Visual Pigments, Ocular Filters and the Evolution of Snake Vision

Bruno F. Simões,*[,1] Filipa L. Sampaio,[1] Ronald H. Douglas,[2] Ullasa Kodandaramaiah,[3] Nicholas R. Casewell,[4] Robert A. Harrison,[4] Nathan S. Hart,[5] Julian C. Partridge,[6] David M. Hunt,[6,7] and David J. Gower*[,1]

[1]Department of Life Sciences, The Natural History Museum, London, United Kingdom

[2]Department of Optometry and Visual Science, City University London, London, United Kingdom

[3]School of Biology, Indian Institute of Science Education and Research Thiruvananthapuram, Thiruvananthapuram, India

[4]Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Liverpool, United Kingdom

[5]Department of Biological Sciences, Macquarie University, North Ryde, NSW, Australia

[6]School of Animal Biology and the Oceans Institute, The University of Western Australia, Perth, WA, Australia

[7]Lions Eye Institute, University of Western Australia, Perth, Australia

***Corresponding author:** E-mail: bruno.simoes@me.com; d.gower@nhm.ac.uk.

Associate editor: Nicholas Vidal

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 analyze 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*, and *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 inferred to be under purifying selection, though dN/dS varies with respect to some lineages, ecologies, and retinal anatomy. Positive selection was inferred 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, whereas 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 exceptionally diverse compared with all other extant tetrapod orders.

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 (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 color 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 A_1 or A_2: Enright et al. 2015) and gene expression (Hofmann and Carleton 2009; Carleton et al. 2010) underlie adaptations to differing ecological and behavioral selection pressures. Visual opsins in some vertebrates have been studied intensely over the past 20 years, to the extent that changes in specific ("spectral tuning") amino acid sites

Article

Fast Track

are known to change the peak absorbance wavelength (λ_{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 those 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; Schott et al. 2016; Simões et al. 2016).

The approximately 3,500 species of living snakes are distributed across all continents except Antarctica (Wallach et al. 2014). They are very diverse ecologically (Greene 1997) and include burrowing, arboreal, gliding, fully aquatic, nocturnal and diurnal species. Some have small eyes lying under typical head scales, whereas 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 humor. 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 uncolored lenses in nocturnal species. However, these observations were qualitative, using the UV-insensitive human visual system, such that the spectral characteristics of both colored and transparent lenses in the UV are unknown, with the exception of two species of sea snake whose lenses transmit substantial 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 snake retinal photoreceptor anatomy, visual opsin and ocular media transmission linked in a predictable way? (3) To what extent is snake 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 data set 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 to have been 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.

Materials and 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 (supplementary table S1, Supplementary Material online) aimed to maximize 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 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 500 ng 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 min. For the following species freshly synthesized cDNA was dehydrated, stored at ambient temperature for 24 h, and returned to −20 °C and rehydrated after a further 24 h before subsequent amplification: *Melanophidium* sp., *Uropeltis* cf. *macrolepis*, *Gongylophis conicus*, *Pareas monticola*, *Amphiesma stolata*, *Xenochrophis piscator*, *Xylophis captaini*, *Boiga forsteni*, and *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): 1× PCR buffer (Invitrogen), 1.5 mmol (mM) of $MgCl_2$ (Invitrogen), 50 µmol/l of deoxynucleotides (Bioline), 0.4 µmol/l of each primer and 1 unit Platinum Taq Polymerase (Invitrogen) and 100 ng of cDNA. PCR products were amplified by touchdown PCR with the following cycling parameters: initial denaturation at 95 °C for 5 min; 20 cycles of 1 min at 95 °C (denaturation), 30 s at 60 °C (annealing), and 1 min at 72 °C (extension) with a decrease of 0.5 °C per cycle; 15 cycles of 1 min at 95 °C (denaturation), 30 s at 50 °C (annealing), and 1 min at 72 °C (extension) followed by a final extension at 72 °C for 5 min. 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 1 ml of 2% X-GAL at 37 °C. Sixteen white colonies were picked and used as DNA template in 25 µl PCR reactions: 1× PCR buffer (Bioline), 1 mmol (mM) of $MgCl_2$ (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 2 µl of DNA (1 colony twirled in 50 µl of ultra-pure water). The PCR had the following cycling parameters: initial denaturation at 95 °C for 10 min; 30 cycles of 15 s at 95 °C (denaturation), 30 s at 58 °C (annealing), and 1 min and 30 s at 72 °C (extension) and a final extension at 72 °C for 1.5 min. 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 KX237782-KX237922 (supplementary table S1, Supplementary Material online).

Barcoding

Genomic DNA (gDNA) was extracted from each eye tissue sample using the DNA layer in Trizol of the RNA extraction, following the Trizol manufacturer's instructions and/or from muscle tissue stored in ethanol using the Qiagen blood and tissue kit. We generated mitochondrial *16s rRNA* "barcodes" for most specimens (supplementary table S1, Supplementary Material online) using universal primers (Palumbi 1996) in 25 µl PCR reactions: 1× PCR buffer (Invitrogen), 1 mmol (mM) of $MgCl_2$ (Invitrogen), 50 µmol/l of deoxynucleotides (Bioline), 0.4 µmol/l of each primer and 1 unit Platinum Taq Polymerase (Invitrogen) and 100 ng of gDNA. The PCR cycling parameters were: initial denaturation at 95 °C for 10 min; 30 cycles of 15 s at 95 °C (denaturation), 30 s at 55 °C (annealing), and 1 min at 72 °C (extension) and a final extension at 72 °C for 1 min. All successfully amplified products were sequenced in both directions using the same primers used for PCR, in an automated DNA sequencer. The barcodes were assembled in Geneious R8 and are deposited in GenBank, accession numbers KX277230-KX277272 (see supplementary table S1, Supplementary Material online).

Phylogenetic Analysis

Visual opsin gene cDNA sequences were aligned with published sequences from other reptiles including other snakes (supplementary table S1, Supplementary Material online) with MAFFT (Katoh et al. 2002) (settings: algorithm; auto; gap penalty: 3; off-set value: 0.1) implemented in Geneious R8, Muscle (Edgar 2004) (default settings: 15 interactions), and PRANK (Löytynoja and Goldman 2005) (HKY model with empirical base frequencies and kappa = 2). These alignments were inspected by eye for both nucleotides and amino acids and were adjusted manually to ensure nucleotides were in-frame and that indels did not include partial codons. The final alignments based on the results of all three programs were identical. jModelTest 2 (Darriba et al. 2012) was used to ascertain the best-fit model of sequence evolution for each alignment according to their AIC and BIC scores. GTR + G+I was the best-fitting model for the three visual opsin genes amplified. Given concerns about incorrectly estimating G when including I in the model (Yang 2006), we also ran analyses under GTR + G. Phylogenetic analyses were conducted using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. ML analyses were run with RAxML v8 (Stamatakis 2014) using majority rule bootstopping criteria (Pattengale et al. 2009), randomized MP starting trees, and a fast hill-climbing algorithm. BI analyses were run with Mr. Bayes v3.1.2 (Huelsenbeck and Ronquist 2001) for 1,000,000 generations with chains sampled every 100 generations (after 25% of trees were discarded as burn-in), random starting trees, 4 chains (3 hot and 1 cold), and convergence was assumed when the standard deviation of split frequencies fell <0.01. Gekkotans were used as the outgroup to root the *sws1* and *lws* trees, and other nonsnake squamate visual opsin gene sequences were used to root the *rh1* tree (supplementary table S1, Supplementary Material online).

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 nonsynonymous (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 ω 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 value across the tree, whereas the more complex free-ratio model allows independent ω for each branch. Branch models were 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 figure 1 and supplementary table S2, Supplementary Material online. Given the substantial diversity of retinal morphology, ecology and density of our sampling within the family, we also applied branch models to Colubridae alone. The fit of branch models was assessed using the Likelihood Ratio Test (LRT), with the simpler model (one-ratio) rejected where $P < 0.05$. Branch models were also applied to a subset of taxa for which the photoreceptor cell complement is known (supplementary table S2, Supplementary Material online) to test for possible links between molecular evolution and the presence/absence of double cones and 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; M7β 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 their simpler corresponding alternative 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 determine whether positive selection at sites could be inferred for various major groups of snakes (Colubridae, snakes with transmuted, rod-like cones and snakes that are primarily fossorial, arboreal, aquatic/semiaquatic and diurnal). In each case, 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 (Delport et al. 2010) to estimate amino acid exchangeability (as in BEB) and also to identify 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. (2005). CMS (Delport 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) (fig. 1). Although the monophyly of the colubrid clades Colubrinae, Natricinae and Dipsadinae are well supported (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 (McDowell 1987), we consider the similarity to the Sri Lankan *Aspidura* also suggestive of phylogenetic affinity (Gans and Fetcho 1982; Gower and Winkler 2007) and 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 among 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.

Functionality and Spectral Sensitivity

In many cases it has proved possible to predict peak absorbance (λ_{max}) of visual pigments from amino acid sequences of their constituent opsins. Such predictions are possible because strong correlations exist between amino acid sequences of opsins and λ_{max} of corresponding pigments where that has been measured directly in photoreceptors or where opsin genes have been cloned and pigments regenerated in vitro. The spectral location of the λ_{max} of a pigment depends on the constituent chromophore as well as the amino acid residues present at so-called tuning sites in the opsin protein (see Yokoyama 2008 and references cited therein). We made predictions of λ_{max} by assuming a vitamin A_1 chromophore because an A_2 chromophore has not been reported in snakes (Sillman et al. 1997; Davies et al. 2009; Hart et al. 2012; Simões et al. 2015, 2016; Schott et al. 2016), though is known to occur in some lizards (Martin et al. 2015). For the three visual pigments found in snakes, spectral tuning sites are generally highly conserved and similar to those found in other vertebrates, such that it is possible to predict the λ_{max} for RH1 (Nathans 1990; Hunt et al. 1996, 2001; Fasick and Robinson 2000; Simões et al. 2016), SWS1 (Hunt et al. 2009; Sekharan et al. 2013; Hunt and Peichl 2013; Simões et al. 2016), and LWS (Yokoyama 1995; Yokoyama and Radlwimmer 1998; Davies et al. 2012; Simões et al. 2016) based on the particular amino

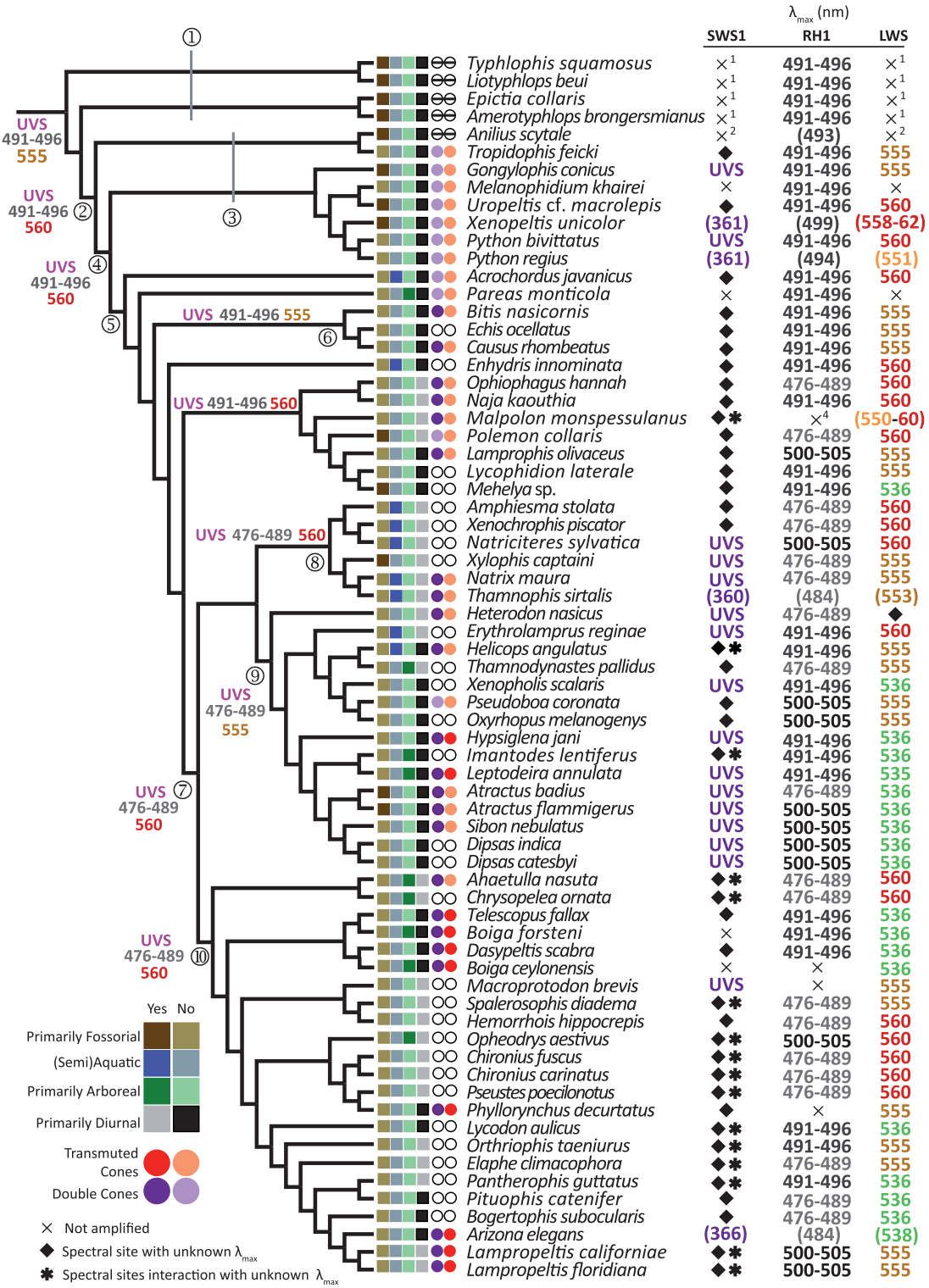

Fig. 1. Snake species tree and phenotypic classifications (see Materials and Methods section for more information) used in analyses of opsin gene evolution. Numbers within circles represent snake higher taxa: (1) Scolecophidia (not recovered in some molecular phylogenies); (2) Alethinophidia; (3) Henophidia (not recovered in molecular phylogenies); (4) Afrophidia; (5) Caenophidia; (6) Viperidae; (7) Colubridae; (8) Natricinae; (9) Dipsadinae; and (10) Colubrinae. Phenotype classifications are 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 (λ_{max}) values for each visual pigment are those predicted from cDNA sequences except where values have been determined spectrally (in parentheses)—see Results section and supplementary tables S3–S5, Supplementary Material online. Ancestral pigment λ_{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); and (3) No visual pigment with an RH1-like λ_{max} was detected by MSP for *Malpolon* (Govardovskii and Chkheidze 1989). UVS, ultraviolet sensitive (λ_{max} c. 360 nm).

acids occupying the tuning sites. Predicting λ_{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). However, the limited microspectrophotometry (MSP) data published thus far for snake visual pigments generally match predictions based on known tuning sites in other vertebrates (Davies et al. 2009; Simões et al. 2015, 2016). We were unable to make confident λ_{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 λ_{max} has not been measured.

Ocular Media Spectral Transmission

We examined spectral transmission of lenses and spectacles. Corneas and humors 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 humors (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 (supplementary table S22, Supplementary Material online), limiting the amount of light transmitted through the measuring system, and the use of 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 51 nm) using Matlab R2011a (The MathWorks Inc, MA). 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 not primarily diurnal species using the package ggplots2 (Wickham 2010) implemented in R (R Core Team 2014).

Results

We sequenced ~1100 bp 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 4 and 14 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 data set 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 supplementary tables S3–S5, Supplementary Material online. For vertebrate *rh1* sequences, 12 tuning sites have been reported (Yokoyama et al. 2008). Of these however, only sites 83 and 292 vary across the sampled snakes: substitutions N83D and A292S are widespread across snake evolutionary history with multiple independent origins and reversals in Colubridae, Elapidae and Lamprophiidae (supplementary table S3, Supplementary Material online). The ancestral snake is reconstructed as having N83 and A292, and the λ_{max} values for pigments with opsins with identical sets of the 12 tuning site residues to those inferred for the ancestral snake have been determined spectrally in other vertebrates to be 491–496 nm (Nathans 1990; Hunt et al. 1996; Yokoyama et al. 2008). Inferred ancestral colubrine and colubrid *rh1* sequences encode a combination of N83 and S292 (seen also in several extant colubroids: fig. 1), a combination seen in vertebrates with RH1 λ_{max} of 476–489 nm (Hunt et al. 1996, 2001; Yokoyama et al. 2008). Finally, the combination of D83 and A292 occurring in many snakes is expected to give a λ_{max} of 500–505 nm based on studies of other vertebrates (Hunt et al. 1996; Yokoyama et al. 2008). These predicted λ_{max} values have been incorporated into figure 1.

The vertebrate SWS1 pigment exists in two spectrally very distinct forms, a UV-sensitive (UVS) pigment with λ_{max} of ~360 nm and a violet-sensitive (VS) pigment with λ_{max} generally >410 nm (reviewed in Hunt and Peichl 2014). This shift is caused primarily by substitution at site 86, with F86 present in UVS pigments and Y86, S86 or V86 in VS pigments (Cowing et al. 2002; Parry et al. 2004; Yokoyama 2005). Other reported tuning substitutions at sites 93, 97, 113 and 118 in vertebrate SWS1 are correlated with λ_{max} variation only within VS pigments (Yokoyama et al. 2006; Hunt and Peichl 2014). Inferred ancestral *sws1* sequences suggest a UVS SWS1 pigment in the ancestor of several major snake clades, including Alethinophidia, Afrophidia and Colubridae (fig. 1 and supplementary table S4, Supplementary Material online). Six sampled snake species have S86 or V86 (supplementary table S4, Supplementary Material online) and would be expected therefore to have VS pigments, although such a prediction is uncompelling in these cases because these species also have combinations of residues at potential tuning sites

that are not known in other vertebrates for which SWS1 λ_{max} has been determined spectrally.

For *lws*-based pigments, the major spectral tuning sites have been identified as 180, 197, 277, 185 and 308 from studies of many vertebrate species and confirmed by MSP and spectral analysis of in vitro expressed pigments (reviewed by Hunt and Collin 2014). At these, snake LWS pigments vary only at sites 180, 285 and 308 (supplementary table S5, Supplementary Material online). The substitution S180A has occurred several times within snakes to give multiple shifts between predicted λ_{max} values of 560 and 555 nm (fig. 1 and supplementary table S5, Supplementary Material online), and a T285A substitution in combination with S180A has likely shifted the λ_{max} further to 536 nm in several snake lineages, notably within colubrine and dipsadine colubroids (fig. 1 and supplementary table S5, Supplementary Material online). In the terminal branch leading to *Heterodon nasicus*, an A308S substitution occurred; 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 we predict that the presence of S308 in *H. nasicus* produces an equivalent short-wave shift. Ancestors of most major snake lineages are reconstructed as having an LWS pigment λ_{max} of 555 nm, with likely shifts to shorter wavelength λ_{max} occurring independently on multiple occasions within Colubridae (fig. 1 and supplementary table S5, Supplementary Material online).

Where the location of λ_{max} has been determined spectrally for snake pigments, predictions of λ_{max} made here based on tuning site amino acids and knowledge of λ_{max} in other vertebrates (supplementary tables S3–S5, Supplementary Material online) are very similar in almost all cases. Of the 12 comparisons that can be made, 8 are within the predicted range and 3 are only 1–3 nm outside the predicted ranges. The single notable departure is the 551 nm (Davies et al. 2009) *Python regius* LWS pigment λ_{max} that is predicted to be 560 nm (Sillman et al. 1999).

Phylogenetics

The inferred *rh1* and *sws1* trees (supplementary figs. S6–S11, Supplementary Material online) 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 nonmonophyly 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) (supplementary table S12, Supplementary Material online) suggest that all three visual opsin genes are under purifying selection ($\omega_{RH1} = 0.237$; $\omega_{SWS1} = 0.107$; $\omega_{LWS} = 0.312$), 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 notably different (data not shown), indicating that these results are robust with respect to this phylogenetic uncertainty.

For the *sws1* opsin gene, branch models (supplementary table S12, Supplementary Material online) suggest that nonfossorial (0.112), nonarboreal (0.108), nonaquatic (0.108) 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 data set 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 (0.098 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 (0.116, 0.284, 0.268 and 0.296, respectively). Colubrids have notably higher ω values (0.422) than non-colubrids (0.193). Higher ω values occur in species with transmuted cones (0.543 vs. 0.273) and in species with double cones (0.387 vs. 0.248). The free-ratio ω ranges from 0.001 to 7.1 (supplementary table S12, Supplementary Material online).

The *rh1* ω ratios among nonfossorial (0.252), nonarboreal (0.240), aquatic/semiaquatic (0.253) and non-diurnal (0.240) snake lineages are higher than for their counterparts (0.161, 0.212, 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 (0.295 vs. 0.189) (supplementary table S12, Supplementary Material online). The free-ratio ω ranges from 0.001 to 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 (supplementary table S12, Supplementary Material online).

Site models results infer several instances of positive selection at the codon level across the three visual opsin genes present in snakes (supplementary table S13, Supplementary Material online). Models 2a and M8 (β&ω) are a significantly better fit than the simpler models M1a and M7, respectively (supplementary table S13, Supplementary Material online).

According to Bayes Empirical Bayes (BEB) implemented in site models M2a and M8 (β&ω) there are two and seven *sws1*

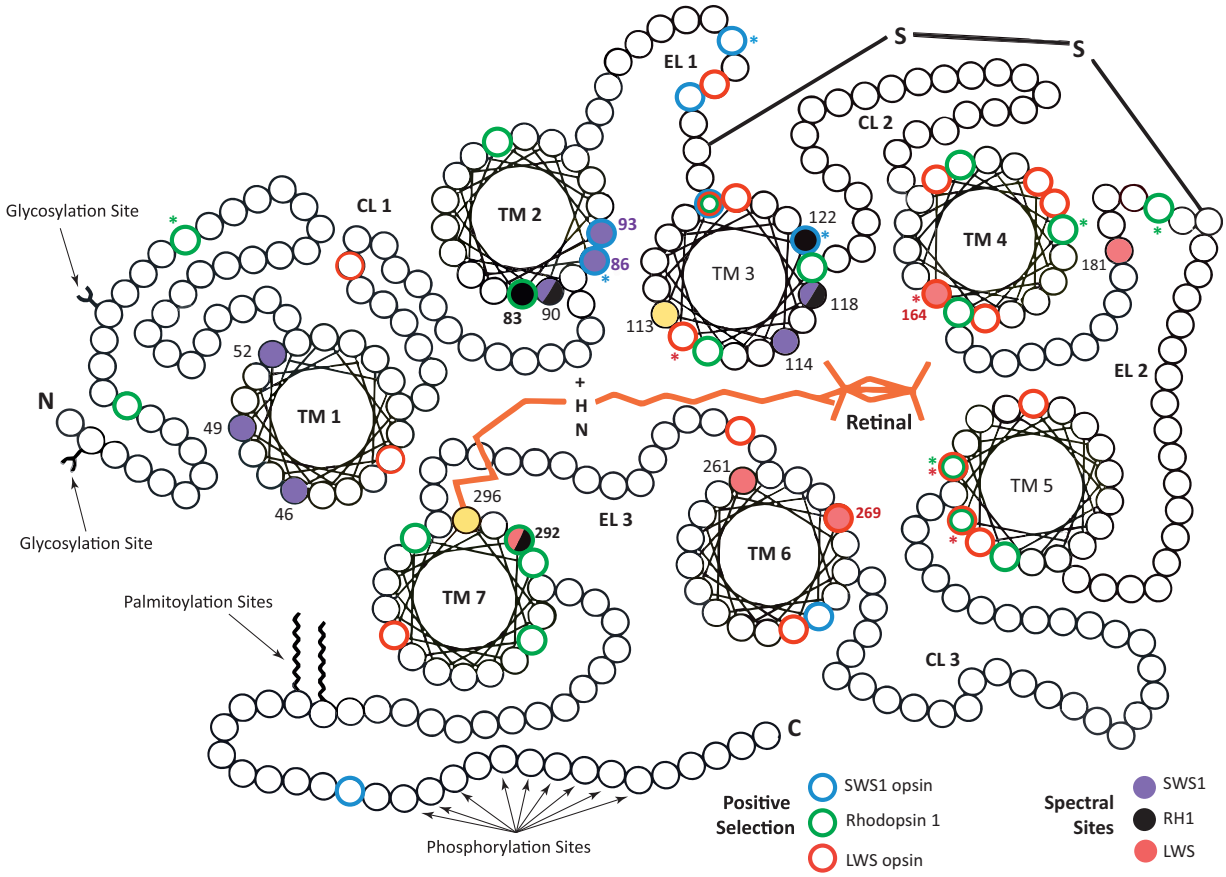

Fig. 2. Two-dimensional diagram illustrating the arrangements of the seven transmembrane (TM) domains in visual opsins around the retinal chromophore (based on Bowmaker and Hunt 2006). Numbering of amino acid sites is based on bovine rhodopsin. Sites known to dictate in vertebrates spectral tuning are 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 $\beta\&\omega$). Sites inferred to be under positive selection associated with biochemical changes (detected by PRIME, supplementary tables S16–S21, Supplementary Material online) are marked with an asterisk (*). EL and CL are extra- and intracellular loops, respectively.

amino acid sites that can be inferred to be under positive selection, respectively (supplementary table S14, Supplementary Material online). 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 and supplementary table S14, Supplementary Material online). 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 (supplementary tables S16–S21, Supplementary Material online), 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 and supplementary table S22, Supplementary Material online). All spectacles transmitted the UVA well (fig. 3B), corroborating recent work on 42 snake species by van Doorn and Sivak (2015). All nocturnal species

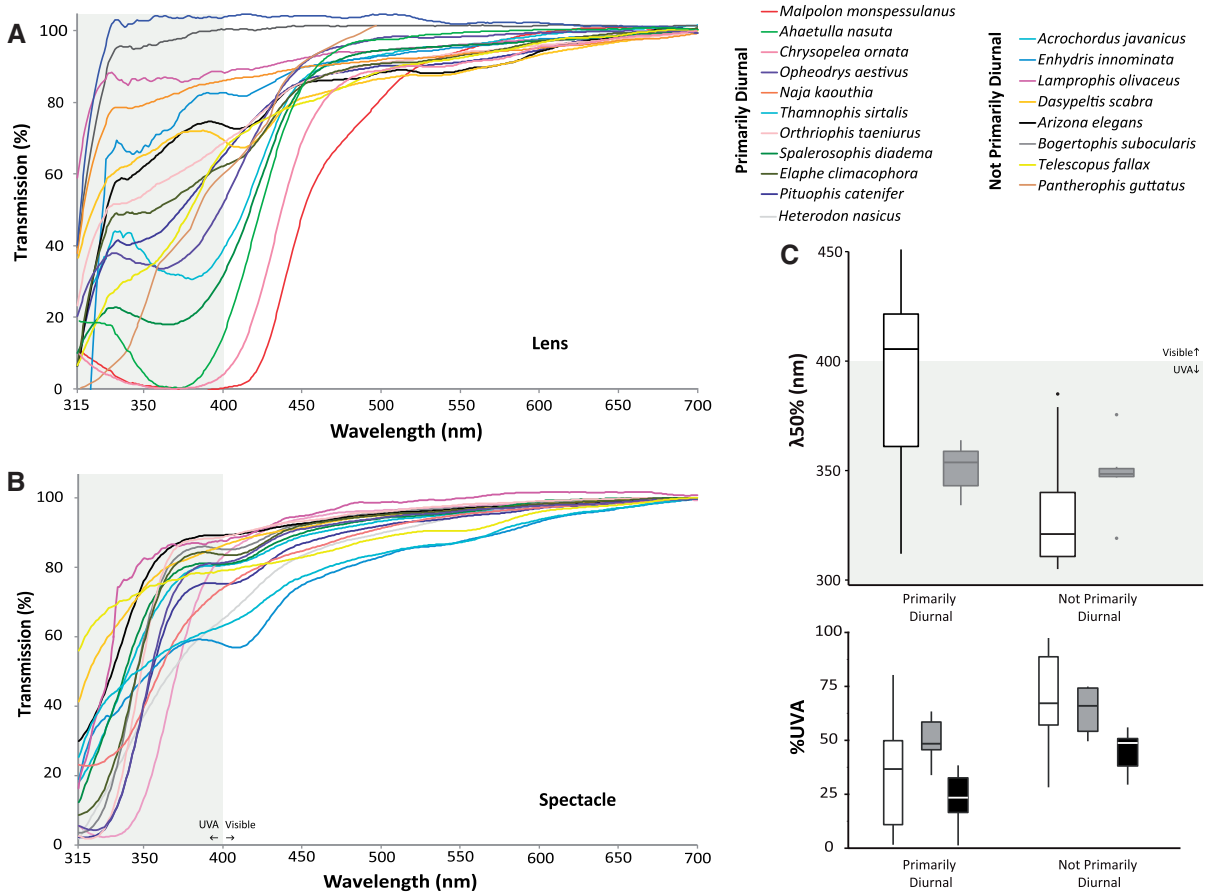

Fig. 3. Spectral transmission curves for sampled snakes for (*A*) lenses and (*B*) spectacles, and (*C*) box-plots showing wavelength at which ocular media transmit 50% of the incident illumination (λ50%, top), and the proportion of UVA (315–400 nm) transmission (%UVA, bottom). The box plots summarize 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 end of the box; outliers more distant than this are shown individually. All data newly generated for this study except for *Pantherophis guttatus* (data from Thorpe 1991).

have very UVA transmissive lenses, whereas 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, 1970) 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*, and *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 *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, 1970) and a previous microspectrophotometric (MSP) study (Govardovskii and Chkheidze 1989) failed to find any visual pigments with a λ_{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 (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 (Gower DJ, personal observation) 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.'s (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 and B; supplementary table S22, Supplementary Material online), the SWS1 pigment λ_{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 (Cowing et al. 2002; Parry et al. 2004; Yokoyama et al. 2005; Carvalho et al. 2012; Hunt and Peichl 2014) suggests that the replacement of F86 by V or S is sufficient to shift the λ_{max} from UV to violet. Consequently, only 6 of the 60 snakes listed in supplementary table S4, Supplementary Material online, 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 color 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 specialized area of the retina.

Based on ancestral state reconstruction for the *sws1* opsin gene (and predictions of λ_{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 (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 λ_{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. (2012) found (using MSP) that the probable SWS1-based pigments in two sea snakes are not maximally sensitive in the UV, with λ_{max} of c. 429 nm. Although many snakes have previously unknown *sws1* tuning site substitutions, there is evidence that some sequences discovered here produce substantial changes in SWS1 λ_{max}. Mutations at site 86 are known to cause major shifts in SWS1 λ_{max}, with F86Y (Fasick and Robinson 1998; Cowing et al. 2002) and F86S (Shi et al. 2001) short-wave shifting λ_{max} by 66 and 51 nm (Yokoyama 2005), respectively, and the latter mutation is observed in the snakes *Malpolon monspessulanus* and *Pantherophis guttatus*. In the colubrid snakes *Ahaetulla nasuta*, *Chrysopelea ornata*, *Helicops angulatus* and *Chironius* spp. F86V is observed. The guinea pig has a 86V substitution and an SWS1 λ_{max} of 420 nm and, furthermore, the V86F substitution produces one of the most substantial known shifts towards the UV with a decrease of 53 nm in the SWS1 pigment λ_{max} (Parry et al. 2004). Given the filtering out of UV light by the lens, a short-wave shifted λ_{max} would seem very unlikely, otherwise SWS1 would not function as an efficient visual pigment in these snakes.

In the dipsadine colubrid *Helicops angulatus*, cloning the *sws1* opsin gene revealed polymorphism at site 86 with either valine or phenylalanine. The exact impact on 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.

The substitution T93V widespread among snakes (supplementary table S4, Supplementary Material online) has not been reported elsewhere in vertebrate *sws1*. The T93A substitution found in the vipers *Echis ocellatus* and *Bitis nasicornis* has previously been reported but only in the distantly related snake *Tropidophis feicki* (Simões et al. 2015). The precise effects of these substitutions on SWS1 λ_{max} are unknown, but substitutions involving sites 86, 93 and 118 in other vertebrates are known to generate substantial shifts in the λ_{max} of VS SWS1 pigments (Parry et al. 2004; Carvalho et al. 2012; Yokoyama et al. 2014 Carvalho et al. 2012).

In contrast to RH1 and SWS1, the LWS spectral site amino acids 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 λ_{max} shifts between long (555–560 nm) and medium wavelengths (536 nm) within Caenophidia. Substitutions are particularly common in Dipsadinae and Colubrinae, with most shifts to predicted shorter wavelength λ_{max} values occurring in nocturnal taxa, thereby providing a possible adaptation to maximize photon capture and potentially color vision in low light conditions. In a study of forest mammals, Veilleux and Cummings (2012) found that SWS1 spectral tuning appeared to be strongly associated with foraging target and LWS tuning to dominant light field characteristics. Although the shorter wavelength shifted LWS λ_{max} values

of nocturnal snakes match this, we are unable to address whether snake SWS1 is more tuned to foraging targets because SWS1 λ_{max} is not known for most snakes (see above), dietary classification for snakes is nontrivial, 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. First, unlike some other studies of vertebrate visual opsins (Yokoyama et al. 2008) we infer multiple sites as under positive selection in all three visual opsins and some of these are 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). Second, shifts in the molecular evolution (functional constraint) of 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 (Schott et al. 2016; 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 (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 (supplementary table S12, Supplementary Material online) and possibly indicating possibly greater purifying selection than in LWS and RH1. This is consistent with the relatively few tuning sites identified in vertebrate SWS1 opsins. Indeed, two of the seven *sws1* sites inferred to be under positive selection in snakes are the spectral tuning sites 86 and 93 known to impart substantial λ_{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 these mediate important changes in λ_{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. Color vision has yet to be demonstrated behaviorally 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 and 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 color vision.

Most of the amino acid sites inferred to be under positive selection in the three visual opsin genes found in snakes occur in transmembrane domains (fig. 3), and most observed changes at these sites are nonconservative in terms of amino acid properties (fig. 2 and supplementary table S13, Supplementary Material online). 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 substantial impact 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.

Supplementary Material

Supplementary tables S1–S5, S12–S14, S16–S22 and figures S6–S11 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

This work was funded by a grant from the Leverhulme Trust (RPG-342 to D.J.G., N.S.H., D.M.H. and J.C.P.). Permits for research and export were granted by Direction de l'Environment de l'Aménagement et du Logement and the Direction des Services Vétérinaires de la Guyane, Cayenne, French Guiana, and by the Ministry of Forestry and 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 U S A*. 102:6395–6400.

Bowmaker J, Hunt D. 2006. Evolution of vertebrate visual pigments. *Curr Biol*. 16:R484–R849.

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.

Carvalho LS, Davies WL, Robinson PR, Hunt DM. 2012. Spectral tuning and evolution of primate short-wavelength-sensitive visual pigments. *Proc. R. Soc. B.* 279(1727): 387–393.

Conant GC, Wagner GP, Stadler PF. 2007. Modeling amino acid substitution patterns in orthologous and paralogous genes. *Mol Phylogenet 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 heuristics and parallel computing. *Nat Methods* 9:772.

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

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.

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

Delport W, Scheffler K, Botha G, Gravenor MB, Muse SV, Kosakovsky Pond SL. 2010. CodonTest: modeling amino acid substitution preferences in coding sequences. *PLoS Comput Biol*. 6:e1000885.

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

Douglas RH, Marshall NJ. 1999. A review of vertebrate and invertebrate ocular filters. In: Archer SN, Djamgoz MBA, Loew ER, Partridge JC, Vallerga S, editors. Adaptive mechanisms in the ecology of vision. Dordrecht: Springer Netherlands. p. 95–162.

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

Dulai K, Dornum M, Mollon J, Hunt D. 1999. The evolution of trichromatic color vision by opsin gene duplication in new world and old world primates. *Genome Res*. 9:629–638.

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

Enright JM, Toomey MB, Sato S-Y, Temple SE, Allen JR, Fujiwara R, Kramlinger VM, Nagy LD, Johnson KM, Xiao Y, et al. 2015. Cyp27c1 red-shifts the spectral sensitivity of photoreceptors by converting vitamin A1 into A2. *Curr Biol*. 25:1–10.

Fasick JI, Robinson PR 2000. Spectral-tuning mechanisms of marine mammal rhodopsins and correlations with foraging depth. *Vis Neurosci.* 17(5):781–788.

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

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

Govardovskii VI, Chkheidze NI. 1989. Retinal photoreceptors and visual pigments in certain snakes. *Biol Abstr*. 90:1036.

Gower DJ, Winkler JD. 2007. Taxonomy of the Indian snake Xylophis Beddome (Serpentes: Caenophidia), with description of a new species. *Hamadryad* 31:315–329.

Greene HW. 1997. Snakes: the evolution of mystery in nature. Berkeley: University of California. pp. 351.

Hart NS, Coimbra JP, Collin SP, Westhoff G. 2012. Photoreceptor types, visual pigments, and topographic specializations in the retinas of hydrophiid sea snakes. *J Comp Neurol*. 520:1246–1261.

Hauser FE, van Hazel I, Chang BSW. 2014. Spectral tuning in vertebrate short wavelength-sensitive 1 (SWS1) visual pigments: can wavelength sensitivity be inferred from sequence data? *J Exp Zoolog B Mol Dev Evol*. 322:529–539.

Hofmann CM, Carleton KL. 2009. Gene duplication and differential gene expression play an important role in the diversification of visual pigments in fish. *Integr Comp Biol*. 49:630.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.

Hughes AL. 2008. The origin of adaptive phenotypes. *Proc Nat Acad Sci USA*. 105(36):13193–13194.

Hunt DM, Collin SP. 2014. The evolution of photoreceptors and visual pigments in vertebrates. In: Hunt DM, Hankins MW, Collin SP, Marshall NJ, editors. Evolution of visual and non-visual pigments. Boston (MA): Springer. p. 276.

Hunt DM, Peichl L. 2014. S cones: evolution, retinal distribution, development, and spectral sensitivity. *Vis. Neurosci.* 31: 115–138.

Hunt DM, Carvalho LS, Cowing JA, & Davies WL. 2009. Evolution and spectral tuning of visual pigments in birds and mammals. *Phil. Trans. R. Soc. B.* 364(1531): 2941–2955.

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.

Hunt DM, Fitzgibbon J, Slobodyanyuk SJ, Bowmaker JK. 1996. Spectral tuning and molecular evolution of rod visual pigments in the species flock of cottoid fish in Lake Baikal. *Vision Res*. 36(9): 1217–1224.

Jacobs G, II J, Tan Y, Li W-H. 2002. Opsin gene and photopigment polymorphism in a prosimian primate. *Vision Res*. 42:11–18.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*. 30:3059–3066.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649.

Kobilka BK. 2007. G protein coupled receptor structure and activation. *BBA—Biomembranes* 1768:794–807.

Kondrasiv SL, Gamburzena AG, Gnjibkina VP, Orlov OJ, My PT. 1986. Coloration of corneas in fish. A list of species. *Vision Res*. 26:287–290.

Land MF. 1981. Optics and vision in invertebrates. In: Autrum HJ, editor. Handbook of sensory physiology. Vol. VII/6B. Berlin: Springer. p. 471–592.

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

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

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

Martin M, Le Galliard J-F, Meylan S, Loew ER. 2015. The importance of ultraviolet and near-infrared sensitivity for visual discrimination in two species of lacertid lizards. *J Exp Biol*. 218:458–465.
McDowell SB. 1987. Systematics. In: Seigel RA, Collins JT, Novak SS, editors. Snakes: ecology and evolutionary biology. New York: MacMillan. p. 3–50.
Nathans J. 1990. Determinants of visual pigment absorbance: role of charged amino acids in the putative transmembrane segments. *Biochemistry* 29:937.
Newman L, Robinson P. 2006. Cone visual pigments of aquatic mammals. *Vis. Neurosci.* 22: 873–879.
Nickle B, Robinson PR. 2007. The opsins of the vertebrate retina: insights from structural, biochemical, and evolutionary studies. *Cell Mol Life Sci*. 64:2917–2932.
Nilsson DE. 1996. Eye ancestry: old genes for new eyes. *Curr Biol*. 6:39–42.
Palumbi SR. 1996. The Polymerase Chain Reaction. In: Hillis DM, Moritz C & Mable BK, editors. Molecular Systematics. Sunderland: Sinauer Associates, Inc. p. 205–247.
Parry J, Poopalasundaram S, Bowmaker J, Hunt D. 2004. A novel amino acid substitution is responsible for spectral tuning in a rodent violet-sensitive visual pigment. *Biochemistry* 43:8014–8020.
Pattengale ND, Alipour M, Bininda-Emonds ORP, Moret BME, Stamatakis A. 2009. How many bootstrap replicates are necessary? Heidelberg: Springer-Verlag. p. 184–200.
Pignatelli V, Champ C, Marshall J, Vorobyev M. 2010. Double cones are used for colour discrimination in the reef fish, Rhinecanthus aculeatus. *Biol Lett*. 6:537–539.
Pyron R, Burbrink FT, Wiens JJ. 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol Biol*. 13:93.
Rasmussen JB. 1990. The retina of Psammodynastes pulverulentus (Boie, 1827) and Telescopus fallax (Fleischmann, 1831) with a discussion of their phylogenetic significance (Colubroidea, Serpentes). *J Zool Syst Evol*. 28:269–276.
R Core Team. 2014. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.
Reeder TW, Townsend TM, Mulcahy DG, Noonan BP, Wood PL, Sites JW, Wiens JJ. 2015. Integrated analyses resolve conflicts over squamate reptile phylogeny and reveal unexpected placements for fossil taxa. *PLoS One* 10:e0118199.
Roth LSV, Kelber A. 2004. Nocturnal colour vision in geckos. *Proc. R. Soc. Lond. B* 271, S485–S487.
Schott RK, Müller J, Yang CGY, Bhattacharyya N, Chan N, Xu M, Morrow JM, Ghenu A-H, Loew ER, Vincent T et al. 2016. Evolutionary transformation of rod photoreceptors in the all-cone retina of a diurnal garter snake. *Proc Nat Acad Sci USA*. 113:356–361.
Sekharan S, Mooney VL, Rivalta I, Kazmi MA, Neitz M, Neitz J, Sakmar TP, Yan EC, Batista VS. 2013. Spectral tuning of ultraviolet cone pigments: an interhelical lock mechanism. *J. Am. Chem. Soc.* 135:19064–19067.
Shi Y, Radlwimmer FB, Yokoyama S. 2001. Molecular genetics and the evolution of ultraviolet vision in vertebrates. *Proc Natl Acad Sci USA*. 98:11731–11736.
Siebeck UE, Marshall NJ. 2000. Transmission of ocular media in labrid fishes. *Philos Trans R Soc Lond B Biol Sci*. 355:1257–1261.
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(14): 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 BF, Sampaio FL, Loew ER, Sanders KL, Fisher RN, Hart NS, Hunt DM, Partridge JC, Gower DJ. 2016. Multiple rod-cone and cone-rod photoreceptor transmutations in snakes: evidence from visual opsin gene expression. *Proc R Soc B*. 283:1823.
Socha JJ, Sidor CA. 2005. *Chrysopelea ornata*, *C. paradisi* (Flying snakes): Behavior. *Herpetol Rev* 36:190–191.
Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30:1312–1313.
Thorpe A. 1991. Spectral transmission and short–wave absorbing pigments in the lenses of fish and other animals. London: City University.
Underwood G. 1967. A contribution to the classification of snakes. London: 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. p. 1–97.
van Doorn K, Sivak JG. 2015. Spectral transmittance of the spectacle scale of snakes and geckos. *Contrib Zool*. 8:1–12.
Veilleux CC, Cummings ME. 2012. Nocturnal light environments and species ecology: implications for nocturnal color vision in forests. *J Exp Biol*. 215:4085–4096.
Wald G. 1968. The molecular basis of visual excitation. *Nature* 219:800–807.
Wallach V, Williams, KL & Boundy, J. 2014. Snakes of the World. Boca Raton: CRC Press.
Walls GL. 1931. The occurrence of colored lenses in the eyes of snakes and squirrels, and their probable significance. *Copeia* 1931:125–127.
Walls GL. 1934. The reptilian retina. I. A new concept of visual cell evolution. *Am J Ophthalmol*. 17:892–915.
Walls GL. 1942. The vertebrate eye and its adaptive radiation. New York: Fafner Publishing Company.
Wickham H. 2010. ggplot2. New York (NY): Springer.
Wiens JJ, Hutter CR, Mulcahy DG, Noonan BP, Townsend TM, Sites JW, Reeder TW. 2012. Resolving the phylogeny of lizards and snakes (Squamata) with extensive sampling of genes and species. *Biol Lett*. 8:1043–1046.
Yang Z. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Mol Biol Evol*. 15:568–573.
Yang Z. 2006. Computational molecular evolution. Oxford: Oxford University Press.
Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol Biol Evol*. 24:1586–1591.
Yang ZH, Nielsen R, Goldman N, Pedersen A. 2000. Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* 155:431–449.
Yang ZH, Wong W, Nielsen R. 2005. Bayes empirical Bayes inference of amino acid sites under positive selection. *Mol Biol Evol*. 22:1107.
Yokoyama S. 1995. Amino acid replacements and wavelength absorption of visual pigments in vertebrates. *Mol Biol Evol.* 12(1): 53–61.
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. 2005. Elephants and human color-blind deuteranopes have identical sets of visual pigments. *Genetics* 170:335–344.
Yokoyama S. 2008. Evolution of dim-light and color vision pigments. *Annu Rev Genomics Hum Genet*. 9:259–282.
Yokoyama S, Blow NS. 2001. Molecular evolution of the cone visual pigments in the pure rod-retina of the nocturnal gecko, Gekko gekko. *Gene* 276:117–125.
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 U S A*. 105:13480–13485.
Yokoyama S, Xing J, Liu Y, Faggionato D, Altun A, Starmer WT. 2014. Epistatic adaptive evolution of human color vision. *PLoS Genet*. 10:e1004884.
Zhang J. 2005. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Mol Biol Evol*. 22:2472–2479.