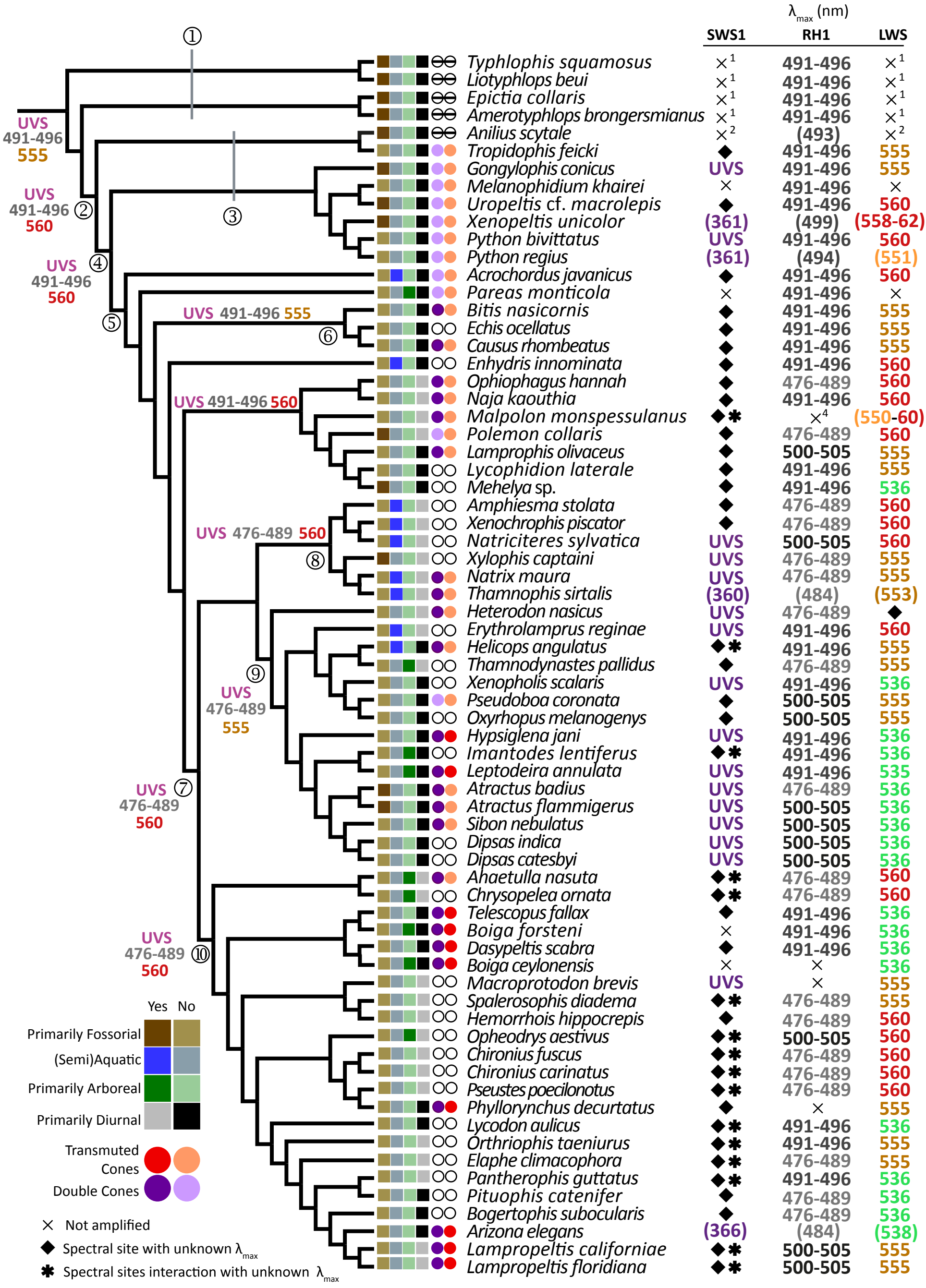
818 Zhang J. 2005. Evaluation of an Improved Branch-Site Likelihood Method for Detecting
819 Positive Selection at the Molecular Level. *Mol Biol Evol.* 22:2472–2479.

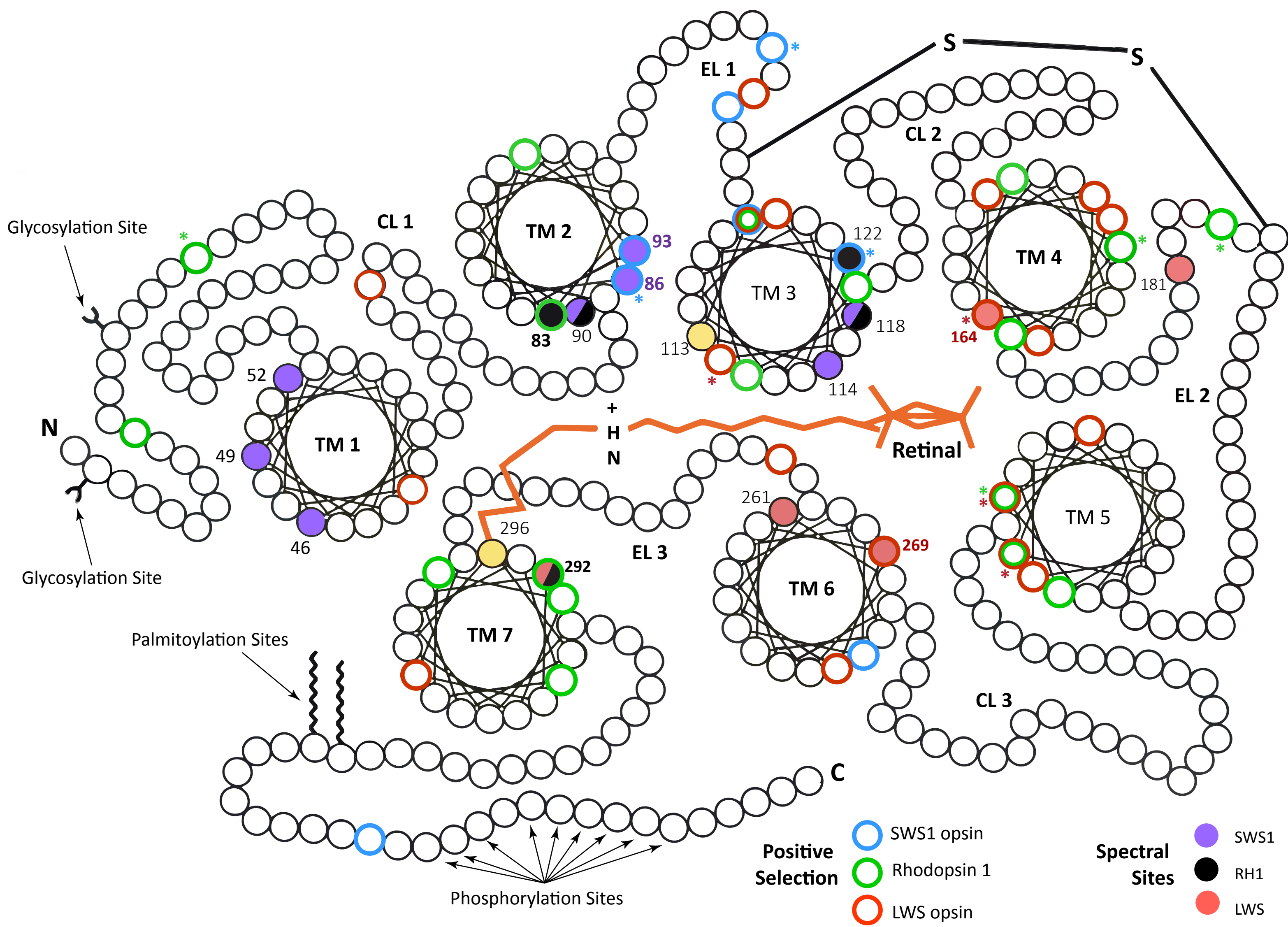
820

821

822

823





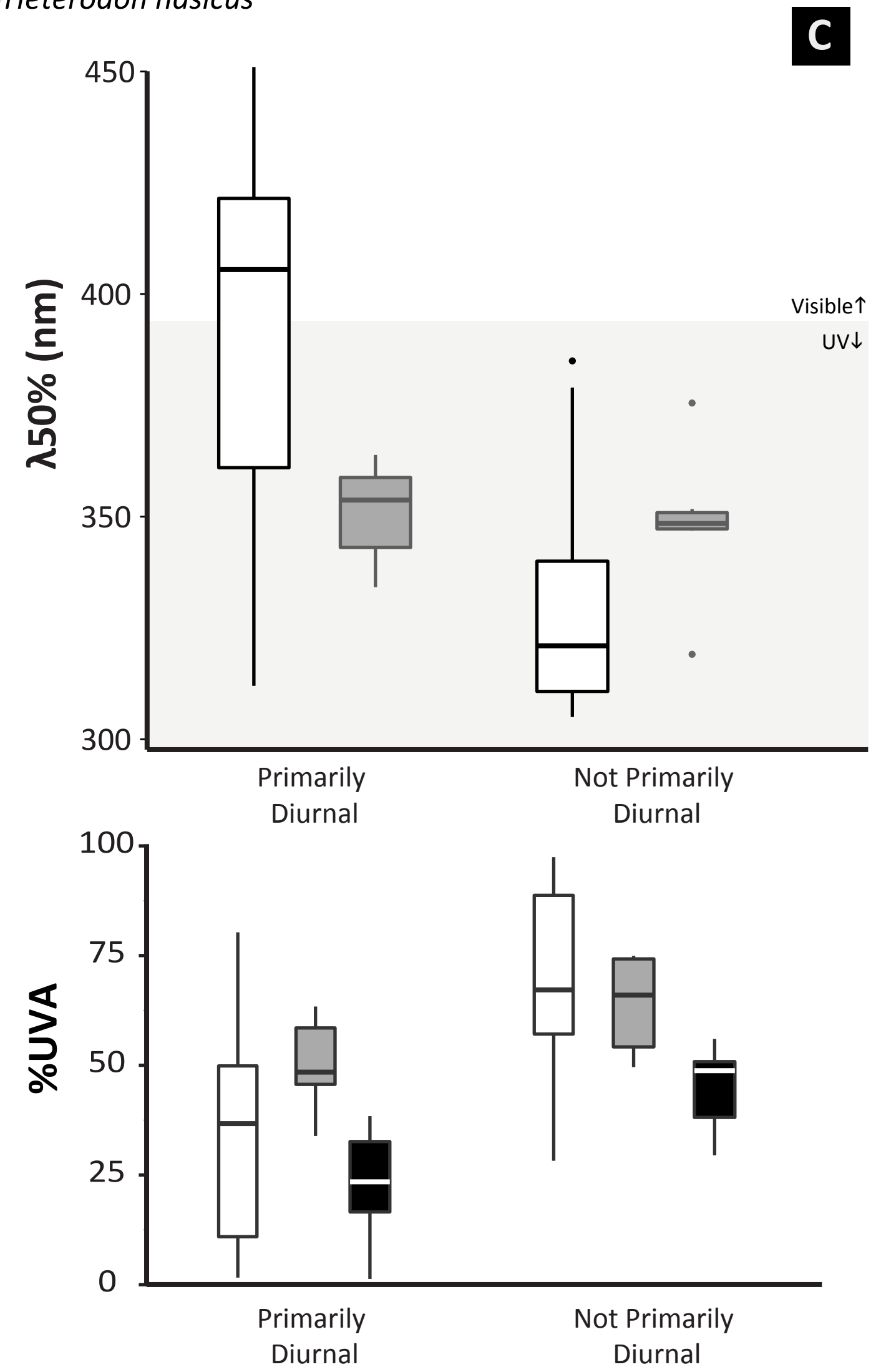
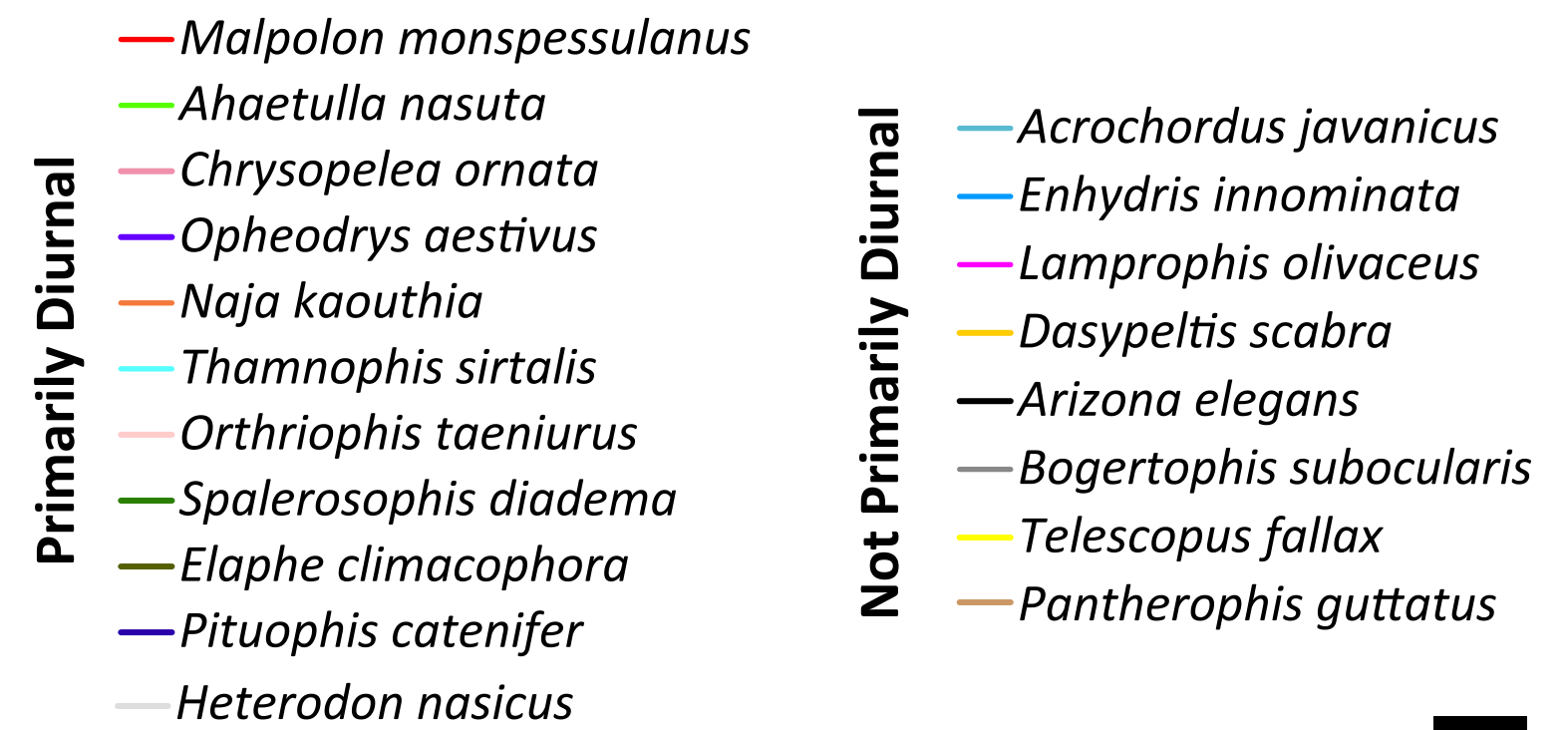
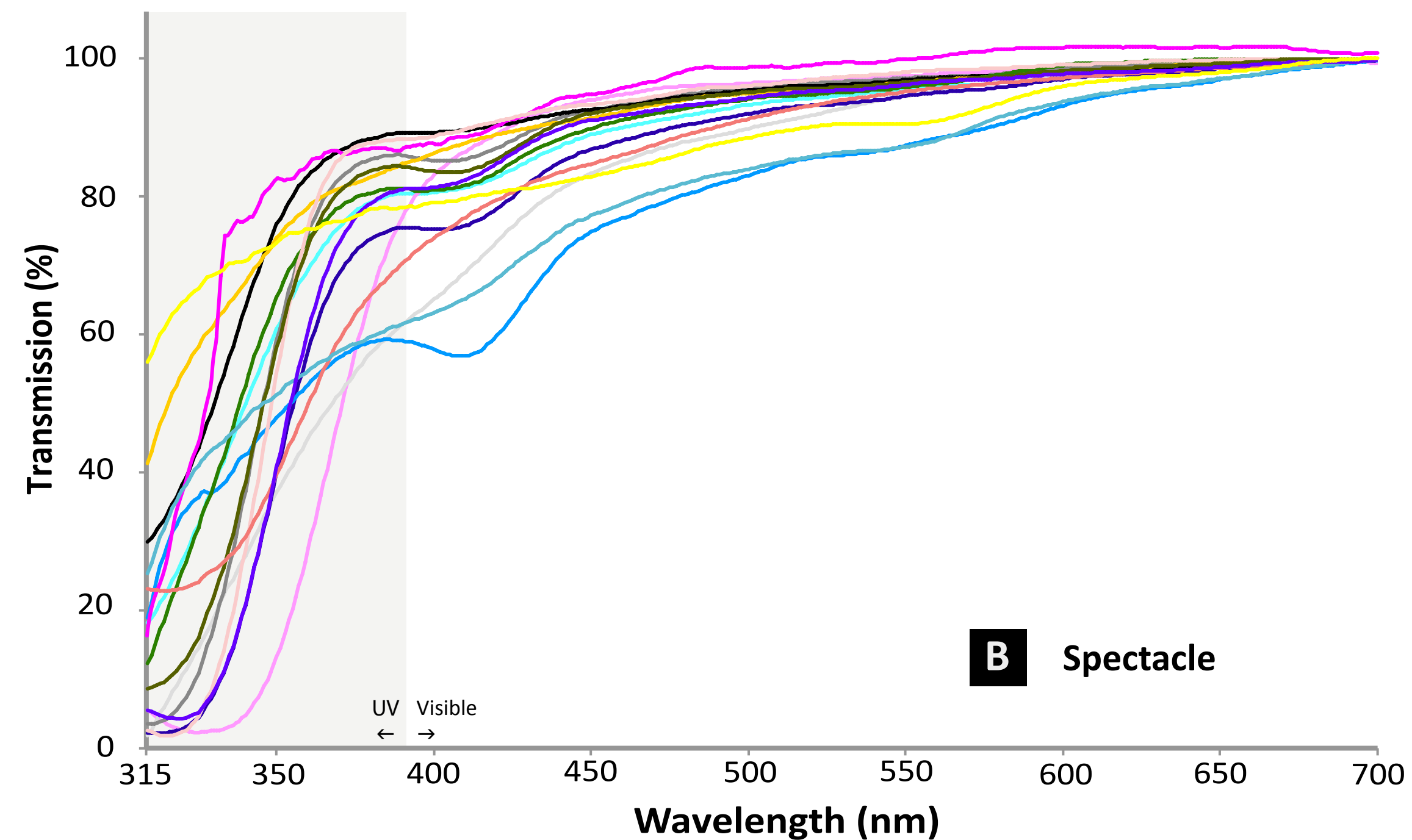
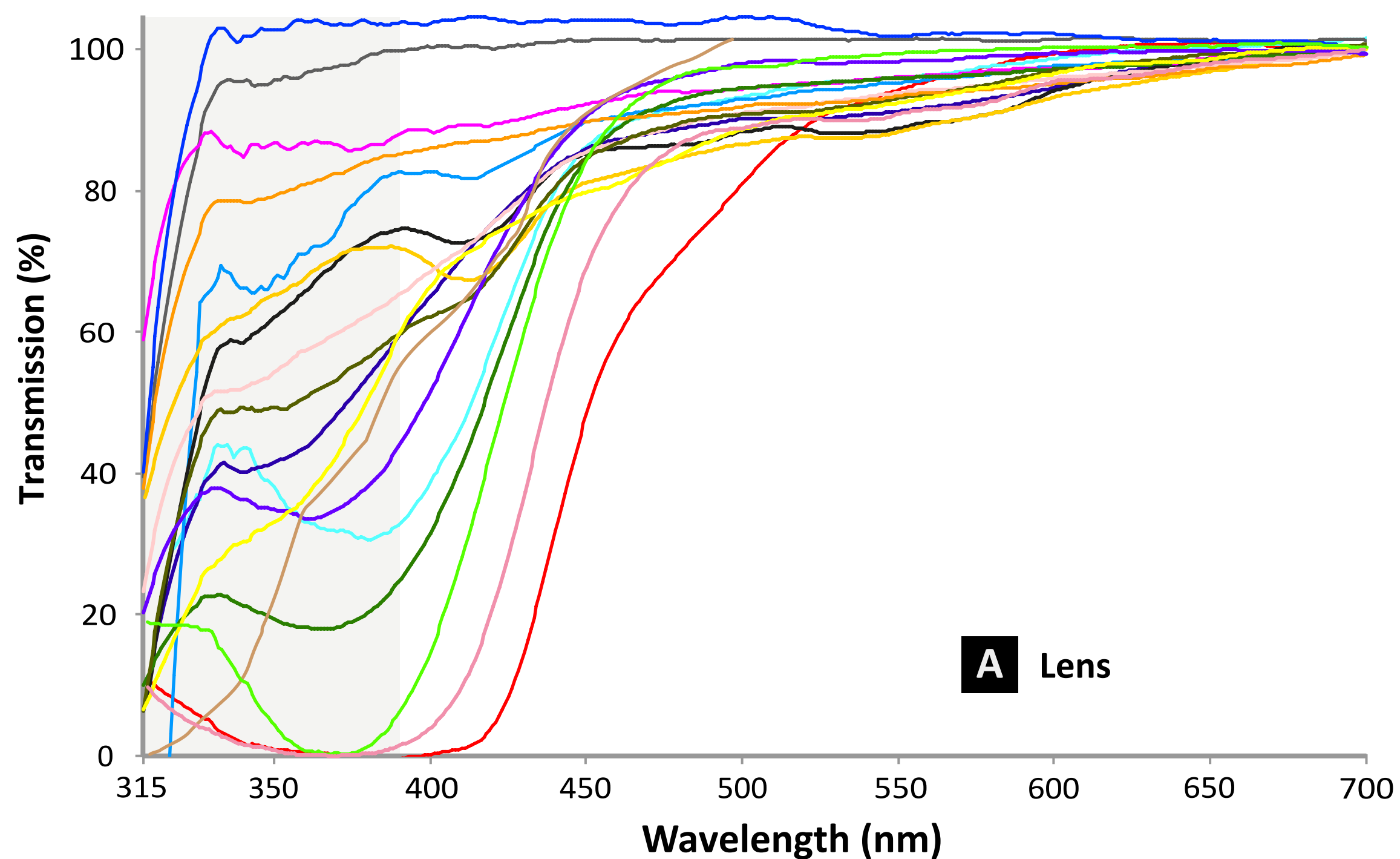


FIGURE CAPTIONS

Figure 1. Snake species tree and phenotypic classifications (see Material and Methods for more information) used in analyses of opsin gene evolution. Numbers within circles represent snake higher taxa: ① Scolecophidia (not recovered in some molecular phylogenies); ② Alethinophidia; ③ Henophidia (not recovered in molecular phylogenies); ④ Afrophidia; ⑤ Caenophidia; ⑥ Viperidae; ⑦ Colubridae, ⑧ Natricinae ⑨ Dipsadinae; ⑩ Colubrinae. Phenotype classifications shown for ecology (squares) and visual cell patterns (circles), with empty circles representing species for which state is unknown and strikethrough circles species with retinas with no cones. Visual pigment peak absorbance ($\lambda_{\max}$) values for each visual pigment are those predicted from cDNA sequences except where indicated. Ancestral pigment $\lambda_{\max}$ values are shown at selected internal branches in order SWS1-RH1-LWS. 1) SWS1 and LWS pigments have not been detected by MSP for any scolecophidian, and no cones have been found in anatomical studies (see Simões et al. 2015); 2) Anatomical studies have not been carried out for *Anilius scytale* but MSP in this species detected only a single visual pigment (RH1: Simões et al. 2015); 3) No visual pigment with an RH1-like $\lambda_{\max}$ was detected by MSP for *Malpolon* (Govardovski & Chkheidze 1989).

Figure 2. Two-dimensional diagram illustrating the arrangements of the seven transmembrane (TM) domains in visual opsins around the retinal chromophore (based on Bowmaker and Hunt 2006). Numbering of amino acid sites is based on bovine rhodopsin. Sites known to dictate spectral tuning are shown for each of the three visual pigments found in snakes, as well sites inferred to be under positive selection estimated by Bayes Empirical Bayes (model M8 β & ω). Sites inferred to be under positive selection associated with biochemical changes (detected by PRIME, SI Table S16-21) are marked with an asterisk (*). EL and CL are extra- and intracellular loops, respectively.

Figure 3. Spectral transmission curves for sampled snakes for (A) lenses and (B) spectacles, and (C) box-plots showing wavelength at which ocular media transmit 50% of the incident illumination ($\lambda_{50\%}$, top), and the proportion of UVA (315–400 nm) transmission (%UVA, bottom). The box plots summarise data for the lens (white), spectacle (grey) and lens + spectacle (black). Boxes extend from first (Q1) to third quartile (Q3); median is indicated as a horizontal line; whiskers extend to the observation that is closest to, but not more than, a distance of 1.5 (Q3 – Q1) from the

859 end of the box; outliers more distant than this are shown individually. All data newly
860 generated for this study except for *Pantherophis guttatus* (data from Thorpe 1991).

Supplementary Information

Table S1 . Identification and GenBank accession numbers of the samples used in this study.

Table S2. Ecological and retinal morphology classifications used in dN/dS analysis.

Table S3. Known amino acid spectral tuning sites for *rh1* opsin gene and predicted peak absorbance ($\lambda_{\max}$) for snakes.

Table S4. Known amino acid spectral tuning sites for *sws1* opsin gene and predicted peak absorbance ($\lambda_{\max}$) for snakes.

Table S5. Known amino acid spectral tuning sites for *lws* opsin gene and predicted peak absorbance ($\lambda_{\max}$) for snakes.

Figure S6. *rh1* opsin gene phylogenetic tree under GTR+G+I model of sequence evolution.

Figure S7. *sws1* opsin gene phylogenetic tree under GTR+G+I model of sequence evolution.

Figure S8. *lws* opsin gene phylogenetic tree under GTR+G+I model of sequence evolution.

Figure S9. *rh1* opsin gene phylogenetic tree under GTR+G model of sequence evolution.

Figure S10. *sws1* opsin gene phylogenetic tree under GTR+G model of sequence evolution.

Figure S11. *lws* opsin gene phylogenetic tree under GTR+G model of sequence evolution.

Table S12. Ratio of synonymous to non-synonymous substitutions (dN/dS or ω) for snake visual opsin gene sequences under branch models.

Table S13. Ratio of synonymous to non-synonymous substitutions (dN/dS or ω) for snake visual opsin gene sequences under site models.

Table S14. Amino acid sites inferred as under positive selection (using Bayes Empirical Bayes), identified under site models in three visual opsin genes in snakes.

Table S15. Amino acid sites inferred as being under positive selection (using Bayes Empirical Bayes), identified under branch-site models for the three visual opsin genes in specific ecologies/lineages (foreground branch) in snakes.

Table S16. PRIME analysis for Atchley properties for the *sws1* opsin gene.

Table S17. PRIME analysis for Conant-Stadler properties for the *sws1* opsin gene.

Table S18. PRIME analysis for Atchley properties for the *lws* opsin gene.

Table S19. PRIME analysis for Conant-Stadler properties for the *lws* opsin gene.

Table S20. PRIME analysis for Atchley properties for the *rh1* opsin gene.

Table S21. PRIME analysis for Conant-Stadler properties for the *rh1* opsin gene.

Table S22. λ 50% light cut-off and %UVA transmittance in lenses and spectacles in snakes.

Table S1. Identification and GenBank accession numbers of the samples used in this study.

Clade/Higher Taxon	Family	Species	Accession codes			
			<i>16S</i>	<i>rh1</i>	<i>sws1</i>	<i>lws</i>
Scolecopidia	Typhlopidae	<i>Amerotyphlops brongersmianus</i>	KR815889	KR336737	×	×
	Leptotyphlopidae	<i>Epictia collaris</i>	KR815892	KR336735	×	×
	Anomalepididae	<i>Liotyphlops beui</i>	KR815891	KR336734	×	×
	Anomalepididae	<i>Typhlophis squamosus</i>	KR815890	KR336733	×	×
Serpentes	Aniliidae	<i>Anilius scytale</i>	KR815894	KR336736	×	×
	Tropidophiidae	<i>Tropidophis feicki</i>	KR815893	KR336738	KR336723	KR336709
	Xenopeltidae	<i>Xenopeltis unicolor</i>	NA	J49723	FJ497234	FJ497235
	Pythonidae	<i>Python regius</i>	NA	FJ497236	FJ4977237	FJ4977238
		<i>Python bivittatus</i>	NA		PRJNA238085	
	Boidae	<i>Gongylophis conicus</i>	xxxxxxxx	KX237870	KX237877	KX237782
	Uropeltidae	<i>Melanophidium khairi</i>	xxxxxxxx	KX237871	-	-
		<i>Uropeltis cf. macrolepis</i>	xxxxxxxx	KX237872	KX237878	KX237783
	Pareatidae	<i>Pareas monticola</i>	xxxxxxxx	KX237868	-	-
	Alethinophidia	<i>Bitis nasicornis</i>	xxxxxxxx	KX237873	KX237880	KX237785
		<i>Echis ocellatus</i>	xxxxxxxx	KX237874	KX237881	KX237786
		<i>Causus rhombeatus</i>	xxxxxxxx	KX237876	KX237882	KX237787
		<i>Acrochordus javanicus</i>	xxxxxxxx	KX237831	KX237879	KX237784
	Homolopsidae	<i>Enhydris innominata</i>	xxxxxxxx	KX237832	KX237883	KX237789
	Lamprophiidae	<i>Polemon collaris</i>	KR815896	KR336739	KR336724	KR336710
		<i>Lamprophis olivaceus</i>	xxxxxxxx	KX237859	KX237886	KX237827
		<i>Malpolon monspessulanus</i>	xxxxxxxx	×	KX237885	KX237790
		<i>Lycophidion laterale</i>	xxxxxxxx	KX237860	KX237887	KX237828
		<i>Mehelya</i> sp.	xxxxxxxx	KX237861	KX237888	KX237829
	Elapidae	<i>Ophiophagus hannah</i>	NA		PRJNA201683	

Colubridae – Natricinae	<i>Naja kaouthia</i>	xxxxxxx	KX237830	KX237884	KX237788
	<i>Amphiesma stolata</i>	xxxxxxx	KX237866	KX237889	KX237792
	<i>Xenochrophis piscator</i>	xxxxxxx	KX237865	KX237890	KX237801
	<i>Natriciteres sylvatica</i>	xxxxxxx	KX237833	KX237891	KX237802
	<i>Xylophis captaini</i>	-	KX237869	KX237892	KX237791
	<i>Natrix maura</i>	KU323977	KU324002	KU323993	KU323982
Colubridae – Dipsadinae	<i>Thamnophis sirtalis</i>	KU323978	KU323978	KU323994	KU323983
	<i>Atractus flammigerus</i>	KR815897	KR336740	KR336726	KR336712
	<i>Atractus badius</i>	xxxxxxx	KX237842	KX237902	KX237809
	<i>Heterodon nasicus</i>	xxxxxxx	KX237850	KX237893	KX237793
	<i>Erythrolamprus reginae</i>	xxxxxxx	KX237855	KX237894	KX237800
	<i>Helicops angulatus</i>	xxxxxxx	KX237836	KX237895	KX237806
	<i>Thamnodynastes pallidus</i>	xxxxxxx	KX237864	KX237896	KX237805
	<i>Xenopholis scalaris</i>	xxxxxxx	KX237834	KX237897	KX237810
	<i>Pseudoboa coronata</i>	xxxxxxx	KX237837	KX237898	KX237803
	<i>Oxyrhopus melanogenys</i>	xxxxxxx	KX237838	KX237899	KX237804
	<i>Hypsiglena jani</i>	KU323975	KU324007	KU323998	KU323988
	<i>Imantodes lentiferus</i>	xxxxxxx	KX237841	KX237900	KX237807
	<i>Leptodeira annulata</i>	xxxxxxx	KX237840	KX237901	KX237808
	<i>Sibon nebulatus</i>	xxxxxxx	KX237843	KX237902	KX237811
	<i>Dipsas indica</i>	xxxxxxx	KX237849	KX237904	KX237813
	<i>Dipsas catesbyi</i>	xxxxxxx	KX237848	KX237905	KX237812
Colubridae – Colubrinae	<i>Ahaetulla nasuta</i>	xxxxxxx	KX237852	KX237906	KX237798
	<i>Chrysopelea ornata</i>	xxxxxxx	KX237851	KX237907	KX237799
	<i>Telescopus fallax</i>	KU323974	KU324005	KU323995	KU323984
	<i>Boiga forsteni</i>	xxxxxxx	KX237867	-	KX237818
	<i>Boiga ceylonensis</i>	xxxxxxx	-	-	KX237819
	<i>Dasypeltis scabra</i>		KX237856	KX237908	KX237821
	<i>Macroprotodon brevis</i>	xxxxxxx	--	KX237909	KX237815

			<i>Spalerosophis diadema</i>	xxxxxxx	KX237853	KX237910	KX237814
			<i>Hemorrhois hippocrepis</i>	xxxxxxx	KX237835	KX237911	KX237796
			<i>Opheodrys aestivus</i>	xxxxxxx	KX237839	KX237912	KX237797
			<i>Chironius fuscus</i>	xxxxxxx	KX237845	KX237913	KX237794
			<i>Chironius carinatus</i>	xxxxxxx	KX237846	KX237914	KX237795
			<i>Phyllorhynchus decurtatus</i>	KU323979	--	KU323996	KU323985
			<i>Lycodon aulicus</i>	xxxxxxx	KX237875	KX237915	KX237820
			<i>Orthriophis taeniurus</i>	xxxxxxx	KX237862	KX237916	KX237816
			<i>Elaphe climacophora</i>	xxxxxxx	KX237845	KX237917	KX237817
			<i>Pantherophis guttatus</i>	xxxxxxx	KX237863	KX237918	KX237824
			<i>Pituophis catenifer</i>	xxxxxxx	KX237854	KX237919	KX237823
			<i>Bogertophis subocularis</i>	xxxxxxx	KX237844	KX237920	KX237822
			<i>Arizona elegans</i>	KU323973	KU324006	KU323997	KU323986
			<i>Lampropeltis californiae</i>	xxxxxxx	KX237858	KX237921	KX237825
			<i>Lampropeltis floridiana</i>	xxxxxxx	KX237857	KX237922	KX237826
			<i>Pseustes poecilonotus</i>	KR815895	KR336741	KR336725	KR336711
Non-snake squamates	Lacertoidea	Amphisbaenidae	<i>Amphisbaena infraorbitale</i>	KR815886	KR336730	KR336719	KR336704
			<i>Amphisbaena alba</i>	KR815887	KR336729	KR336720	KR336705
			<i>Amphisbaena</i> sp.	KR815888	KR336728	KR336721	KR336706
		Lacertidae	<i>Takydromus sexlineatus</i>	KR815885	KR336727	KR336722	KR336707
		Gymnophthalmidae	<i>Bachia</i> cf. <i>flavescens</i>	KR815884	KR336731	KR336715	KR336703
	Scincoidea	Scincidae	<i>Melanoseps occidentalis</i>	KR815882	KR336743	KR336718	KR336713
		Scincidae	<i>Feylinia</i> sp.	KR815883	KR336742	KR336717	KR336714
	Anguimorpha	Diploglossidae	<i>Ophiodes striatus</i>	KR815881	KR336732	KR336716	KR336708
	Iguania	Dactyloidae	<i>Anolis carolinensis</i>	NA	Ensembl v75		
		Phrynosomatidae	<i>Uta stansburiana</i>	NA	DQ100323	DQ100325	DQ129869

Table S2. Ecology and retinal morphology classifications for sampled snakes. Y = yes, N = no, ? = not known, -- = inapplicable character. Cited references are generally not primary sources. The aim was to score as many cells as possible where ‘reasonable’ evidence was considered available. In some cases for retinal morphology we have extrapolated evidence from congeners. In a few cases we have extrapolated from information available for members of suprageneric taxa. Thus, uropeltid (*Melanophidium*, *Uropeltis*) retinal characters were scored based on data for the uropeltid *Rhinophis*; *Polemon* retinal characters were scored based on data for other atractaspidids; *Ophiophagus* and *Naja* scored for retinal characters based on data for other elapids.

Species	Primarily Fossorial	Primarily Arboreal	Aquatic or Semiaquatic	Primarily Diurnal	Double Cones Present	Transmuted Cones Present	Ecological source data	Retinal source data
<i>Typhlophis squamosus</i>	Y	N	N	N	--	--	Starace and Lambert 2013	Underwood 1967
<i>Liotyphlops beui</i>	Y	N	N	N	--	--	Kley 2003a	Underwood 1967
<i>Epictia collaris</i>	Y	N	N	N	--	--	Starace and Lambert 2013	Underwood 1967
<i>Amerotyphlops brongersmianus</i>	Y	N	N	N	--	--	Kley 2003b	Underwood 1967
<i>Anilius scytale</i>	Y	N	N	N	--	--	Starace and Lambert 2013	Underwood 1967
<i>Tropidophis feicki</i>	N	N	N	N	N	N	Schwartz 1957	Walls 1942; Underwood 1967
<i>Xenopeltis unicolor</i>	Y	N	N	N	N	N	Whitaker et al. 2004	Underwood 1967
<i>Python bivittatus</i>	N	N	N	N	N	N	Whitaker et al. 2004	Sillman et al. 1999
<i>Python regius</i>	N	N	N	N	N	N	www.toxinology.com	Sillman et al. 1999
<i>Melanophidium khairei</i>	Y	N	N	N	N	N	Gower et al. 2016	Baumeister 1908
<i>Uropeltis cf. macrolepis</i>	Y	N	N	N	N	N	Whitaker et al. 2004	Baumeister 1908
<i>Gongylophis conicus</i>	Y	N	N	N	N	N	Whitaker et al. 2004	Underwood 1967
<i>Echis ocellatus</i>	N	N	N	N	?	?	Chirio and LeBreton 2007	-
<i>Causus rhombeatus</i>	N	N	N	N	Y	N	Spawls et al. 2006	Underwood 1967
<i>Bitis nasicornis</i>	N	N	N	N	Y	N	Spawls et al. 2006	Walls 1942
<i>Pareas monticola</i>	N	Y	N	N	N	N	Ahmed et al. 2009	Underwood 1967, 1970
<i>Acrochordus javanicus</i>	N	N	Y	N	N	N	Das, 2015	Underwood 1967
<i>Enhydrys innominata</i>	N	N	Y	N	Y	N	Murphy 2007	Underwood 1966
<i>Polemon collaris</i>	Y	N	N	N	?	N	Chirio and LeBreton 2007	Underwood 1967; Underwood & Kochva 1993
<i>Lycophidion laterale</i>	N	N	N	N	?	?	www.toxinology.com	-
<i>Mehelya sp.</i>	Y	N	N	N	?	?	Shine et al. 1996	-
<i>Lamprophis olivaceus</i>	N	N	N	N	Y	N	Spawls et al. 2006	Underwood 1967
<i>Malpolon monspessulanus</i>	N	N	N	Y	Y	N	Arnold and Oviden 2002	Underwood 1967
<i>Ophiophagus hannah</i>	N	N	N	Y	Y	N	Ahmed et al. 2009	Underwood 1967, 1970
<i>Naja kaouthia</i>	N	N	N	Y	Y	N	www.thailandsnakes.com	Underwood 1967, 1970
<i>Xenochrophis piscator</i>	N	N	Y	Y	?	?	www.toxinology.com	-
<i>Natriciteres sylvatica</i>	N	N	Y	Y	?	?	www.toxinology.com	-
<i>Amphiesma stolata</i>	N	N	Y	Y	?	?	Whitaker et al. 2004	-
<i>Natrix maura</i>	N	N	Y	Y	Y	N	Arnold and Oviden 2002	Underwood 1967
<i>Thamnophis sirtalis</i>	N	N	Y	Y	Y	N	Ernst and Ernst 2011	Sillman et al. 1997
<i>Imantodes lentiferus</i>	N	Y	N	N	?	?	Starace and Lambert 2013	-
<i>Leptodeira annulata</i>	N	Y	N	N	Y	Y	Starace and Lambert 2013	Underwood 1967, 1970
<i>Atractus badius</i>	Y	N	N	N	Y	N	Starace and Lambert 2013	Underwood 1970

<i>Atractus flammigerus</i>	Y	N	N	N	Y	N	Starace and Lambert 2013	Underwood 1970
<i>Dipsas indica</i>	N	Y	N	N	?	?	Starace and Lambert 2013	-
<i>Dipsas catesbyi</i>	N	Y	N	N	?	?	Starace and Lambert 2013	-
<i>Sibon nebulatus</i>	N	Y	N	N	Y	N	Starace and Lambert 2013	Underwood 1970
<i>Hypsiglena jani</i>	N	N	N	N	Y	Y	Starace and Lambert 2013	Walls 1942
<i>Erythrolamprus reginae</i>	N	N	Y	Y	?	?	Starace and Lambert 2013	-
<i>Xenopholis scalaris</i>	N	N	N	N	?	?	Starace and Lambert 2013	-
<i>Pseudoboa coronata</i>	N	N	N	N	Y	N	Starace and Lambert 2013	Underwood 1970
<i>Oxyrhopus melanogenys</i>	N	N	N	N	?	?	Starace and Lambert 2013	-
<i>Helicops angulatus</i>	N	N	Y	N	Y	N	Starace and Lambert 2013	Underwood 1970
<i>Thamnodynastes pallidus</i>	N	Y	N	N	?	?	Starace and Lambert 2013	-
<i>Heterodon nasicus</i>	N	N	N	Y	Y	N	Ernst and Ernst 2011	Underwood 1967
<i>Ahaetulla nasuta</i>	N	Y	N	Y	Y	N	Whitaker et al. 2004	Underwood 1967
<i>Chrysopelea ornata</i>	N	Y	N	Y	?	?	Whitaker et al. 2004	-
<i>Telescopus fallax</i>	N	N	N	N	Y	Y	Arnold and Ovenden 2002	Munk and Rasmussen 1993
<i>Boiga forsteni</i>	N	Y	N	N	Y	Y	Whitaker et al. 2004	Underwood 1967
<i>Boiga ceylonensis</i>	N	Y	N	N	Y	Y	Whitaker et al. 2004	Underwood 1967
<i>Dasypeltis scabra</i>	N	N	N	N	Y	Y	Spawls et al. 2006	Underwood 1967
<i>Opheodrys aestivus</i>	N	Y	N	Y	?	?	Ernst and Ernst 2011	-
<i>Phyllorhynchus decurtatus</i>	N	N	N	N	Y	Y	Ernst and Ernst 2011	Walls 1942
<i>Pseustes poecilonotus</i>	N	Y	N	Y	?	?	Starace and Lambert 2013	-
<i>Chironius fuscus</i>	N	Y	N	Y	?	?	Starace and Lambert 2013	-
<i>Chironius carinatus</i>	N	Y	N	Y	?	?	Starace and Lambert 2013	-
<i>Lycodon aulicus</i>	N	N	N	N	?	?	Whitaker et al. 2004	-
<i>Orthriophis taeniurus</i>	N	N	N	Y	?	?	www.toxinology.com	-
<i>Elaphe climacophora</i>	N	N	N	Y	?	?	www.toxinology.com	-
<i>Pantherophis guttatus</i>	N	N	N	N	?	?	Ernst and Ernst 2011	-
<i>Pituophis catenifer</i>	N	N	N	Y	?	?	Ernst and Ernst 2011	-
<i>Arizona elegans</i>	N	N	N	N	Y	Y	Ernst and Ernst 2011	Walls 1942
<i>Lampropeltis californiae</i>	N	N	N	Y	Y	Y	Ernst and Ernst 2011	Walls 1942
<i>Lampropeltis floridiana</i>	N	N	N	Y	Y	Y	Ernst and Ernst 2011	Walls 1942
<i>Bogertophis subocularis</i>	N	N	N	N	?	?	Ernst and Ernst 2011	-
<i>Macroprotodon brevis</i>	N	N	N	Y	?	?	www.afpmb.org	-
<i>Spalerosophis diadema</i>	N	N	N	Y	?	?	www.toxinology.com	-
<i>Hemorrhois hippocrepis</i>	N	N	N	Y	?	?	Arnold and Ovenden 2002	-
<i>Xylophis captaini</i>	Y	N	N	N	?	?	Gower and Winkler 2007	-

Table S3. Known amino acid spectral tuning sites for *rh1* (Yokoyama 2008; Hunt et al. 2001) and predicted peak absorbance ($\lambda_{\max}$) of RH1-based visual pigment for snakes. Site values in first row represent amino acid positions numbered with respect to bovine rhodopsin. All $\lambda_{\max}$ values are predicted based on amino acid sequences (for a review see Yokoyama et al. 2008) except those in parentheses (measured using MSP or *in vitro* expression). When two mutations are present and the $\lambda_{\max}$ shift of the interaction is not known, the individual shift of each mutation is shown.

Species	83	90	113	118	122	164	180	261	265	269	285	292	$\lambda_{\max}$ (nm)
<i>Bachia flavescens</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Ophiodes striatus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Takydromus sexlineatus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Phelsuma madagascariensis</i>	N	S	E	T	Q	A	P	F	W	A	V	A	-6 -20
<i>Anolis carolinensis</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496 (491 ¹)
<i>Feylinia</i> sp.	N	G	K	T	E	A	P	F	W	A	P	A	-6 ?
<i>Melanoseps occidentalis</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Amphisbaena</i> sp.	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Amphisbaena alba</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Amphisbaena infraorbitale</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Amerotyphlops brongersmianus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Typhlophis squamosus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Liotyphlops beui</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Epictia collaris</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Anilius scytale</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496 (493 ²)
<i>Tropidophis feicki</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Python regius</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496 (494 ³)
<i>Python bivittatus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Xenopeltis unicolor</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496 (499 ⁴)
<i>Gongylophis conicus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Melanophidium khairi</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Uropeltis</i> cf. <i>macrolepis</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Naja kaouthia</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Ophiophagus hannah</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Lamprophis olivaceus</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Enhydryis innominata</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Acrochordus javanicus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Lycophidion laterale</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Polemon collaris</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Mehelya</i> sp.	N	G	E	T	E	A	P	F	W	A	P	A	491-496

Species	83	90	113	118	122	164	180	261	265	269	285	292	$\lambda_{\max}$ (nm)
<i>Pareas monticola</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Xylophis captaini</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Echis ocellatus</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Bitis nasicornis</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Causus rhombeatus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Natriciteres sylvatica</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Thamnophis sirtalis</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489 (482 ⁵)
<i>Natrix maura</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Amphiesma stolata</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Xenochrophis piscator</i>	N	G	E	T	E	A	Q	F	W	A	P	S	476-489
<i>Chrysopelea ornata</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Ahaetulla nasuta</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Spalerosophis diadema</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Hemorrhois hippocrepis</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Dasypeltis scabra</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Telescopus fallax</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Boiga forsteni</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Ophoeodrys aestivus</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Chironius carinatus</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Chironius fuscus</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Pseustes poecilonotus</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Imantodes lentiferus</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Hypsiglena jani</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Leptodeira annulata</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Atractus flammigerus</i>	N	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Atractus badius</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Sibon nebulatus</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Dipsas catesbyi</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Dipsas indica</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Helicops angulatus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Oxyrhopus melanogenys</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Heterodon nasicus</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Lycodon aulicus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Pseudoboa coronata</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Xenopholis scalaris</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496

Species	83	90	113	118	122	164	180	261	265	269	285	292	$\lambda_{\max}$ (nm)
<i>Bogertophis subocularis</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Elaphe climacophora</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Pituophis catenifer</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Erythrolampus reginae</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Lampropeltis californiae</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Lampropeltis floridiana</i>	D	G	E	T	E	A	P	F	W	A	P	A	500-505
<i>Arizona elegans</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489 (484 ⁶)
<i>Orthriophis taeniurus</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
<i>Pantherophis guttatus</i>	N	G	E	T	E	A	P	F	W	A	P	A	491-496
<i>Thamnodynastes pallidus</i>	N	G	E	T	E	A	P	F	W	A	P	S	476-489
Ancestor Serpentes	N	G	E	T	E	A	P	F	W	A	P	A	491-496
Ancestor Alethinophidia	N	G	E	T	E	A	P	F	W	A	P	A	491-496
Ancestor Afrophidia	N	G	E	T	E	A	P	F	W	A	P	A	491-496
Ancestor Elapidae + Lamprophidae + Homolopsidae	N	G	E	T	E	A	P	F	W	A	P	A	491-496
Ancestor Viperidae	N	G	E	T	E	A	P	F	W	A	P	A	491-496
Ancestor Colubridae	N	G	E	T	E	A	P	F	W	A	P	S	476-489
Ancestor Colubrinae	N	G	E	T	E	A	P	F	W	A	P	S	476-489
Ancestor Natricinae	N	G	E	T	E	A	P	F	W	A	P	S	476-489
Ancestor Dipsadinae	N	G	E	T	E	A	P	F	W	A	P	S	476-489

¹Yokoyama 2000; ²Simões et al. 2015; ³Davies et al. 2009; ⁴Sillman et al. 1999; ⁵Sillman et al. 1997; ⁶Simões et al. 2016

Table S4. Known amino acid spectral tuning sites for *sws1* (Yokoyama et al. 2006) and predicted peak absorbance ($\lambda_{\max}$) of SWS1-based visual pigment for snakes. Site values in first row represent amino acid positions numbered with respect to bovine opsin. Underline indicates amino acids with stronger effects on spectral tuning (Cowing et al. 2002; Babu et al. 2001; Asenjo et al. 1994 and Fasick et al. 2002). All $\lambda_{\max}$ values are predicted based on amino acid sequences (for a review see Yokoyama 2008) except those in parentheses (measured using MSP or *in vitro* expression). When two mutations are present and the $\lambda_{\max}$ shift of the interaction is unknown, the individual shift of each mutation is shown. An asterisk (*) marks a possible case of trichromacy. UVS = ultraviolet light sensitive ($\lambda_{\max}$ c. 360nm).

Species	46	49	52	86	90	93	97	<u>113</u>	114	116	118	265	$\lambda_{\max}$ (nm)
<i>Bachia flavescens</i>	F	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Ophiodes striatus</i>	A	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Takydromus sexlineatus</i>	F	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Phelsuma madagascariensis</i>	F	F	T	F	S	T	S	E	A	L	S	Y	UVS
<i>Anolis carolinensis</i>	F	F	T	F	S	T	A	E	A	L	S	Y	UVS (359 ¹)
<i>Feylinia</i> sp.	F	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Melanoseps occidentalis</i>	F	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Amphisbaena</i> sp.	F	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Amphisbaena alba</i>	F	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Amphisbaena infraorbitale</i>	F	F	T	F	S	T	A	E	A	L	S	Y	UVS
<i>Tropidophis feicki</i>	L	F	T	F	A	A	S	E	A	L	S	Y	UVS
<i>Python regius</i>	L	F	T	F	A	T	A	E	A	L	S	Y	UVS (361 ²)
<i>Python bivittatus</i>	L	F	T	F	A	T	A	E	A	L	S	Y	UVS
<i>Xenopeltis unicolor</i>	L	F	T	F	A	T	A	E	A	L	S	Y	UVS (360 ³)
<i>Gongylophis conicus</i>	L	F	T	F	A	T	A	E	A	L	S	Y	UVS
<i>Uropeltis</i> cf. <i>macrolepis</i>	L	F	T	F	S	T	A	E	A	L	S	Y	?
<i>Naja kaouthia</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Ophiophagus hannah</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Lamprophis olivaceus</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Enhydris innominata</i>	F	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Acrochordus javanicus</i>	L	F	T	F	A	V	C	E	A	L	T	Y	?
<i>Malpolon monspessulanus</i>	L	L	T	S	A	V	T	E	A	L	S	Y	419 ?
<i>Polemon collaris</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Mehelya</i> sp.	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Lycophidion laterale</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?

Species	46	49	52	86	90	93	97	113	114	116	118	265	$\lambda_{\max}$ (nm)
<i>Xylophis captaini</i>	L	F	T	F	A	T	S	E	A	L	S	Y	UVS
<i>Echis ocellatus</i>	L	F	T	F	A	A	S	E	A	L	S	Y	?
<i>Bitis nasicornis</i>	L	F	T	F	A	A	S	E	A	L	T	Y	?
<i>Causus rhombeatus</i>	L	C	T	F	A	T	S	E	A	V	S	Y	UVS
<i>Natriciteres sylvatica</i>	L	F	T	F	A	T	S	E	A	L	S	Y	UVS
<i>Thamnophis sirtalis</i>	L	F	T	F	A	T	S	E	A	L	S	Y	UVS (360 ⁴)
<i>Natrix maura</i>	L	F	T	L	A	T	S	E	A	L	S	Y	UVS
<i>Amphiesma stolata</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Xenochrophis piscator</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Chrysopelea ornata</i>	L	F	T	V	A	V	S	E	A	L	T	Y	?
<i>Ahaetulla nasuta</i>	L	F	T	V	A	V	S	E	A	L	T	Y	?
<i>Macroprotodon brevis</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Spalerosophis diadema</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Hemorrhois hippocrepis</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Dasypeltis scabra</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Telescopus fallax</i>	L	F	T	F	A	V	C	E	A	L	T	Y	?
<i>Ophoeodrys aestivus</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Chironius carinatus</i>	L	F	T	V	A	V	S	E	A	L	T	Y	?
<i>Chironius fuscus</i>	L	F	T	V	A	V	S	E	A	L	T	Y	?
<i>Pseustes poecilonotus</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Phyllorhynchus decurtatus</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Imantodes lentiferus</i>	L	F	T	F	A	A	S	D	A	L	T	Y	-4 ?
<i>Hypsiglena jani</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Leptodeira annulata</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Atractus flammigerus</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Atractus badius</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Sibon nebulatus</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Dipsas catesbyi</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Dipsas indica</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Helicops angulatus</i>	L	F	T	VF	A	V	S	E	A	L	T	Y	?*
<i>Oxyrhopus melanogenys</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Heterodon nasicus</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Lycodon aulicus</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Pseudoboa coronata</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Xenopholis scalaris</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS

Species	46	49	52	86	90	93	97	113	114	116	118	265	$\lambda_{\max}$ (nm)
<i>Bogertophis subocularis</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Elaphe climacophora</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Pituophis catenifer</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
<i>Erythrolampus reginae</i>	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
<i>Lampropeltis californiae</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Lampropeltis floridiana</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Arizona elegans</i>	L	F	T	F	A	V	S	E	A	L	T	Y	? (366 ⁵)
<i>Orthriophis taeniurus</i>	L	F	T	F	A	V	S	E	A	L	T	Y	?
<i>Pantherophis guttatus</i>	L	L	T	S	A	V	T	E	A	L	S	Y	419 ?
<i>Thamnodynastes pallidus</i>	L	F	T	F	A	V	S	E	A	L	S	Y	?
Ancestor Serpentes/ Alethinophidia	L	F	T	F	A	A	S	E	A	L	S	Y	UVS
Ancestor Afrophidia	L	F	T	F	A	A	S	E	A	L	S	Y	UVS
Ancestor Elapidae + Lamprophidae + Homolopsidae	L	F	T	F	A	V	S	E	A	L	T	Y	UVS
Ancestor Viperidae	L	F	T	F	A	A	S	E	A	L	T	Y	UVS
Ancestor Colubridae	L	F	T	F	A	V	S	E	A	L	T	Y	UVS
Ancestor Colubrinae	L	F	T	F	A	V	S	E	A	L	T	Y	UVS
Ancestor Natricinae	L	F	T	F	A	T	S	E	A	L	T	Y	UVS
Ancestor Dipsadinae	L	F	T	F	A	T	S	E	A	L	T	Y	UVS

¹Yokoyama 2000; ²Davies et al. 2009; ³Sillman et al. 1999; ⁴Sillman et al. 1997; ⁵Simões et al. 2016

Table S5. Known amino acid spectral tuning sites for *lws* (Yokoyama and Radlwimmer 1998) and predicted peak absorbance ($\lambda_{\max}$) of LWS-based visual pigment for snakes. Site values in first row represent amino acid positions numbered with respect to bovine rhodopsin. Underline indicates amino acids with stronger effects on spectral tuning (Cowing et al. 2002; Babu et al. 2001; Asenjo et al. 1994 and Fasick et al. 2002). All $\lambda_{\max}$ values are predicted based on amino acid sequences (for a review see Yokoyama 2008) except those in parentheses (measured using MSP or *in vitro* expression).

Species	<u>180</u>	<u>197</u>	<u>277</u>	<u>285</u>	<u>308</u>	$\lambda_{\max}$ (nm)
<i>Bachia flavescens</i>	A	H	Y	T	A	555
<i>Ophiodes striatus</i>	S	H	Y	T	A	560
<i>Takydromus sexlineatus</i>	S	H	Y	T	A	560
<i>Phelsuma madagascariensis</i>	S	H	Y	T	A	560
<i>Anolis carolinensis</i>	S	H	Y	T	A	560 (560 ¹)
<i>Feylinia</i> sp.	A	H	Y	T	A	555
<i>Melanoseps occidentalis</i>	S	H	Y	T	A	560
<i>Amphisbaena</i> sp.	S	H	Y	T	A	560
<i>Amphisbaena alba</i>	S	H	Y	T	A	560
<i>Amphisbaena infraorbitale</i>	S	H	Y	T	A	560
<i>Tropidophis feicki</i>	A	H	Y	T	A	555
<i>Python regius</i>	S	H	Y	T	A	560 (551 ²)
<i>Python bivittatus</i>	S	H	Y	T	A	560
<i>Xenopeltis unicolor</i>	S	H	Y	T	A	560 (558-562 ³)
<i>Gongylophis conicus</i>	A	H	Y	T	A	555
<i>Uropeltis</i> cf. <i>macrolepis</i>	A	H	Y	T	A	555
<i>Naja kaouthia</i>	S	H	Y	T	A	560
<i>Ophiophagus hannah</i>	S	H	Y	T	A	560
<i>Lamprophis olivaceus</i>	A	H	Y	T	A	555
<i>Enhydris innominata</i>	S	H	Y	T	A	560
<i>Acrochordus javanicus</i>	S	H	Y	T	A	560
<i>Malpolon monspessulanus</i>	S	H	Y	T	A	560 (550-560 ⁴)
<i>Polemon collaris</i>	S	H	Y	T	A	560
<i>Mehelya</i> sp.	A	H	Y	A	A	536
<i>Lycophidion laterale</i>	A	H	Y	T	A	555
<i>Xylophis captaini</i>	A	H	Y	T	A	555
<i>Echis ocellatus</i>	A	H	Y	T	A	555
<i>Bitis nasicornis</i>	A	H	Y	T	A	555
<i>Causus rhombeatus</i>	A	H	Y	T	A	555
<i>Natriciteres sylvatica</i>	S	H	Y	T	A	560
<i>Thamnophis sirtalis</i>	A	H	Y	T	A	555 (553 ⁵)
<i>Natrix maura</i>	A	H	Y	T	A	555
<i>Amphiesma stolata</i>	S	H	Y	T	A	560
<i>Xenochrophis piscator</i>	S	H	Y	T	A	560
<i>Chrysopelea ornate</i>	S	H	Y	T	A	560
<i>Ahaetulla nasuta</i>	S	H	Y	T	A	560
<i>Spalerosophis diadema</i>	A	H	Y	T	A	555
<i>Hemorrhois hippocrepis</i>	A	H	Y	T	A	560
<i>Dasypeltis scabra</i>	A	H	Y	A	A	536
<i>Telescopus fallax</i>	A	H	Y	A	A	536
<i>Boiga forsteni</i>	A	H	Y	A	A	536
<i>Boiga ceylonensis</i>	A	H	Y	A	A	536
<i>Macroprotodon brevis</i>	A	H	Y	T	A	555
<i>Ophoeodrys aestivus</i>	S	H	Y	T	A	560
<i>Chironius carinatus</i>	S	H	Y	T	A	560
<i>Chironius fuscus</i>	S	H	Y	T	A	560
<i>Pseustes poecilonotus</i>	S	H	Y	T	A	560

Species	180	197	277	285	308	$\lambda_{\max}$ (nm)
<i>Phyllorhynchus decurtatus</i>	A	H	Y	T	A	555
<i>Imantodes lentiferus</i>	A	H	Y	A	A	536
<i>Hypsiglena jani</i>	A	H	Y	A	A	536
<i>Leptodeira annulata</i>	A	H	Y	A	A	536
<i>Atractus flammigerus</i>	A	H	Y	A	A	536
<i>Atractus badius</i>	A	H	Y	A	A	536
<i>Sibon nebulatus</i>	A	H	Y	A	A	536
<i>Dipsas catesbyi</i>	A	H	Y	A	A	536
<i>Dipsas indica</i>	A	H	Y	A	A	536
<i>Helicops angulatus</i>	A	H	Y	T	A	555
<i>Oxyrhopus melanogenys</i>	A	H	Y	T	A	555
<i>Heterodon nasicus</i>	A	H	Y	A	S	?
<i>Lycodon aulicus</i>	A	H	Y	A	A	536
<i>Pseudoboa coronata</i>	A	H	Y	T	A	555
<i>Xenopholis scalaris</i>	A	H	Y	A	A	536
<i>Bogertophis subocularis</i>	A	H	Y	A	A	536
<i>Elaphe climacophora</i>	A	H	Y	T	A	555
<i>Pituophis catenifer</i>	A	H	Y	A	A	536
<i>Erythrolampus reginae</i>	S	H	Y	T	A	560
<i>Lampropeltis californiae</i>	A	H	Y	T	A	555
<i>Lampropeltis floridiana</i>	A	H	Y	T	A	555
<i>Arizona elegans</i>	A	H	Y	A	A	536 (538 ⁶)
<i>Orthriophis taeniurus</i>	A	H	Y	T	A	555
<i>Pantherophis guttatus</i>	A	H	Y	A	A	536
<i>Thamnodynastes pallidus</i>	A	H	Y	T	A	555
Ancestor Serpentes/Alethinophidia	A	H	Y	T	A	555
Ancestor Afrophidia	S	H	Y	T	A	560
Ancestor Elapidae + Lamprophidae + Homolopsidae	S	H	Y	T	A	560
Ancestor Viperidae	A	H	Y	T	A	555
Ancestor Colubridae	S	H	Y	T	A	560
Ancestor Colubrinae	S	H	Y	T	A	560
Ancestor Natricinae	S	H	Y	T	A	560
Ancestor Dipsadinae	A	H	Y	T	A	555

¹Yokoyama 2000; ²Sillman et al. 1999; ³Davies et al. 2009; ⁴Govardovskii & Chkheidze 1989; ⁵Sillman et al. 1997;

⁶Simões et al. 2016

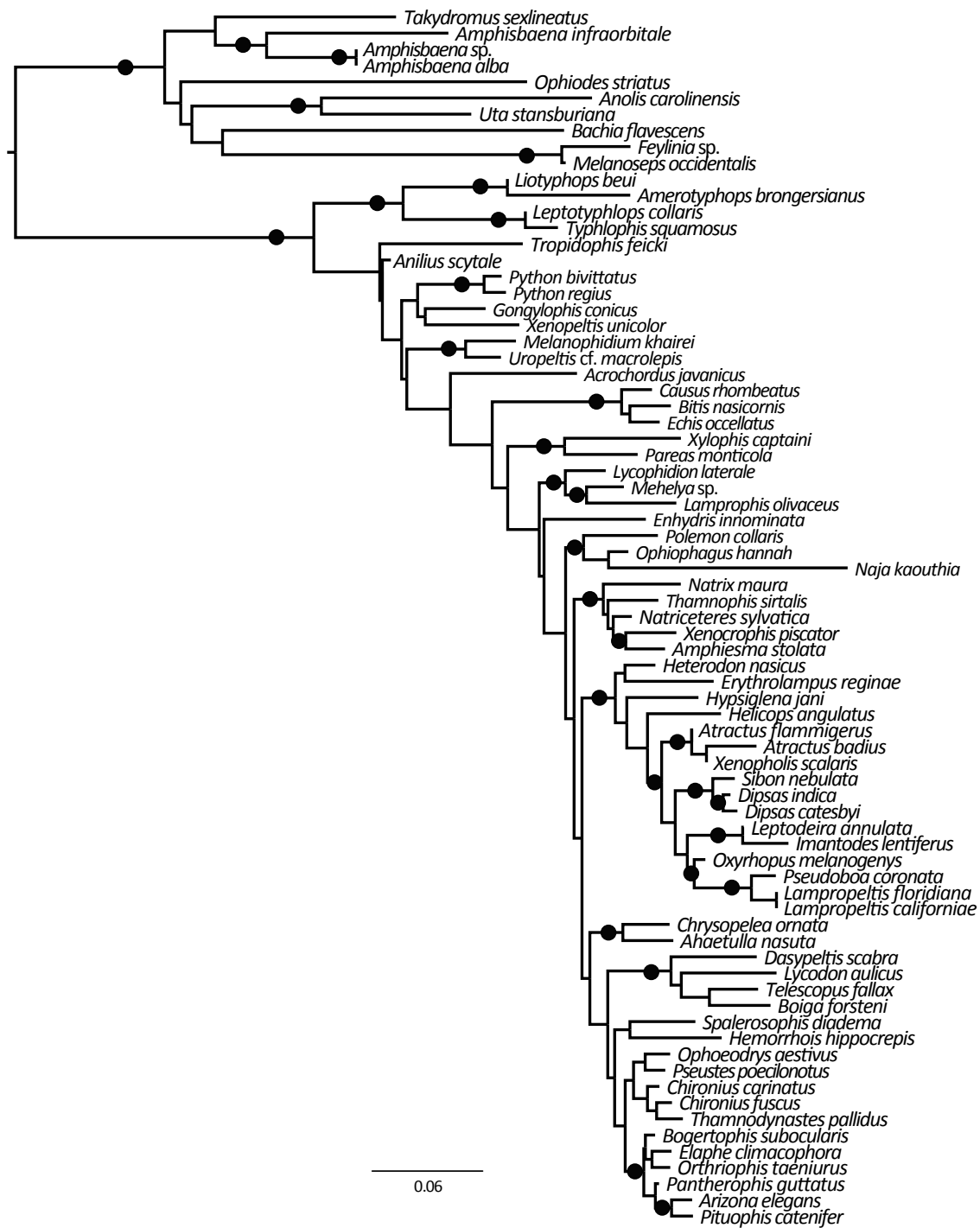


Figure S6. Maximum Likelihood rhodopsin 1 (*rh1*) gene phylogenetic tree for squamates estimated by RAXML based on GTR+G+I model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

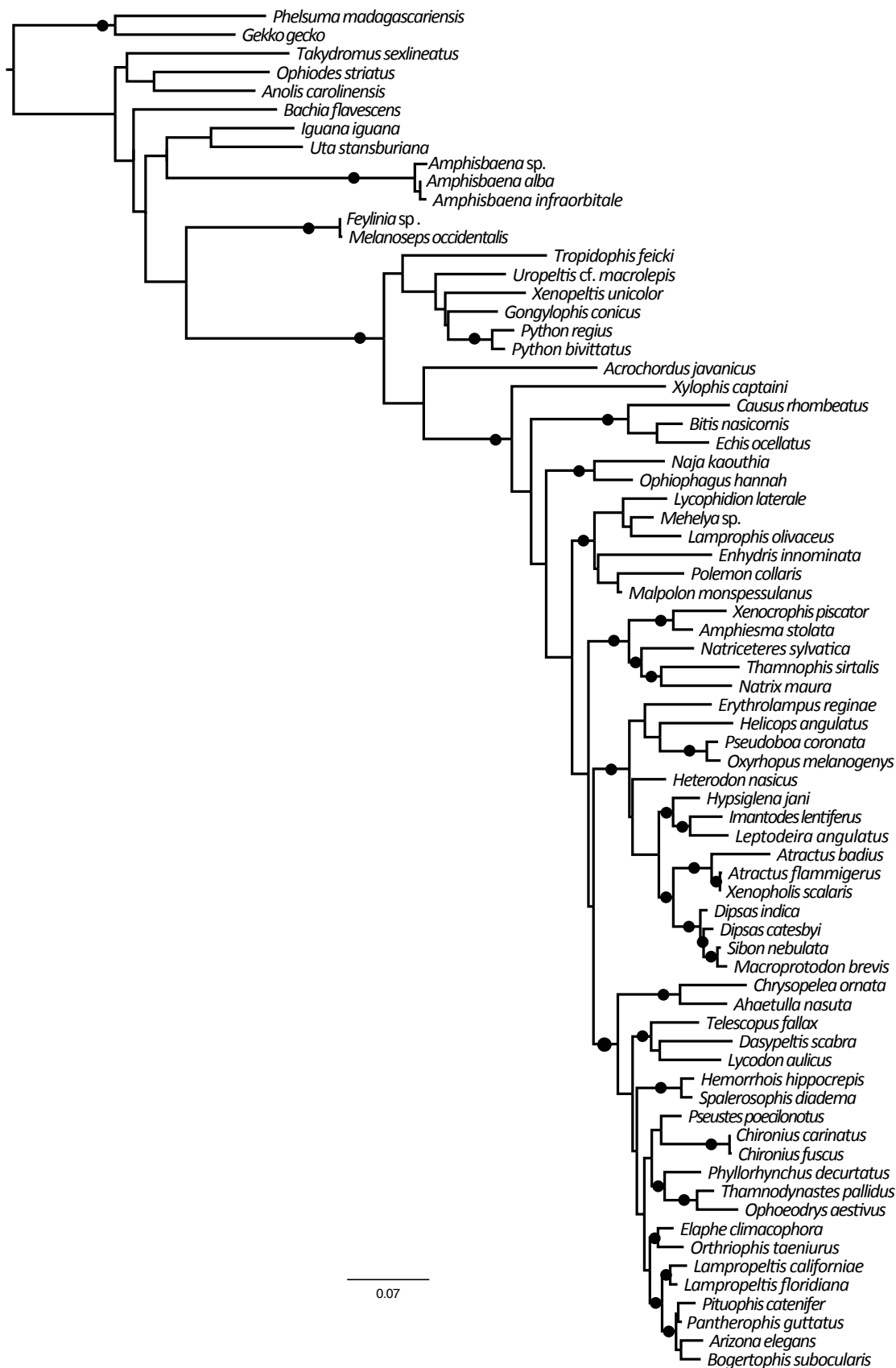


Figure S7. Maximum Likelihood short-wavelength opsin 1 (*sws1*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G+I model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

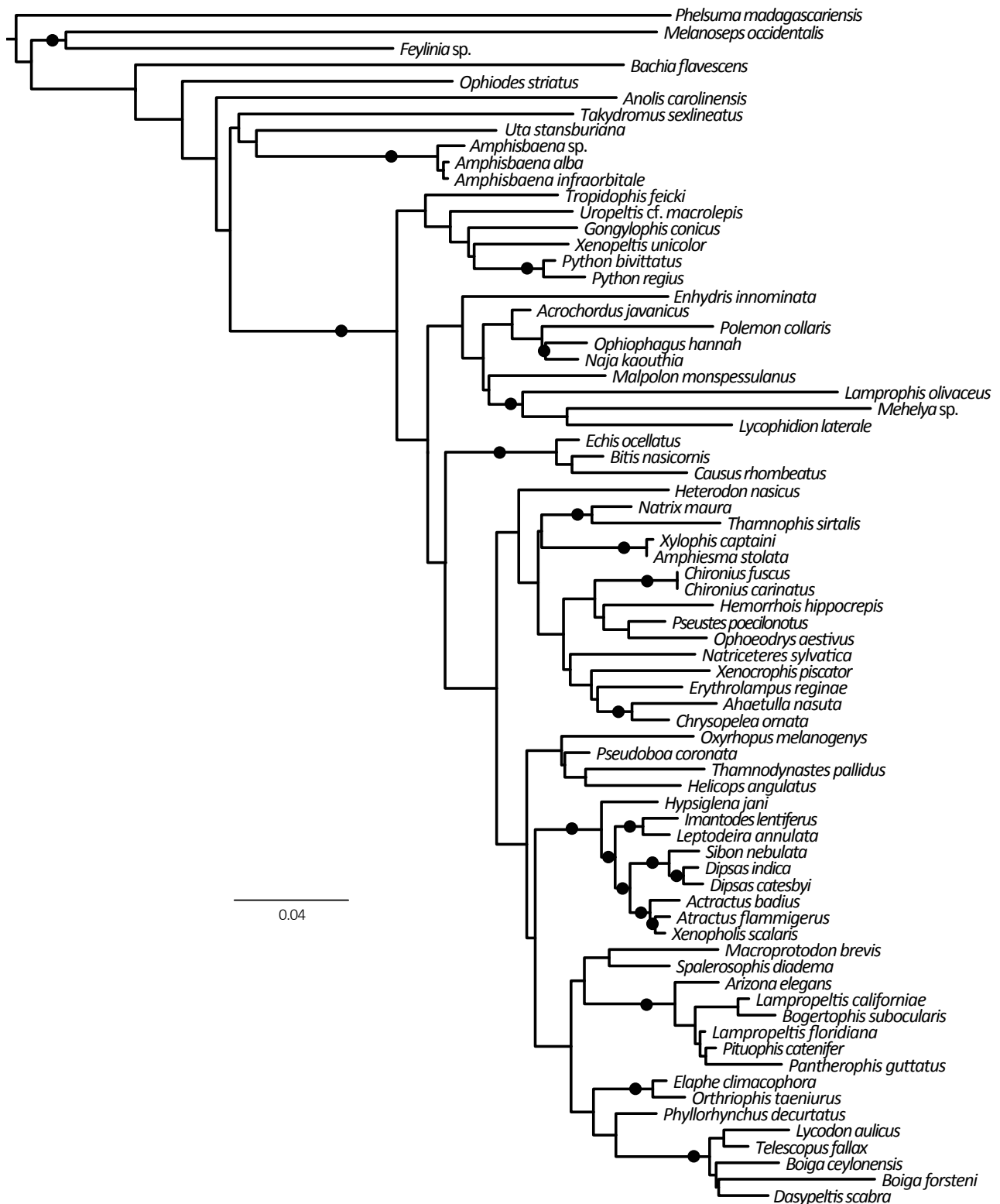


Figure S8. Maximum Likelihood medium-to-long wavelength opsin gene (*lws*) phylogenetic tree for squamates estimated by RAXML based on GTR+G+I model of sequence evolution. Numbers on the major internal branches are ML bootstrap support and Bayesian posterior probabilities, respectively.

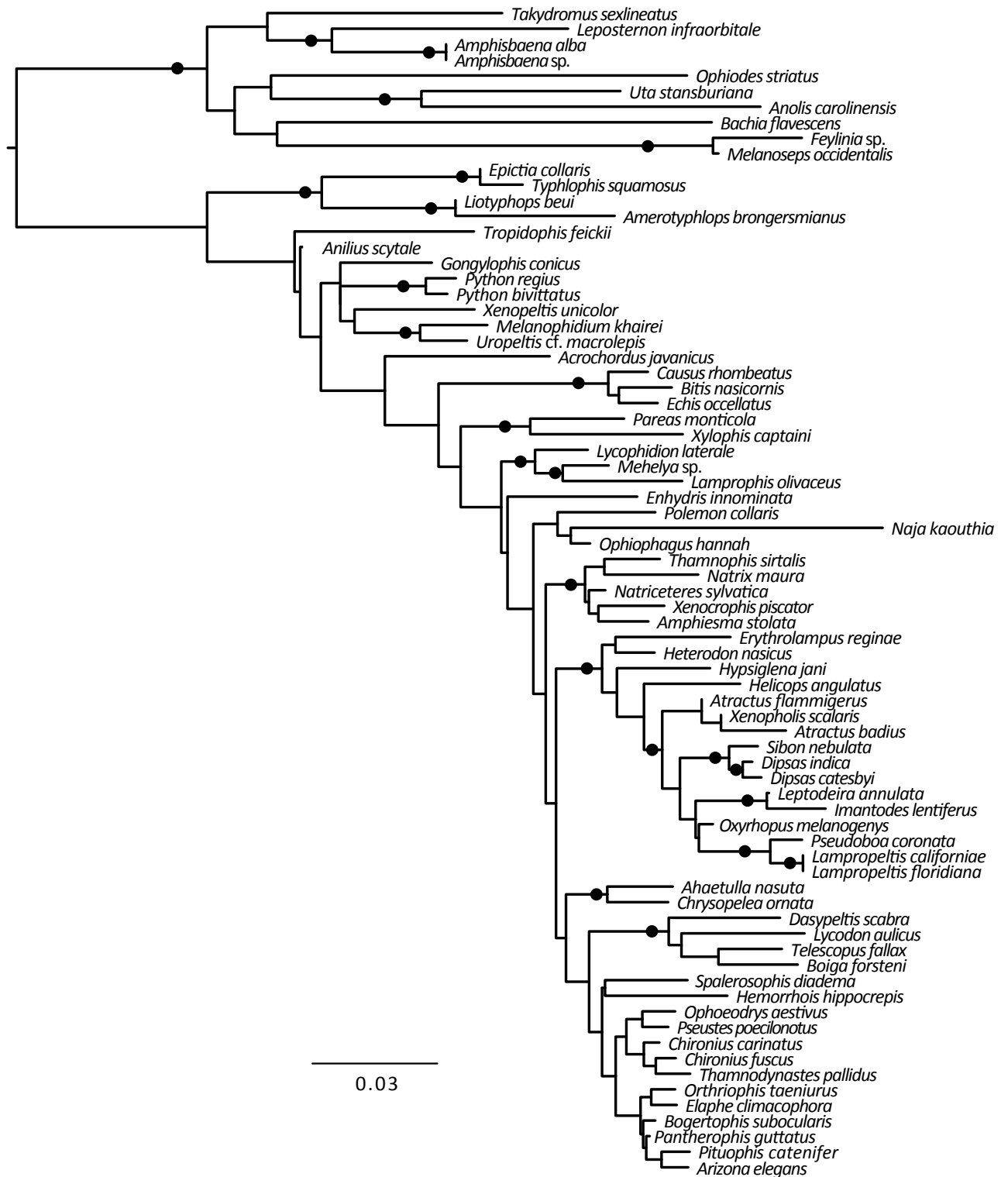


Figure S9. Maximum Likelihood rhodopsin 1 (*rh1*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

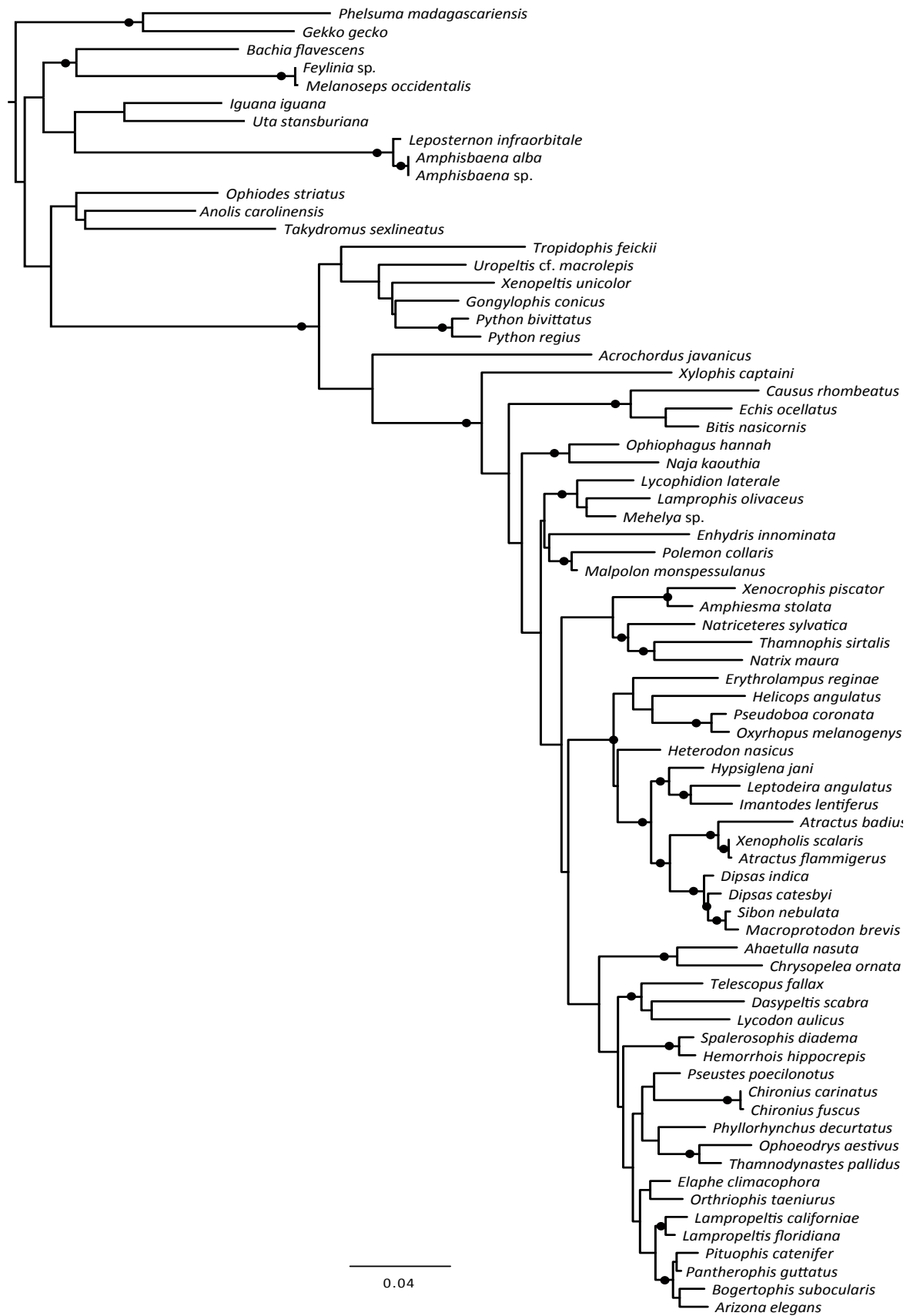


Figure S10. Maximum Likelihood short-wavelength opsin 1 (*sws1*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

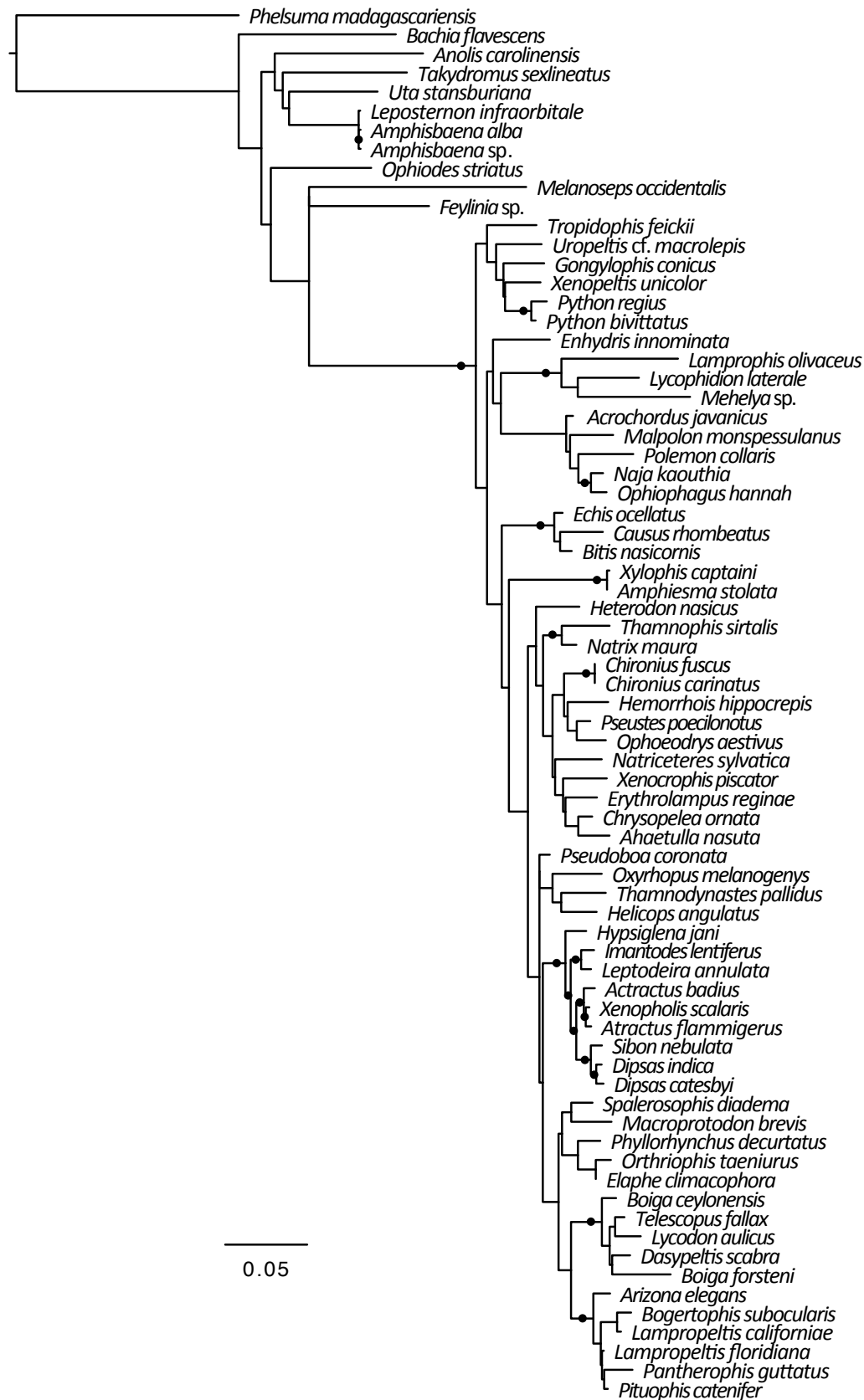


Figure S11. Maximum Likelihood medium-to-long wavelength opsin (*lws*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

Table S12. Ratio of synonymous to non-synonymous substitutions ($dN/dS = \omega$) for snake visual opsin gene sequences under branch models. $2\Delta l$ = twice the difference logarithm of the likelihood value for the models; D.F. = degrees of freedom used to compare the models (corresponding with the number of free parameters).

Models	ω (d_N/d_S)	D.F.	Models Compared	2Δ (ln L)	P
1. <i>sws1</i> opsin gene					
A. All branches have one ω	$\omega = 0.107$	-	-	-	-
B. Fossorial have ω_1 ; Non-fossorial ω_2	$\omega_1 = 0.062$; $\omega_2 = 0.112$	1	B vs. A	112.9	2.3 ⁻²⁶
C. Arboreal have ω_1 ; Non-arboreal ω_2	$\omega_1 = 0.097$; $\omega_2 = 0.108$	1	C vs. A	125.3	4.4 ⁻²⁹
D. (Semi)aquatic have ω_1 ; Non-aquatic ω_2	$\omega_1 = 0.092$; $\omega_2 = 0.108$	1	D vs. A	124.5	6.7 ⁻²⁹
E. Diurnal have ω_1 ; Nocturnal ω_2	$\omega_1 = 0.149$; $\omega_2 = 0.099$	1	E vs. A	117.9	1.8 ⁻²⁷
F. Colubrids have ω_1 ; Non-colubrids ω_2	$\omega_1 = 0.119$; $\omega_2 = 0.088$	1	F vs. A	117.4	2.7 ⁻²⁷
H. Every branch has its own ω	Variable by branch	117	H vs. A	254.6	3.5 ⁻¹²
1.1 <i>sws1</i> opsin gene (pruned)					
A1. All branches have one ω (double cones)	$\omega = 0.105$	-	-	-	-
B1. All branches have one ω (transmuted)	$\omega = 0.105$	-	-	-	-
C1. Double cone taxa have ω_1 ; others ω_2	$\omega_1 = 0.098$; $\omega_2 = 0.110$	1	C1 vs. A1	7.64	0.002
D1. Transmuted cone taxa have ω_1 ; others ω_2	$\omega_1 = 0.117$; $\omega_2 = 0.101$	1	D1 vs. B1	7.92	0.004
2. <i>lws</i> opsin gene					
I. All branches have one ω	$\omega = 0.312$	-	-	-	-
J. Fossorial have ω_1 ; Non-fossorial ω_2	$\omega_1 = 0.116$; $\omega_2 = 0.340$	1	J vs. I	121.3	3.4 ⁻²⁸
K. Arboreal have ω_1 ; Non-arboreal ω_2	$\omega_1 = 0.398$; $\omega_2 = 0.284$	1	K vs. I	150.8	1.1 ⁻³⁴
L. (Semi)aquatic have ω_1 ; Non-aquatic ω_2	$\omega_1 = 0.268$; $\omega_2 = 0.308$	1	L vs. I	155.3	1.2 ⁻³⁵
M. Diurnal have ω_1 ; Nocturnal ω_2	$\omega_1 = 0.342$; $\omega_2 = 0.296$	1	M vs. I	155.3	1.2 ⁻³⁵
N. Colubrids have ω_1 ; Non-colubrids ω_2	$\omega_1 = 0.422$; $\omega_2 = 0.193$	1	N vs. I	105.5	9.4 ⁻²⁵
P. Every branch has its own ω	Variable by branch	119	P vs. I	316.9	6.5 ⁻²⁰
2.1 <i>lws</i> opsin gene (pruned)					
I1. All branches have one ω (double cones)	$\omega = 0.311$	-	-	-	-
J1. All branches have one ω (transmuted)	$\omega = 0.305$	-	-	-	-
K1. Double cone taxa have ω_1 ; others ω_2	$\omega_1 = 0.387$; $\omega_2 = 0.248$	1	K1 vs. I1	66.18	4.9 ⁻¹⁶
L1. Transmuted cone taxa have ω_1 ; others ω_2	$\omega_1 = 0.543$; $\omega_2 = 0.273$	1	L1 vs. J1	66.08	4.3 ⁻¹⁶
3. <i>rh1</i> rhodopsin gene					
Q. All branches have one ω	$\omega = 0.237$	-	-	-	-
R. Fossorial have ω_1 ; Non-fossorial ω_2	$\omega_1 = 0.161$; $\omega_2 = 0.252$	1	R vs. Q	183.7	7.6 ⁻⁴²
S. Arboreal have ω_1 ; Non-arboreal ω_2	$\omega_1 = 0.212$; $\omega_2 = 0.240$	1	S vs. Q	192.6	8.7 ⁻⁴⁴
T. (Semi)aquatic have ω_1 ; Non-aquatic ω_2	$\omega_1 = 0.253$; $\omega_2 = 0.229$	1	T vs. Q	193.2	6.4 ⁻⁴⁴
U. Diurnal have ω_1 ; Nocturnal ω_2	$\omega_1 = 0.141$; $\omega_2 = 0.240$	1	U vs. Q	188.4	7.2 ⁻⁴³
V. Colubrids have ω_1 ; Non-colubrids ω_2	$\omega_1 = 0.252$; $\omega_2 = 0.212$	1	V vs. Q	191.0	1.9 ⁻⁴³
W. Scolecophidia have ω_1 ; Alethinophidia ω_2	$\omega_1 = 0.141$; $\omega_2 = 0.244$	1	W vs. Q	193.2	6.4 ⁻⁴⁴
Y. Every branch has its own ω	Variable by branch	127	Y vs. Q	272.2	1.4 ⁻¹²
3.1 <i>rh1</i> rhodopsin gene (pruned)					
Q1. All branches have one ω (double cones)	$\omega = 0.244$	-	-	-	-
R1. All branches have one ω (transmuted)	$\omega = 0.245$	-	-	-	-
S1. Double cone taxa have ω_1 ; others ω_2	$\omega_1 = 0.295$; $\omega_2 = 0.189$	1	S1 vs. Q1	68.22	5.7 ⁻¹⁵
T1. Transmuted cone taxa have ω_1 ; others ω_2	$\omega_1 = 0.388$; $\omega_2 = 0.212$	1	T1 vs. R1	65.23	6.7 ⁻¹⁶

Tabl S13. Ratio of synonymous to non-synonymous substitutions ($dN/dS = \omega$) for snake visual opsin gene sequences under site models. For each gene two pairs of models are compared to test for significant difference in goodness-of-fit to data. $2\Delta l$ = twice the difference logarithm of the likelihood value for the models; D.F. = degrees of freedom used to compare the models (corresponding with the number of free parameters).

Models	Parameters	D.F.	Models Compared	$2\Delta (\ln L)$	P
1. <i>sws1</i> opsin gene					
A. M1a	$\omega_0 = 0.039, \omega_1 = 1,$ $p_0 = 0.829$ ($p_1 = 0.171$)	-	-	-	-
B. M2a	$\omega_0 = 0.039, \omega_1 = 1, \omega_2 = 4.230,$ $p_0 = 0.830, p_1 = 0.168$ ($p_2 = 0.002$)	2	B vs. A	34.36	3.4 ⁻⁸
C. M7	$p = 0.154, q = 0.858$	-	-	-	-
D. M8 ($\beta\&\omega$)	$p_0 = 0.997$ ($p_1 = 0.003$), $p = 0.158, q = 0.923, \omega_s = 3.501$	2	D vs. C	24	6.1 ⁻⁶
2. <i>lws</i> opsin gene					
E. M1a	$\omega_0 = 0.032, \omega_1 = 1,$ $p_0 = 0.807$ ($p_1 = 0.193$)	-	-	-	-
F. M2a	$\omega_0 = 0.036, \omega_1 = 1, \omega_2 = 3.037,$ $p_0 = 0.800, p_1 = 0.140$ ($p_2 = 0.059$)	2	F vs. E	152.5	7.8 ⁻³⁴
G. M7	$p = 0.085, q = 0.317$	-	-	-	-
H. M8 ($\beta\&\omega$)	$p_0 = 0.927$ ($p_1 = 0.073$), $p = 0.111, q = 0.602, \omega_s = 0.693$	2	H vs. G	153.3	5.2 ⁻³⁴
2. <i>rh1</i> rhodopsin 1 gene					
I. M1a	$\omega_0 = 0.032, \omega_1 = 1,$ $p_0 = 0.789$ ($p_1 = 0.211$)	-	-	-	-
J. M2a	$\omega_0 = 0.033, \omega_1 = 1, \omega_2 = 2.46,$ $p_0 = 0.786, p_1 = 0.182$ ($p_2 = 0.032$)	2	J vs. I	29.9	3.2 ⁻⁷
K. M7	$p = 0.103, q = 0.408$	-	-	-	-
L. M8 ($\beta\&\omega$)	$p_0 = 0.935$ ($p_1 = 0.065$), $p = 0.144, q = 0.925, \omega_s = 1.881$	2	L vs. K	42.22	6.8 ⁻¹⁰

Table S14. Amino acid sites inferred to be under positive selection (using Bayes Empirical Bayes), identified under site models for the three visual opsin genes in snakes. Sites in bold are those known to be associated with spectral tuning of the corresponding visual pigment and those marked with an asterisk are associated with stabilization of the chromophore (retinal) pocket.

Models	Sites Under Positive Selection
1. <i>sws1</i> opsin gene	
B. M2a	13 – 152
D. M8 ($\beta\&\omega$)	86 – 93 – 103 – 106 – 110 – 120 – 257
2. <i>lws</i> opsin gene	
F. M2a	55 – 61 – 120 – 128* – 140 – 170 – 174 – 180 – 181 – 229 – 234 – 305
H. M8 ($\beta\&\omega$)	55 – 61 – 65 – 128* – 135 – 140 – 170 – 174 – 180 – 181 – 221 – 224* – 229 – 234 – 285 – 286 – 292 – 305
2. <i>rh1</i> rhodopsin gene	
J. M2a	19 – 81 – 83 – 112 – 119* – 133 – 159 – 213 – 217 – 290 – 299
L. M8 ($\beta\&\omega$)	11 – 19 – 81 – 83 – 112 – 119* – 133 – 158 – 159 – 173 – 209* – 213 – 217 – 290 – 292 – 299

Table S15. Amino acid sites inferred to be under positive selection (using Bayes Empirical Bayes), identified under branch-site models for the three visual opsin genes in particular phenotypic or phylogenetic groups (foreground branch) in snakes. Sites in bold are those known to be associated with spectral tuning.

Gene	Foreground branch	Sites under positive selection	2ΔI	P Value
lws	Colubridae	24 – 28 – 54 – 119 – 127 – 169 – 180 – 181 – 214 – 220 – 229 – 234 – 285 – 305	107.1	5.5 ⁻²⁴
	Aquatic	181 – 229	16.88	0.0002
	Arboreal	31 – 130 – 131 – 198 – 239 – 297	47.88	4.0 ⁻¹¹
	Fossorial	None	16.76	0.0002
	Diurnal	54 – 127 – 180 – 181 – 229 – 291 – 305	81.42	2.0 ⁻¹⁸
sws1	Colubridae	207 – 257 – 280 – 327	20.9	2.89 ⁻⁰⁵
	Aquatic	141	36.68	1.08 ⁻⁰⁸
	Arboreal	98 – 176 – 197	30.34	2.58 ⁻⁰⁷
	Fossorial	50 – 61	25.08	3.58 ⁻⁰⁶
	Diurnal	84 – 98 – 107	27.32	1.16 ⁻⁰⁶
rh1	Colubridae	155 – 213	8.4	0.0145
	Aquatic	48 – 155	8.6	0.0136
	Arboreal	30 – 217 – 323 – 332	6.92	0.0314
	Fossorial	168 – 210 – 241	7.52	0.0233
	Diurnal	4 – 38 – 151 – 155	13.08	0.0014

Table S16. Sites with changing properties under PRIME analysis for Atchley properties for the sws1 opsin gene. Changing properties are marked in bold.

Codon	t	α1	p1	α2	p2	α3	p3	α4	p4	α5	p5
28	1.944	0.840	1.000	2.159	0.824	15.938	0.080	8.291	0.879	-13.97	0.018
40	0.259	3.683	0.211	-2.59	0.011	0.368	1.000	-0.075	1.000	-0.888	0.812
51	0.956	0.855	0.513	0.594	0.755	-0.920	1.000	-6.268	0.008	7.687	0.688
<u>84</u>	0.008	2.414	0.057	-1.195	1.000	1.250	0.875	-4.338	0.031	-0.815	0.175
107	0.160	1.172	1.000	18.280	0.410	-0.617	1.000	-4.653	0.036	-0.086	1.000
177	1.283	1.992	0.518	16.784	0.325	-5.560	0.043	12.932	0.026	0.508	0.446
211	0.078	2.106	0.080	12.148	0.031	-4.740	0.000	5.059	0.005	1.416	0.015
222	1.937	6.047	0.000	-0.874	0.192	0.107	1.000	-1.684	0.031	0.653	0.601
313	0.340	11.350	0.068	-2.392	0.000	0.086	1.000	1.258	1.000	-0.023	1.000
331	5.181	3.327	0.019	-1.538	0.032	-0.016	1.000	6.020	0.017	-0.352	0.246

Table S17. Sites with changing properties under PRIME analysis for Conant-Stadler properties for the sws1 opsin gene. Changing properties are marked in bold.

Codon	t	α1	p1	α2	p2	α3	p3	α4	p4	α5	p5
11	0.317	-1.559	1.000	-1.323	1.000	16.937	1.000	0.968	1.000	-3.556	0.003
13	0.065	-1.387	1.000	-1.894	1.000	20.000	1.000	0.547	1.000	-5.047	0.039
54	0.218	-2.416	0.035	-0.119	1.000	0.074	1.000	14.246	0.335	-0.319	1.000
79	1.127	20.000	0.037	0.300	0.940	0.143	1.000	-20.00	0.012	14.148	0.037
103	1.661	20.000	0.075	1.297	1.000	-0.048	1.000	-20.00	0.008	14.660	0.027
120	0.526	20.000	0.001	-12.97	0.006	11.032	0.007	1.029	0.033	-2.232	0.155
313	0.636	-2.724	0.003	0.497	0.785	0.377	1.000	20.000	0.000	0.493	1.000
331	3.727	-3.055	0.028	2.709	0.038	-1.280	0.034	19.245	0.023	1.076	1.000

Table S18. Sites with changing properties under PRIME analysis for Atchley properties for the *lws* opsin gene. Changing properties are marked in bold.

Codon	t	$\alpha 1$	p1	$\alpha 2$	p2	$\alpha 3$	p3	$\alpha 4$	p4	$\alpha 5$	p5
17	13.425	3.179	1.000	1.524	0.016	2.227	0.002	0.058	1.000	-2.744	0.002
54	1.584	3.743	0.004	-1.881	0.128	-2.216	0.001	-3.667	0.010	9.560	0.000
140	2.181	6.755	0.049	-2.706	0.002	1.363	1.000	1.265	1.000	0.393	1.000
176	0.002	5.478	0.554	-3.017	0.026	-0.316	1.000	-0.239	1.000	-0.008	0.966
<u>180</u>	0.082	8.744	0.638	-1.886	0.009	-0.587	1.000	-0.966	1.000	0.328	1.000
181	3.057	6.106	0.000	-0.479	0.128	0.577	0.080	-3.543	0.002	-0.637	0.139
225	0.494	0.584	1.000	-2.122	0.013	0.194	0.511	0.195	0.836	-0.261	1.000
230	1.173	1.364	0.019	0.313	0.473	1.254	0.019	0.306	1.000	-2.402	0.004

Table S19. Sites with changing properties under PRIME analysis for Conant-Stadler properties for the *lws* opsin gene. Changing properties are marked in bold.

Codon	t	$\alpha 1$	p1	$\alpha 2$	p2	$\alpha 3$	p3	$\alpha 4$	p4	$\alpha 5$	p5
132	3.155	0.268	0.481	-9.982	0.008	0.605	0.573	10.505	0.031	7.309	0.008
168	1.779	20.000	0.024	-20.00	0.007	16.512	0.090	15.514	0.061	1.236	0.464
181	2.422	0.556	0.253	-9.902	0.000	12.121	0.000	19.883	0.000	-0.876	0.327
245	3.728	20.000	0.000	-19.09	0.000	17.237	0.000	11.033	0.000	0.649	0.750

Table S20. . Sites with changing properties under PRIME analysis for Atchley properties for the *rh1* opsin gene. Changing properties are marked in bold.

Codon	t	$\alpha 1$	p1	$\alpha 2$	p2	$\alpha 3$	p3	$\alpha 4$	p4	$\alpha 5$	p5
4	1.629	-1.441	0.096	20.000	0.103	-0.281	1.000	-13.85	0.017	2.576	0.067
19	0.444	1.232	1.000	2.110	0.937	1.314	0.401	-0.308	0.935	-3.16	0.019
30	0.499	0.933	1.000	-1.202	1.000	0.872	1.000	-4.807	0.017	0.481	1.000
57	0.872	19.500	0.788	1.952	0.591	0.764	0.494	-7.650	0.000	0.417	0.445
154	0.474	1.301	0.274	0.536	1.000	-0.732	0.246	-2.963	0.007	2.201	0.076
214	2.458	0.095	1.000	-0.059	1.000	2.288	0.012	1.290	0.748	-2.697	0.041
232	0.033	0.966	1.000	-0.324	1.000	-3.532	0.045	0.796	1.000	9.781	0.005
248	0.520	5.908	0.063	1.156	1.000	-1.086	1.000	-5.570	0.016	0.678	0.672
308	0.880	1.312	1.000	-0.742	1.000	-2.456	0.005	1.340	0.539	3.680	0.032
318	0.619	1.587	1.000	1.296	1.000	-3.349	0.022	0.246	1.000	5.352	0.059
332	0.861	-6.025	0.048	-0.692	1.000	0.232	1.000	10.358	0.010	0.067	1.000

Table S21. Sites with changing properties under PRIME analysis for Conant-Stadler properties for the *rh1* opsin gene. Changing properties are marked in bold.

Codon	t	$\alpha 1$	p1	$\alpha 2$	p2	$\alpha 3$	p3	$\alpha 4$	p4	$\alpha 5$	p5
41	0.504	-4.465	0.006	0.418	0.232	-2.915	0.054	20.000	0.022	5.871	0.033
57	0.675	20.000	1.000	0.665	0.736	10.604	0.591	3.646	1.000	-8.104	0.008
169	0.634	-1.819	0.365	0.937	1.000	-0.111	1.000	20.000	0.001	-0.572	1.000
185	0.774	-1.423	0.572	-11.56	0.034	4.003	0.644	-1.957	0.598	14.370	0.040
217	7.231	3.924	0.562	-3.880	0.024	0.694	0.750	4.338	0.000	-0.419	1.000
270	0.474	20.000	0.210	-20.00	0.008	11.823	0.066	14.749	0.055	0.665	1.000

Table S22. λ 50% light cut-off and %UVA transmission in lenses and spectacles in snakes. All data newly generated for this study except for *Pantherophis guttatus* (data from Thorpe 1991).

Family	Species (number of lenses, spectacles)	Mean Lens Diameter (mm)	%UVA		Total %UVA	λ 50% (nm)	
			Lens	Spectacle		Lens	Spectacle
Acrochordidae	<i>Acrochordus javanicus</i> (1,2)	1.35	97.4	52.8	51.2	310	346
Homolopsidae	<i>Enhydris innominata</i> (2,2)	—	68.1	49.6	34.8	324	355
Lamprophiidae	<i>Malpolon monspessulanus</i> (2,0)	2.13	1.7	—	—	451	—
	<i>Lamprophis olivaceus</i> (1,0)	1.35	87.2	—	—	306	—
Elapidae	<i>Naja kaouthia</i> (2,2)	1.95	80.4	47.7	38.8	312	360
Colubridae	<i>Thamnophis sirtalis</i> (1,1)	1.75	35.8	61.8	21.6	413	340
	<i>Ahaetulla nasuta</i> (2,0)	1.75	7.6	—	—	424	—
	<i>Chrysopelea ornata</i> (2,2)	2.75	1.9	33.9	0.4	438	371
	<i>Telescopus fallax</i> (2,2)	1.58	39.3	74.3	29.5	380	304
	<i>Opheodrys aestivus</i> (2,2)	1.63	37.8	48.5	18.9	398	354
	<i>Spalerosophis diadema</i> (2,2)	2.33	21.3	63.5	13.5	417	338
	<i>Elaphe climacophora</i> (2,2)	1.87	51.2	58.6	31.5	357	346
	<i>Pituophis catenifer</i> (1,2)	2.15	46.2	45.7	23.3	373	355
	<i>Orthriophis taeniurus</i> (2,2)	2.01	57.0	56.5	34.1	327	348
	<i>Bogertophis subocularis</i> (2,2)	1.95	93.2	58.3	55.9	313	345
	<i>Dasypeltis scabra</i> (1,2)	2.20	66.2	74.5	49.4	318	315
	<i>Arizona elegans</i> (2,2)	2.10	63.0	73.5	48.0	327	330
	<i>Heterodon nasicus</i> (1,2)	—	—	40.6	—	—	368
	<i>Pantherophis guttatus</i> (1,0)	2.89	28.2	—	—	385	—

References

- Ahmed MF, Das A, Dutta SK. 2009. Amphibians and Reptiles of Northeast India. Aaranyak: Guwahati
- Arnold N, Ovenden D. 2002. A Field Guide to the Reptiles and Amphibians of Britain and Europe. HarperCollins UK
- Asenjo AB, Rim J, Oprian DD. 1994. Molecular determinants of human red/green color discrimination. *Neuron* 12:1131–1138.
- Babu KR, Dukkupati A, Birge RR, Knox BE. 2001. Regulation of Phototransduction in Short-Wavelength Cone Visual Pigments via the Retinylidene Schiff Base Counterion. *Biochemistry* 40:13760–13766.
- Baumeister L. 1908. Beiträge zur anatomie und physiologie der Rhinophiden. Integument, Drüsen der Mundhöhle, Augen und Skeletsystem. :423–526.
- Chirio L, LeBreton M. 2007. Atlas des reptiles du Cameroun. IRD Editions
- Cowing JA, Poopalasundaram S, Wilkie SE, Robinson PR, Bowmaker JK, Hunt DM. 2002. The molecular mechanism for the spectral shifts between vertebrate ultraviolet- and violet-sensitive cone visual pigments. *Biochem. J.* 367:129.
- Das, I. 2015. A Field Guide To The Reptiles Of South-East Asia. London: Bloomsbury Publishing.
- Davies WL, Cowing JA, Bowmaker JK, Carvalho LS, Gower DJ, Hunt DM. 2009. Shedding Light on Serpent Sight: The Visual Pigments of Henophidian Snakes. *J. Neurosci.* 29:7519–7525.
- Ernst CH, Ernst EM. 2011. Venomous Reptiles of the United States, Canada, and Northern Mexico. JHU Press
- Fasick JI, Applebury ML, Oprian DD. 2002. Spectral tuning in the mammalian short-wavelength sensitive cone pigments. *Biochemistry* 41:6860–6865.
- Govardovskii VI, & Chkheidze NI (1989). Retinal photoreceptors and visual pigments in certain snakes. *Biological Abstracts*, 90: 1036.
- Gower DJ, Giri V, Captain A, Wilkinson M. 2016. A reassessment of *Melanophidium* Günther, 1864 (Reptilia: Serpentes: Uropeltidae) from the Western Ghats of peninsular India, with the description of a new species. *Zootaxa* 4085: 481–503.
- Gower DJ, Winkler JD. 2007. Taxonomy of the Indian snake *Xylophis* Beddome (Serpentes: Caenophidia), with description of a new species. *Hamadryad* 31:315–329.
- Hunt DM, Dulai KS, Partridge JC, Cottrill P, Bowmaker JK. 2001. The molecular basis for spectral tuning of rod visual pigments in deep-sea fish. *J. Exp. Biol.* 204:3333–3344.
- Kley NJ. 2003a. Early blindsnakes (Anomalepididae). In: Hitchins M, Murphy JB, Schlager N, editors. *Grzimeks Animal Life Encyclopedia*. Vol. 7. Farmington Hills, MI. pp. 369–372.
- Kley NJ. 2003b. Blindsnakes (Typhlopidae). In: Hutchins M, Murphy JB, Schlager N, editors. *Grzimeks Animal Life Encyclopedia*. Vol. 7. Farmington Hills, MI: Gale Group. pp. 379–385.

- Munk O, Rasmussen JB. 1993. Note on the Rod-like Photoreceptors in the Retina of the Snake *Telescopus fallax* (Fleischmann, 1831). *Acta Zool.* 74:9–13.
- Murphy JC. 2007. Homalopsid Snakes. Malabar, Florida: Krieger Publishing Company
- Schwartz A. 1957. A new species of boa (genus *Tropidophis*) from western Cuba. *American Museum Novitates*: 8.
- Shine R, Branch WR, Harlow PS, Webb JK. 1996. Sexual dimorphism, reproductive biology, and food habits of two species of African filesnakes (*Mehelya*, Colubridae). *J. Zool. Lond.* 240:327–340.
- Sillman AJ, Carver JK, Loew ER. 1999. The Photoreceptors and Visual Pigments in the Retina of a Boid Snake, the Ball Python (*Python regius*). *J. Exp. Biol.* 202:1931–1938.
- Sillman AJ, Govardovskii VI, Rohlich P, Southard JA, Loew ER. 1997. The photoreceptors and visual pigments of the garter snake (*Thamnophis sirtalis*): a microspectrophotometric, scanning electron microscopic and immunocytochemical study. *J. Comp. Physiol. A* 181:89.
- Simões BF, Sampaio FL, Jared C, Antoniazzi MM, Loew ER, Bowmaker JK, Rodriguez A, Hart NS, Hunt DM, Partridge JC, et al. 2015. Visual system evolution and the nature of the ancestral snake. *J. Evol. Biol.* 28:1309–1320.
- Simões, B. F., Sampaio, F. L., Loew, E. R., Sanders, K. L., Fisher, R. N., Hart, N. S., et al. (2016). Multiple rod-cone and cone-rod photoreceptor transmutations in snakes: evidence from visual opsin gene expression. *Proc. R. Soc. B*, 283 (1823).
- Spawls S, Howell K, Drewes RC. 2006. Pocket Guide to the Reptiles and Amphibians of East Africa. London: Christopher Helm Publishers.
- Starace F, Lambert V. 2013. Serpents et amphibènes de Guyane française. Ibis Rouge
- Thorpe A. 1991. Spectral transmission and short-wave absorbing pigments in the lenses of fish and other animals. City University, London
- Underwood, G. 1966. On the visual-cell pattern of a homalopsine snake. *J. Anat.* 100, 571–575.
- Underwood G. 1967. A Contribution to the Classification of Snakes. British Museum Natural History Publications
- Underwood G. 1970. The Eye. In: Gans C, Parsons TS, editors. *Biology of the Reptilia: Morphology B*. Vol. 2. New York: Academic Press. pp. 1–97.
- Underwood, G., & Kochva, E. (1993). On the affinities of the burrowing asps *Atractaspis* (Serpentes: Atractaspididae). *Zool. J. Linn. Soc.*, 107: 3–64.
- Walls GL. 1942. The Vertebrate Eye and Its Adaptive Radiation. New York: Fafner Publishing Company
- Whitaker R, Captain A, Photographer FA, Chaudhuri S. 2004. Snakes of India. Draco Books
- Yokoyama S, Radlwimmer FB. 1998. The “five-sites” rule and the evolution of red and green color vision in mammals. *Mol. Biol. Evol.* 15:560–567.
- Yokoyama S, Starmer WT, Takahashi Y, Tada T. 2006. Tertiary structure and spectral tuning of UV and violet pigments in vertebrates. *Gene* 365:95–103.

- Yokoyama S, Tada T, Zhang H, Britt L. 2008. Elucidation of phenotypic adaptations: Molecular analyses of dim-light vision proteins in vertebrates. *Proc. Natl. Acad. Sci. USA* 105:13480–13485.
- Yokoyama S. 2000. Molecular Evolution of Vertebrate Visual Pigments. *Prog. Retin. Eye Res.* 19:385–419.
- Yokoyama S. 2008. Evolution of Dim-Light and Color Vision Pigments. *Annu. Rev. Genomics Hum. Genet.* 9:259–282.