

1 Visual pigments, ocular filters and the evolution of snake 2 vision

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

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

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

16

17 **Abstract**

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

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

37

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

40

41 **Introduction**

42

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

54 The fundamentals of vertebrate vision have been particularly well studied in
55 terms of the molecular basis of photoreception and phototransduction. A cornerstone
56 of this is knowledge of the photosensitivity of visual pigments, members of the large
57 family of G-protein-coupled-receptor (GPCR) proteins, which share a common
58 arrangement of an opsin protein linked to a chromophore derived from vitamin A (Wald
59 1968). Visual pigments play a core role in photon detection and colour vision and they
60 are a leading example of how gene duplications (Dulai et al. 1999) and changes in amino
61 acid sequences (Yokoyama 2008), type of chromophore (vitamin A1 or A2: Enright et al.
62 2015) and gene expression (Hofmann and Carleton 2009; Carleton et al. 2010) underlie
63 adaptations to differing ecological and behavioural selection pressures. Visual opsins in
64 some vertebrates have been intensely studied over the past 20 years, to the extent that
65 changes in specific amino acid ('spectral tuning') sites are known to change the peak
66 absorbance wavelength (λ_{\max}) of the visual pigments (Yokoyama 2008; Yokoyama et al.
67 2014). However, there is no universal consensus about the tuning impacts of all such
68 mutations (Hauser et al. 2014), with some data suggesting that additional mechanisms
69 to change spectral sensitivity may exist (Davies et al. 2009; Martin et al. 2015).

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

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

85 The approximately 3,500 species of living snakes are distributed across all
86 continents except Antarctica (Van Wallach et al. 2014). They are very diverse
87 ecologically (e.g., Greene 1997) and include burrowing, arboreal, gliding, fully aquatic,
88 nocturnal and diurnal species. Some have small eyes lying under typical head scales,
89 while others are visual hunters with well developed binocular vision, some of which
90 have horizontal pupils and a fovea (Walls 1942). Since Walls' and Underwood's
91 pioneering anatomical surveys, we have learned that the ancestral snake likely had
92 three of the five visual opsin genes present in the ancestral vertebrate (Davies et al.
93 2009; Simões et al. 2015), but not much more is known.

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

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

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

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

126

127 **Material & Methods**

128 *Taxon sampling and sample storage*

129 Snakes were acquired through fieldwork, the Liverpool school of Tropical Medicine,
130 from hobbyists and the commercial trade. Our sampling (SI Table S1) aimed to maximise
131 taxonomic (phylogenetic), ecological and ocular anatomical diversity. One specimen
132 each of 48 species was newly sampled. The use of animals in this research was
133 conducted using standard protocols approved by the Liverpool school of Tropical
134 Medicine Animal Welfare and Ethical Review Board and the UK Home Office. Following
135 euthanasia, spectacle scales (brilles) were removed and the eyes extracted. After
136 removing the lens, each eye was coarsely macerated and stored in RNAlater (Ambion) at
137 -80°C until the RNA extraction. Where possible, undamaged lenses and spectacles were
138 stored dry at -20°C until measurement of spectral transmission was performed.

139

140 *RNA extraction and cDNA synthesis*

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

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

153

154 *Visual opsin gene amplification and cloning*

155 Here we denote opsin genes in lower case italics and opsin proteins in upper case (e.g.
156 *rh1* and RH1, respectively). We amplified the coding regions of *sws1*, *lws* and *rh1* visual
157 opsin genes using universal primers designed to amplify visual opsin genes across snakes
158 and squamates (Simões et al. 2015). All fragments were amplified in 25 µl Polymerase
159 Chain Reactions (PCR): 1x PCR buffer (Invitrogen), 1.5 mmol (mM) of MgCl₂ (Invitrogen),
160 50 µmol/L of deoxynucleotides (Bioline), 0.4 µmol/L of each primer and 1 unit Platinum
161 Taq Polymerase (Invitrogen) and 100ng of cDNA. PCR products were amplified by
162 touchdown PCR with the following cycling parameters: initial denaturation at 95°C for 5
163 minutes; 20 cycles of 1 minute at 95°C (denaturation), 30 seconds at 60°C (annealing),
164 and 1 minute at 72°C (extension) with a decrease of 0.5°C per cycle; 15 cycles of 1
165 minute at 95°C (denaturation), 30 seconds at 50°C (annealing), and 1 minute at 72°C
166 (extension) followed by a final extension at 72°C for 5 minutes. PCR products were run
167 on a 1% agarose gel, excised in a Blue Light Transilluminator (Safe Imager, Invitrogen)
168 and purified with a PureLink Quick Gel Extraction Kit (Invitrogen). PCR fragments were
169 cloned with a StrataClone PCR Cloning Kit (Agilent) and corresponding chemically
170 competent cells following the manufacturer's protocol. Transformed cells were grown
171 overnight on agar medium treated with 100 mg/ml of Ampicilin (Bioline) and 1ml of 2%
172 X-GAL at 37°C. Sixteen white colonies were picked and used as DNA template in 25µl
173 PCR reactions: 1x PCR buffer (Bioline), 1 mmol (mM) of MgCl₂ (Bioline), 80 µmol/L of
174 deoxynucleotides (Bioline), 0.2 µmol/L of M13F and M13R vector primers and 1 unit of
175 BioTAQ Polymerase (Bioline) and 2µl of DNA (1 colony twirled in 50µl of ultra-pure
176 water). The PCR had the following cycling parameters: initial denaturation at 95°C for 10
177 minutes; 30 cycles of 15 seconds at 95°C (denaturation), 30 seconds at 58°C (annealing),
178 and 1 minute and 30 seconds at 72°C (extension) and a final extension at 72°C for 1.5
179 minutes. Between four and eight positive clones were sequenced in both directions with
180 M13 universal primers in an automated DNA sequencer. Sequences were assembled in
181 Geneious R8 (Kearse et al. 2012) and are deposited in GenBank, accession numbers
182 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

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

221

222 *Analyses of molecular evolution*

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

230 Branch models (Yang 1998) allow the ω ratio to vary across branches in the tree
231 and can be used to infer positive selection ($\omega > 1$) acting in particular lineages. The
232 simplest branch model (one-ratio) allows only one ω ratio value across the tree,
233 whereas the more complex free-ratio model assumes independent ω ratios for each
234 branch. Branch models were also used to estimate ω for two branch categories based
235 on ecotypes (primarily fossorial or not, aquatic/semiaquatic or not, primarily arboreal or
236 not, primarily diurnal or not). The ecological classification applied to each species is
237 reported in Fig. 1 and Table S2. Given the substantial diversity of retinal morphology,
238 ecology and density of our sampling within the family, we also estimated two-ratio
239 branches within Colubridae alone. All branch models were compared using the
240 Likelihood Ratio Test (LRT) and the simpler model (one-ratio) was rejected where
241 $p < 0.05$. Branch models were also carried out for a subset of taxa for which the
242 photoreceptor cell complement is known (SI Table S2) to test for possible links between
243 molecular evolution and the presence/absence of double cones or transmuted (sensu
244 Walls 1934) rod-like cones. Retinal anatomy is not known for all species sampled so we
245 removed such taxa from the data set for corresponding molecular evolution analyses
246 and pruned them from the phylogeny in investigations of the relationship between
247 opsin gene evolution and retinal morphology.

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

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

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

266 For analyses of molecular evolution and ancestral state reconstruction we used a
267 phylogenetic tree congruent with those published by (Wiens et al. 2012; Pyron et al.
268 2013; Reeder et al. 2015) (Species Tree, Figure 1). Although the monophyly of the
269 colubrid clades Colubrinae, Natricinae and Dipsadinae are well supported (e.g., Wiens et
270 al. 2012; Pyron et al. 2013), there is currently no compelling resolution of the
271 relationships among them. Thus, as well as following the weakly supported resolution in
272 the trees of (Pyron et al. 2013) (Colubrinae lying outside Natricinae+Dipsadinae), we
273 accounted for phylogenetic uncertainty and repeated the branch and site model
274 analyses for the two alternative phylogenetic resolutions: ((Dipsidinae, Colubrinae),
275 Natricinae) and ((Natricinae, Colubrinae), Dipsidinae). The Indian snake *Xylophis captaini*
276 or any congeners have not yet been included in molecular phylogenetic analyses.
277 Although some workers have reported similarities between *Xylophis* and xenodermatids
278 (e.g., McDowell 1987), we consider the similarity to the Sri Lankan *Aspidura* suggestive
279 of phylogenetic affinity (e.g., Gans and Fetcho 1982; Gower and Winkler 2007) and so
280 we include it as a correspondingly resolved natricine here.

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

288

289 *Estimating visual pigment λ_{\max}*

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

308

309 *Ocular media spectral transmission*

310 We examined spectral transmission of lenses and spectacles. Corneas and humours
311 were not scanned because, with the exception of some fish corneas (Kondrasiv et al.
312 1986; Douglas and McGuigan 1989; Siebeck and Marshall 2000), the vertebrate lens
313 always removes more shortwave radiation than either the cornea or humours (Douglas
314 and Marshall 1999; Douglas and Jeffery 2014). Lenses, and some spectacle samples
315 were thawed and briefly rinsed in phosphate-buffered saline (PBS) and mounted in
316 purpose-built holders in air in front of a Shimadzu ISR 260 integrating sphere within a
317 Shimadzu UV-2101PC spectrophotometer. Transmission at 700 nm was set to 100% and
318 ocular media scanned at 1 nm intervals from 300 to 700 nm. We averaged the
319 measurements of both eyes unless we had only one usable spectacle scale or lens. The
320 lenses were small, 1–3 mm diameter (SI Table S22), limiting the amount of light
321 transmitted through the measuring system, and the use on an integrating sphere
322 reduced sensitivity further, thus the raw data are noisy at short wavelengths where
323 lamp output is low. Data from scans were therefore smoothed using a cubic Savitzky-
324 Golay filter (data frame length 51nm) using Matlab R2011a (The MathWorks Inc, MA,
325 USA). The 50% cut-off wavelength ($\lambda_{50\%}$), the wavelength at which transmission is 50%,
326 was determined for each sample and rounded to the nearest integer. The proportion of
327 UVA (315–400 nm) transmission was calculated for each lens and spectacle following
328 (Douglas and Jeffery 2014). $\lambda_{50\%}$ and %UVA values were plotted for primarily diurnal and

329 nocturnal species using the package ggplots2 (Wickham 2010) implemented in R (Team
330 2014) (R Core Team, 2014).

331

332 **Results**

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

350

351 *Functionality and spectral sensitivity*

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

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

370

371 *Phylogenetics*

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

383

384 *Adaptive molecular evolution*

385 Values for dN/dS (ω) (SI Table S12) suggest that all three visual opsin genes are
386 under purifying selection ($\omega_{RH1} = 0.237$; $\omega_{SWS1} = 0.107$; $\omega_{LWS} = 0.312$), indicative of strong
387 functional constraint (Li et al. 1985). Additional tests performed with alternative
388 phylogenetic relationships among dipsadine, colubrine and natricine colubrids yield ω
389 estimates that are not significantly different (data not shown), indicating that these
390 results are robust with respect to this phylogenetic uncertainty.

391 For the *sws1* opsin gene, branch models (SI Table S12) suggest that non-fossorial
392 (0.112), non-arboreal (0.08), non-aquatic (0.308) and diurnal (0.149) snake lineages
393 have higher ω values than their counterparts (0.062, 0.097, 0.092 and 0.099,
394 respectively). Colubrids have higher ω values (0.119) than non-colubrids (0.088). In the
395 dataset pruned to species for which retinal anatomy is known, ω values are similar
396 between taxa with (0.117) and without (0.101) transmuted cones, and lower in species
397 with doubles cones (0.097 vs 0.110). The free-ratio model ω values vary between 0.001
398 and 0.409.

399 For the *lws* opsin gene ω values are higher in non-fossorial (0.340), arboreal
400 (0.398), non-aquatic (0.308) and diurnal snakes (0.342) than their counterparts (0.016,
401 0.284, 0.268 and 0.246, respectively). Colubrids have significantly higher ω values
402 (0.422) than non-colubrids (0.193). Higher ω occur in species with transmuted cones
403 (0.543 vs. 0.273) and in species with double cones (0.373 vs 0.245). The free-ratio ω
404 ranges from 0.001–7.1 (SI Table S12).

405 The *rh1* ω ratios among non-fossorial (0.252), non-arboreal (0.240),
406 aquatic/semiaquatic (0.253) and nocturnal (0.240) snake lineages are higher than for
407 their counterparts (0.161, 0.212, 0.0229 and 0.141, respectively). Colubrids have higher
408 (0.252) ω ratios than non-colubrids (0.212) whereas ω ratios are lower for the
409 thoroughly fossorial Scolecophidia (0.141) than their sister group Alethinophidia (0.244).
410 The *rh1* opsin gene is inferred to be under less functional constraint in snakes with
411 transmuted, rod-like cones (0.388 vs. 0.212) and in snakes with double cones (0.283 vs
412 0.190) (SI Table S12).

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

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

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

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

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

444

445 *Ocular media transmission*

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

454

455 **Discussion**

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

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

470 The vast majority of species surveyed express the same three (*rh1*, *sws1*, *lws*)
471 visual opsin genes that were likely to have been present in the ancestral snake (Davies
472 et al. 2009; Simões et al. 2015). A striking feature is however the absence of *rh1* in the
473 *Malpolon monspessulanus*. Given the good quality of the template cDNA available for
474 this species, this is unlikely to be a PCR artefact. *Malpolon monspessulanus*, a highly
475 diurnal species, is reported to have only cones (Underwood 1967; Underwood 1970)
476 and a previous microspectrophotometric (MSP) study (Govardovskii and Chkheidze
477 1989) failed to find any visual pigments with a λ_{\max} close to the c. 500 nm expected for
478 RH1 pigments (typically occurring in rods). The presence of *rh1* in two other colubrids,
479 *Phyllorhynchus decurtatus* and *Macroprotodon brevis* remains unconfirmed.
480 *Phyllorhynchus decurtatus* is nocturnal but its 'rods' have been argued to be transmuted
481 (rod-like) cones (Walls 1934), consistent therefore with the lack of RH1. Among
482 vertebrates, absence of an expressed *rh1* has previously been reported only in another
483 group of squamate reptiles, geckos (e.g. Loew et al. 1996; Yokoyama and Blow 2001).
484 More work examining the physiology of visual pigments and gene expression will be
485 required to test this further for snakes. Among those snakes with three functional visual
486 opsin genes is *Uropeltis cf. macrolepis*. Like all uropeltids, this is a mostly fossorial
487 species, though it is more likely to be seen above ground during daylight (D.J.G., pers.
488 obs.) and has a larger eye than the distantly related, but also burrowing, scolecophidians
489 and *Anilius scytale*, for which Simões et al. (2015) failed to amplify either *sws1* or *lws*.
490 The presence of *sws1* and *lws* in *U. cf. macrolepis* adds support to Simões et al. (2015)
491 conclusion that loss of all visual opsins except *rh1* has occurred in snakes in only the
492 most dedicated of burrowers, and that if the ancestral snake was a burrower it was
493 likely not as fossorial as living scolecophidians.

494 We predict that in snakes where the ocular media filter out most of the UVA (Fig
495 3A&B, SI Table S22), the SWS1 pigment λ_{\max} is long-wave shifted. However, the *sws1*
496 sequences of these species include previously unreported amino acid residues at some
497 key tuning sites and direct measurements of visual pigment absorbance (e.g. by MSP)
498 are currently lacking. Nevertheless, evidence from other studies (Carvalho et al. 2012;
499 Cowing et al. 2002; Hunt and Peichl, 2014; Parry et al. 2004; Yokoyama et al. 2005)
500 suggests that the replacement of is sufficient to shift the λ_{\max} from UV to violet. only six
501 of the 60 snakes listed in Table S4 may have lost a UVS SWS1 pigment. Removal of UV
502 has been linked to increased acuity rather than an adaptation underpinning a particular
503 form of colour vision or protection from harmful UV light (Douglas and Jeffery 2014).
504 This hypothesis receives support here because the snakes with the least transparent
505 lenses are highly visual hunters. These include a gliding species (*Chrysopelea ornata*)
506 known to track distant objects (Socha and Sidor 2005) and a taxon (*Ahaetulla*) with
507 horizontal pupils, binocular vision and a fovea (Walls 1942). The latter structure is

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

510 Based on ancestral state reconstruction for the *sws1* gene (and predictions of
511 λ_{\max}), the most recent common ancestor of living snakes was UV sensitive, and UV vision
512 is also predicted to be present in many nocturnal caenophidians, matching the situation
513 in other vertebrate groups (e.g., Veilleux and Cummings 2012) in which nocturnality is
514 associated with UV sensitivity. Although there is evidence of a substantial amount of
515 evolutionary change in snake *sws1* sequences, it is not possible to predict the λ_{\max} of the
516 SWS1-based visual pigments in 42 of the 63 species for which sequences are available
517 (many of these species are not primarily nocturnal) because of tuning site amino acid
518 substitutions (or combinations of substitutions) not known in other vertebrates. It is
519 very likely (based on lens transmission) that at least some of these species have
520 substantially long-wave shifted SWS1-based visual pigments. Hart et al. (Hart et al.
521 2012) found (using MSP) that the probable SWS1-based pigments in two sea snakes is
522 not maximally sensitive in the UV, with λ_{\max} of c. 429nm. Although many snakes have
523 previously unknown *sws1* tuning site substitutions, there is evidence that some
524 sequences discovered here produce substantial changes in SWS1 λ_{\max} . Mutations at site
525 86 are known to cause major shifts in SWS1 λ_{\max} , with F86Y (Fasick and Robinson 1998;
526 Cowing et al. 2002) and F86S (Shi et al. 2001) short-wave shifting λ_{\max} by 66 and 51 nm
527 (Yokoyama 2005) respectively, and the latter mutation is observed in the snakes
528 *Malpolon monspessulanus* and *Pantherophis guttatus*. In *Ahaetulla nasuta*, *Chrysopelea*
529 *ornata*, *Helicops angulatus* and *Chironius* spp. F86V is observed. The guinea pig has a
530 86V substitution and an SWS1 λ_{\max} of 420nm and, furthermore, the V86F substitution
531 produces one of the most substantial shifts towards the UV with a decrease of 53nm in
532 the SWS1 pigment λ_{\max} (Parry et al. 2004). Given the filtering out of UV light by the lens
533 a short-wave shifted λ_{\max} would seem very unlikely, otherwise SWS1 would not function
534 as an efficient visual pigment in these snakes.

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

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

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

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

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

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

608

609 **Conclusion**

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

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

622

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636

637

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639

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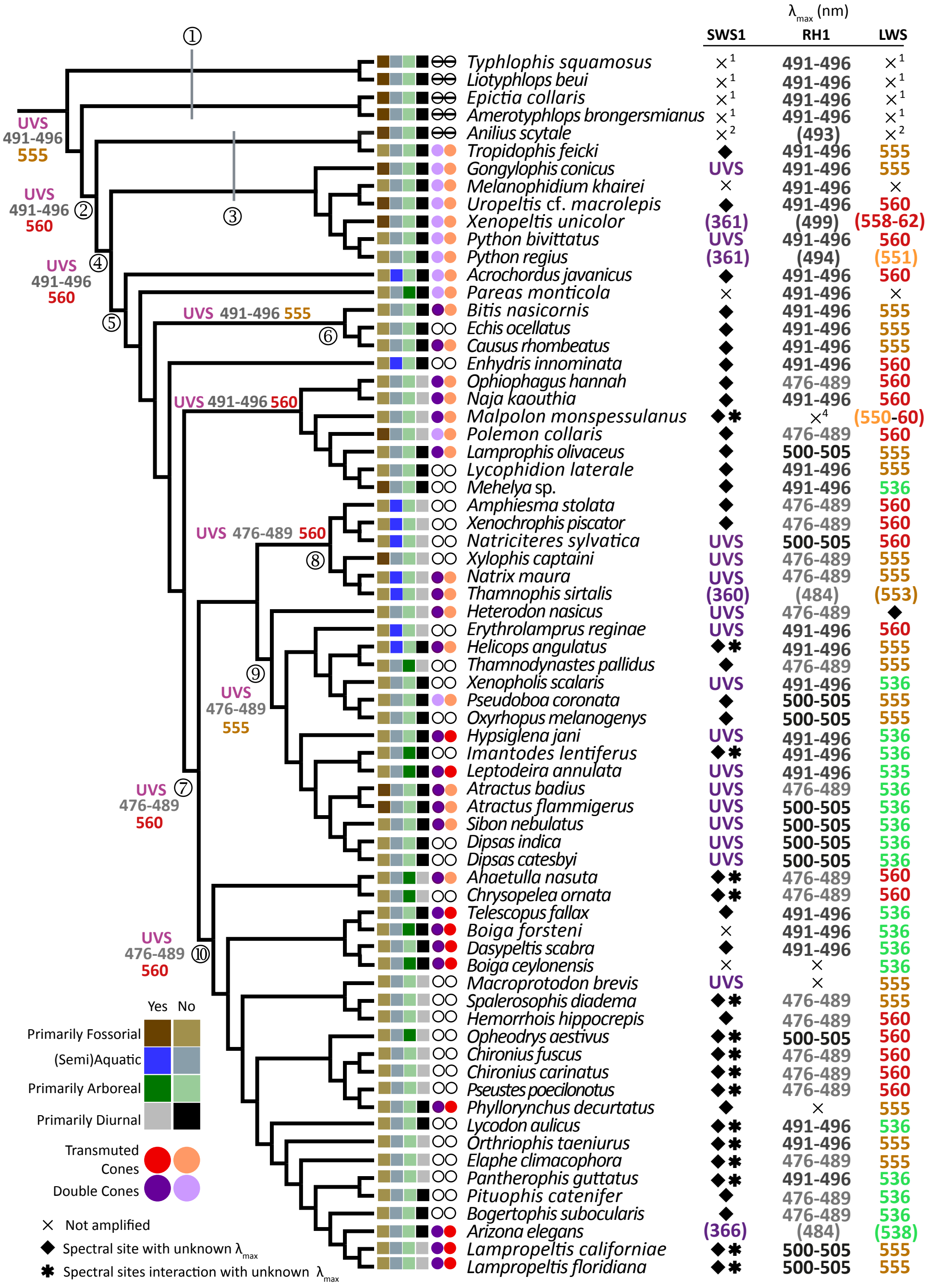
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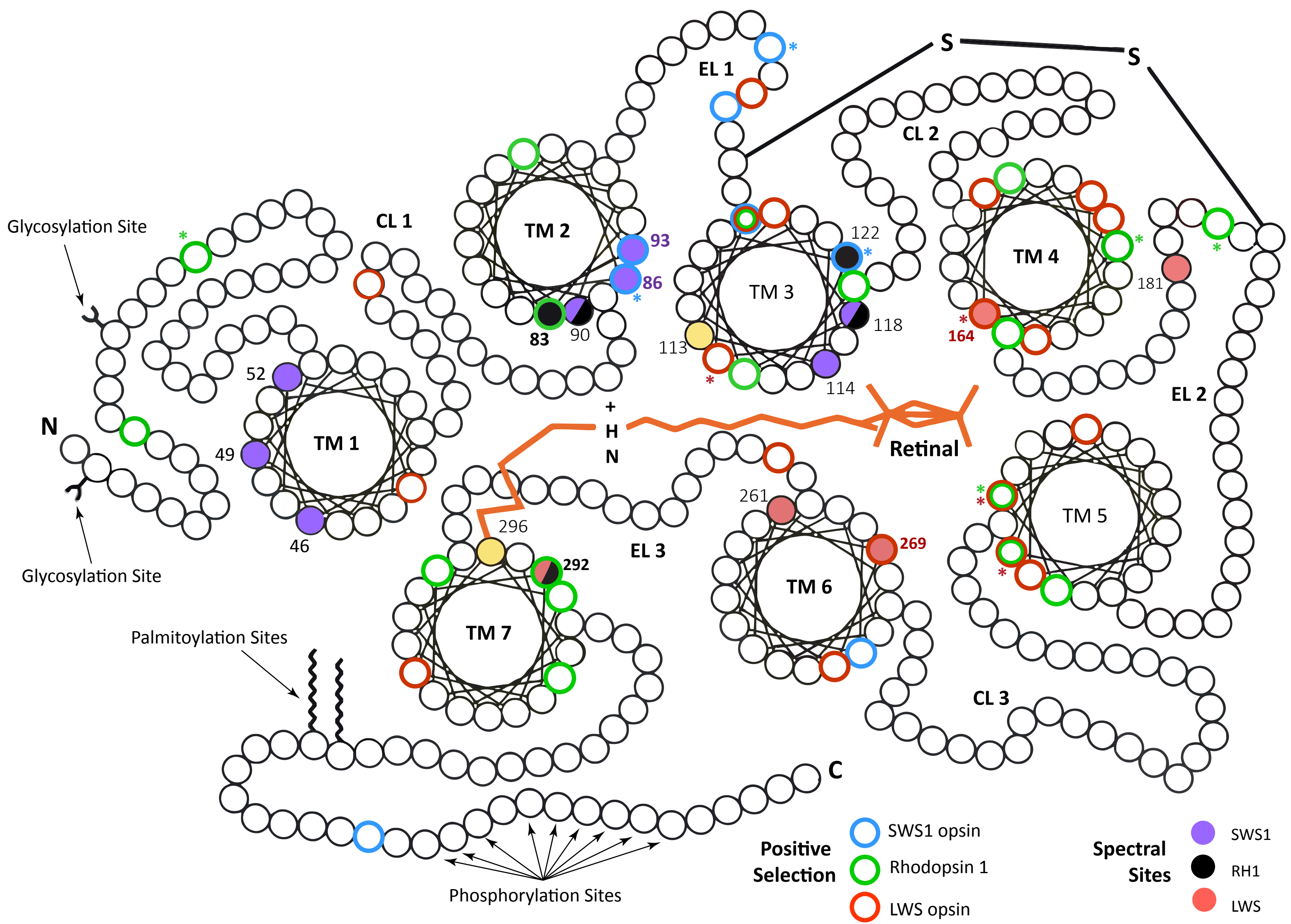
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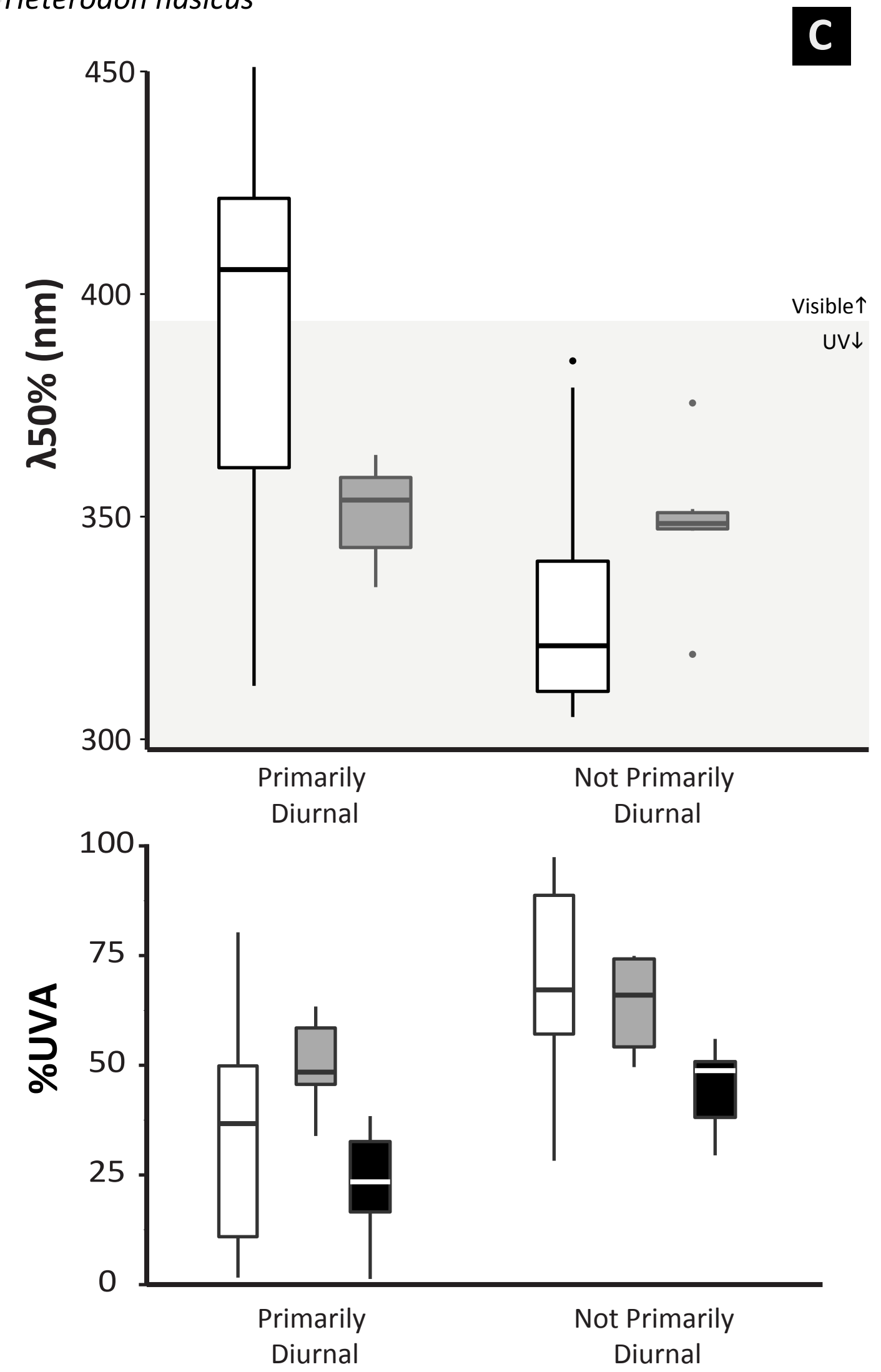
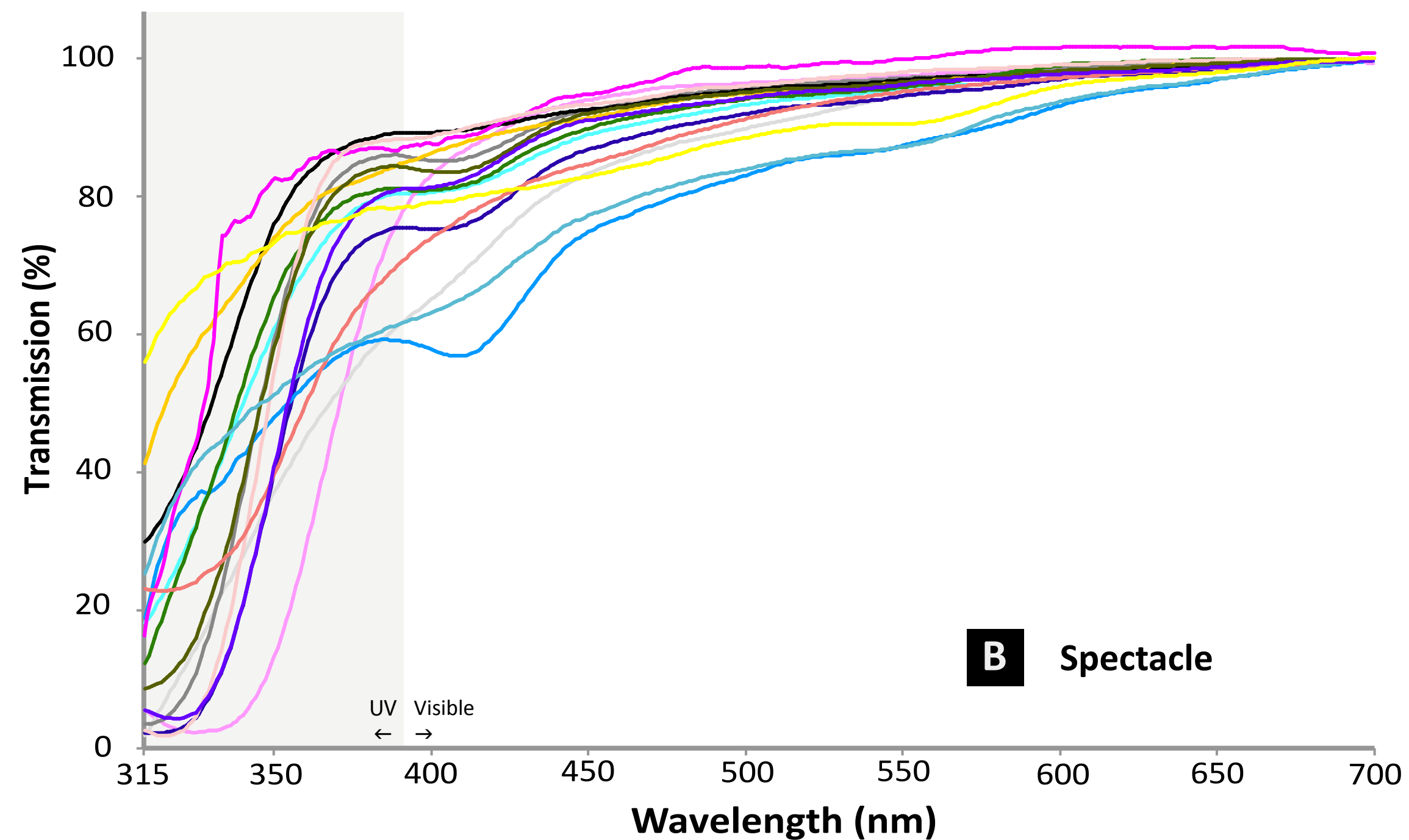
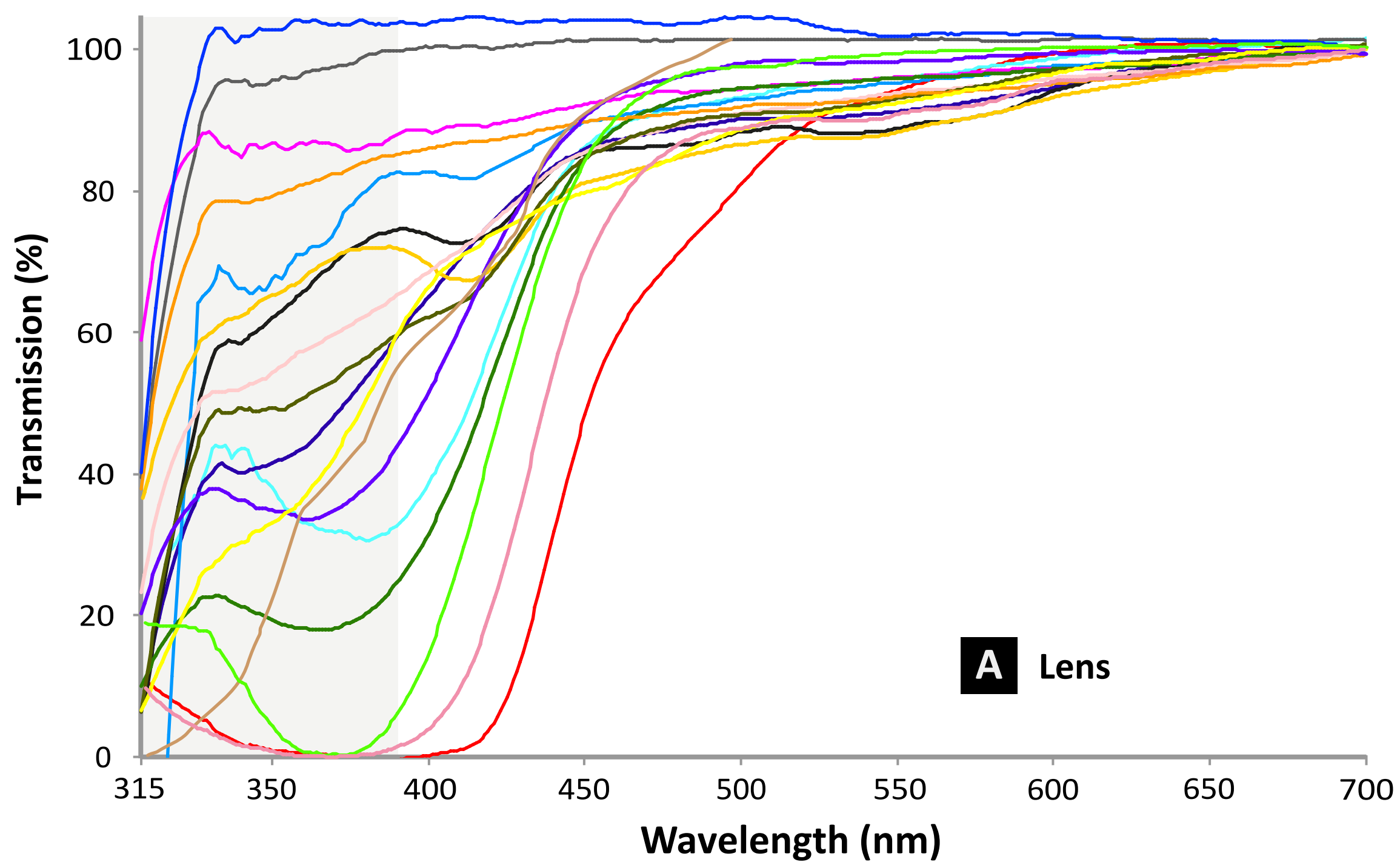
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824 **FIGURE CAPTIONS**

825

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

842

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

851

852 **Figure 3.** Spectral transmission curves for sampled snakes for (A) lenses and (B)
853 spectacles, and (C) box-plots showing wavelength at which ocular media transmit 50%
854 of the incident illumination ($\lambda_{50\%}$, top), and the proportion of UVA (315–400 nm)
855 transmission (%UVA, bottom). The box plots summarise data for the lens (white),
856 spectacle (grey) and lens + spectacle (black). Boxes extend from first (Q1) to third
857 quartile (Q3); median is indicated as a horizontal line; whiskers extend to the
858 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 (λ_{\max}) for snakes.

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

Table S5. Known amino acid spectral tuning sites for *lws* opsin gene and predicted peak absorbance (λ_{\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. $\lambda_{50\%}$ 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>	
Scolecophidia	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	FJ497237	FJ497238	
		<i>Python bivittatus</i>	NA		PRJNA238085		
	Boidae	<i>Gongylophis conicus</i>	xxxxxxx	KX237870	KX237877	KX237782	
	Uropeltidae	<i>Melanophidium khairi</i>	xxxxxxx	KX237871	-	-	
		<i>Uropeltis cf. macrolepis</i>	xxxxxxx	KX237872	KX237878	KX237783	
	Pareatidae	<i>Pareas monticola</i>	xxxxxxx	KX237868	-	-	
	Alethinophidia	Viperidae	<i>Bitis nasicornis</i>	xxxxxxx	KX237873	KX237880	KX237785
			<i>Echis ocellatus</i>	xxxxxxx	KX237874	KX237881	KX237786
			<i>Causus rhombeatus</i>	xxxxxxx	KX237876	KX237882	KX237787
	Acrochordidae	<i>Acrochordus javanicus</i>	xxxxxxx	KX237831	KX237879	KX237784	
	Homolopsidae	<i>Enhydris innominata</i>	xxxxxxx	KX237832	KX237883	KX237789	
		<i>Polemon collaris</i>	KR815896	KR336739	KR336724	KR336710	
	Lamprophiidae	<i>Lamprophis olivaceus</i>	xxxxxxx	KX237859	KX237886	KX237827	
		<i>Malpolon monspessulanus</i>	xxxxxxx	×	KX237885	KX237790	
<i>Lycophidion laterale</i>		xxxxxxx	KX237860	KX237887	KX237828		
<i>Mehelya sp.</i>		xxxxxxx	KX237861	KX237888	KX237829		
Elapidae	<i>Ophiophagus hannah</i>	NA		PRJNA201683			

	<i>Naja kaouthia</i>	xxxxxxx	KX237830	KX237884	KX237788
	<i>Amphiesma stolata</i>	xxxxxxx	KX237866	KX237889	KX237792
	<i>Xenochrophis piscator</i>	xxxxxxx	KX237865	KX237890	KX237801
Colubridae – Natricinae	<i>Natriciteres sylvatica</i>	xxxxxxx	KX237833	KX237891	KX237802
	<i>Xylophis captaini</i>	-	KX237869	KX237892	KX237791
	<i>Natrix maura</i>	KU323977	KU324002	KU323993	KU323982
	<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
Colubridae – Dipsadinae	<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
		<i>Ahaetulla nasuta</i>	xxxxxxx	KX237852	KX237906
	<i>Chrysopelea ornata</i>	xxxxxxx	KX237851	KX237907	KX237799
Colubridae – Colubrinae	<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>Hemorrhais hipocrepis</i>	xxxxxxx	KX237835	KX237911	KX237796	
		<i>Ophedryx 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 sp.</i>	KR815888	KR336728	KR336721	KR336706
		Lacertidae	<i>Takydromus sexlineatus</i>	KR815885	KR336727	KR336722	KR336707
		Gymnophthalmidae	<i>Bachia cf. flavescens</i>	KR815884	KR336731	KR336715	KR336703
	Scincoidea	Scincidae	<i>Melanoseps occidentalis</i>	KR815882	KR336743	KR336718	KR336713
		Scincidae	<i>Feylinia sp.</i>	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>Enhydris 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 (λ_{\max}) of RH1-based visual pigment for snakes. Site values in first row represent amino acid positions numbered with respect to bovine rhodopsin. All λ_{\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 λ_{\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	λ_{\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 khairai</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>Enhydris 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	λ_{\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>Hemorrhoidis 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	λ_{\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 (λ_{\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 λ_{\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 λ_{\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 (λ_{\max} c. 360nm).

Species	46	49	52	86	90	93	97	113	114	116	118	265	λ_{\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	λ_{\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>Hemorrhhis 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>Pseudoboia 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	λ_{\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/ Alethiniphidia	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 (λ_{\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 λ_{\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>	197	<u>277</u>	<u>285</u>	<u>308</u>	λ_{\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>Ophoeodryx 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	λ_{\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/Alethiniphidia	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

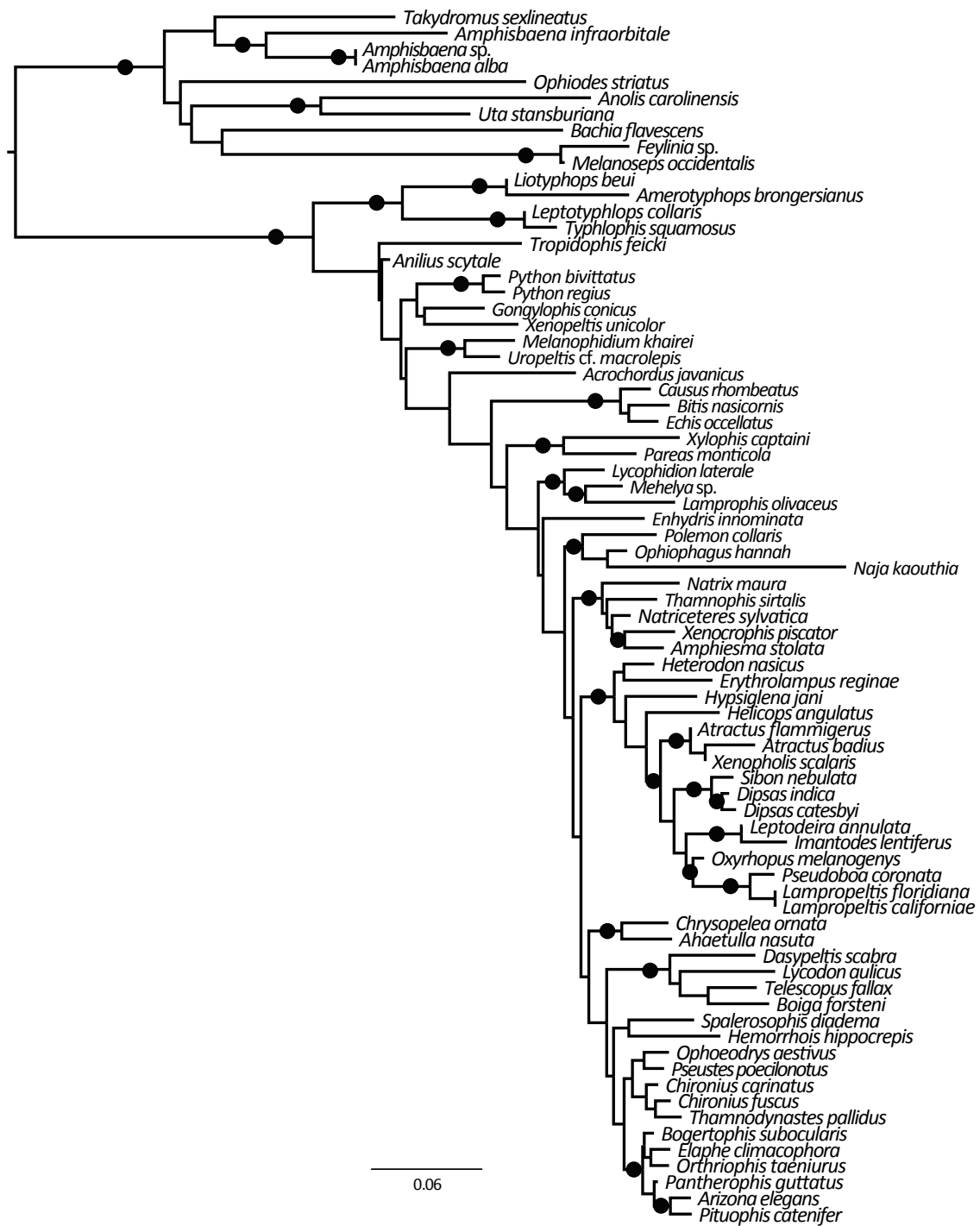


Figure S6. Maximum Likelihood rhodopsin 1 (*rh1*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G+I model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

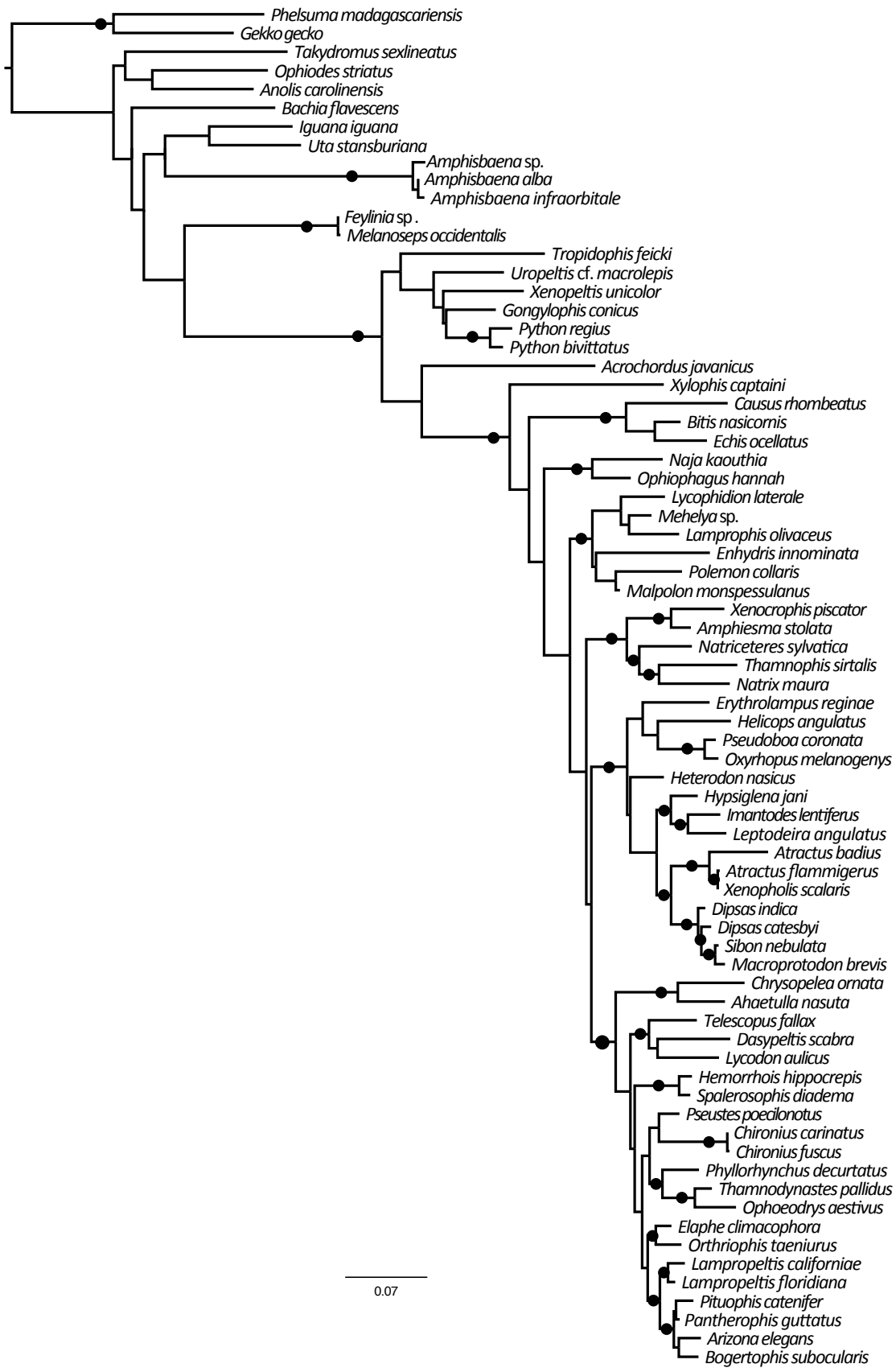


Figure S7. Maximum Likelihood short-wavelength opsin 1 (*sws1*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G+I model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

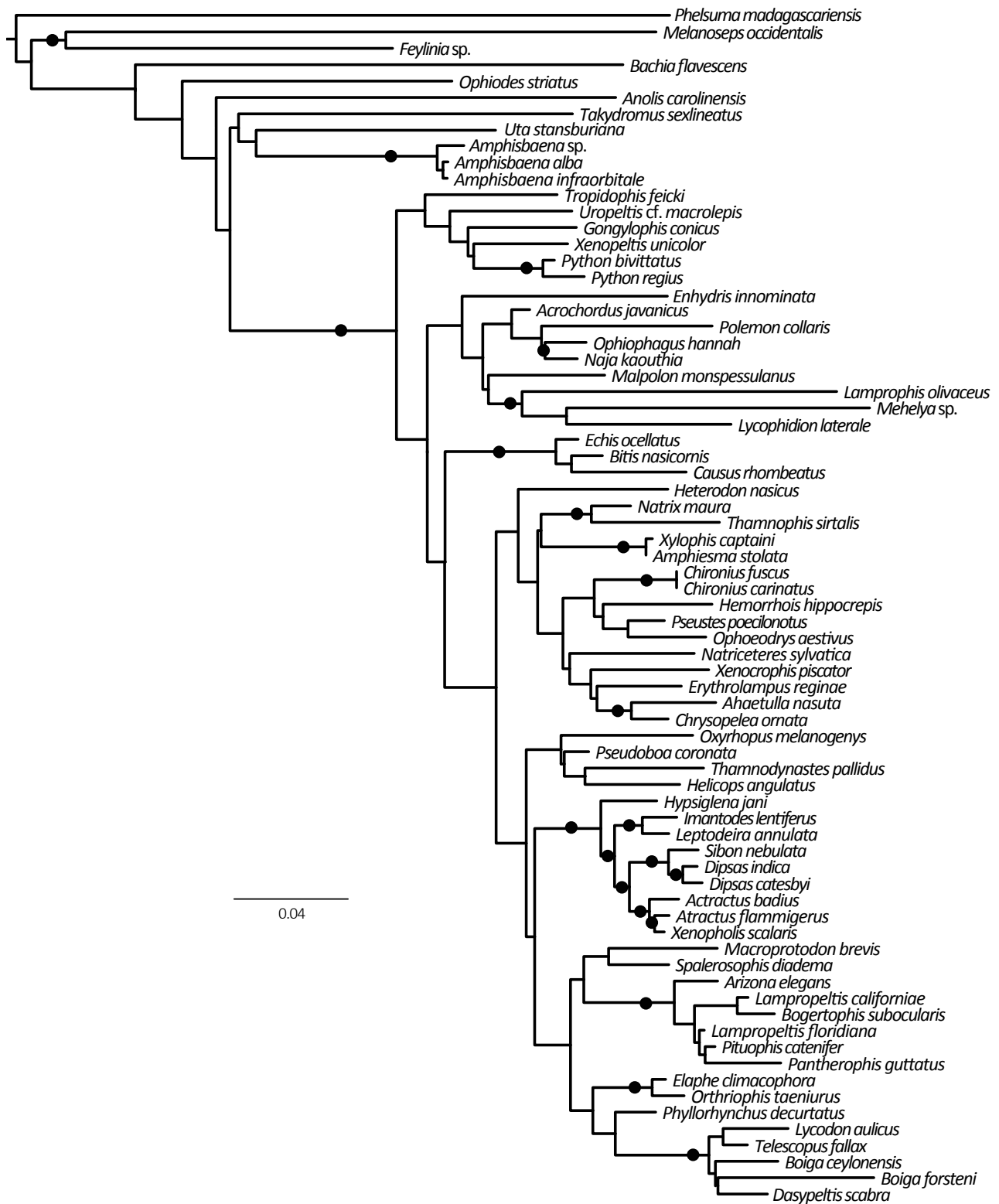


Figure S8. Maximum Likelihood medium-to-long wavelength opsin gene (*lws*) phylogenetic tree for squamates estimated by RAXML based on GTR+G+I model of sequence evolution. Numbers on the major internal branches are ML bootstrap support and Bayesian posterior probabilities, respectively.

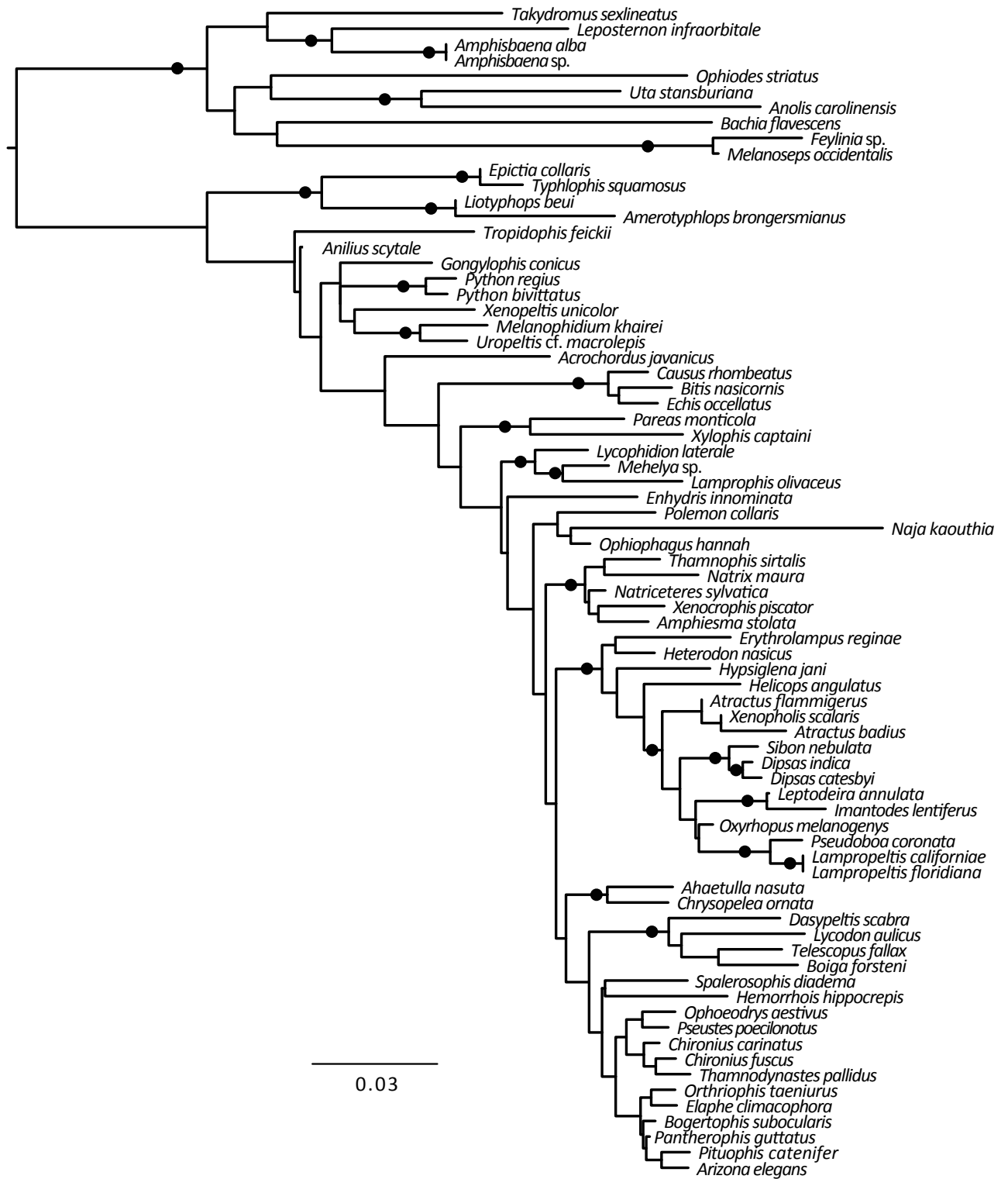


Figure S9. Maximum Likelihood rhodopsin 1 (*rh1*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

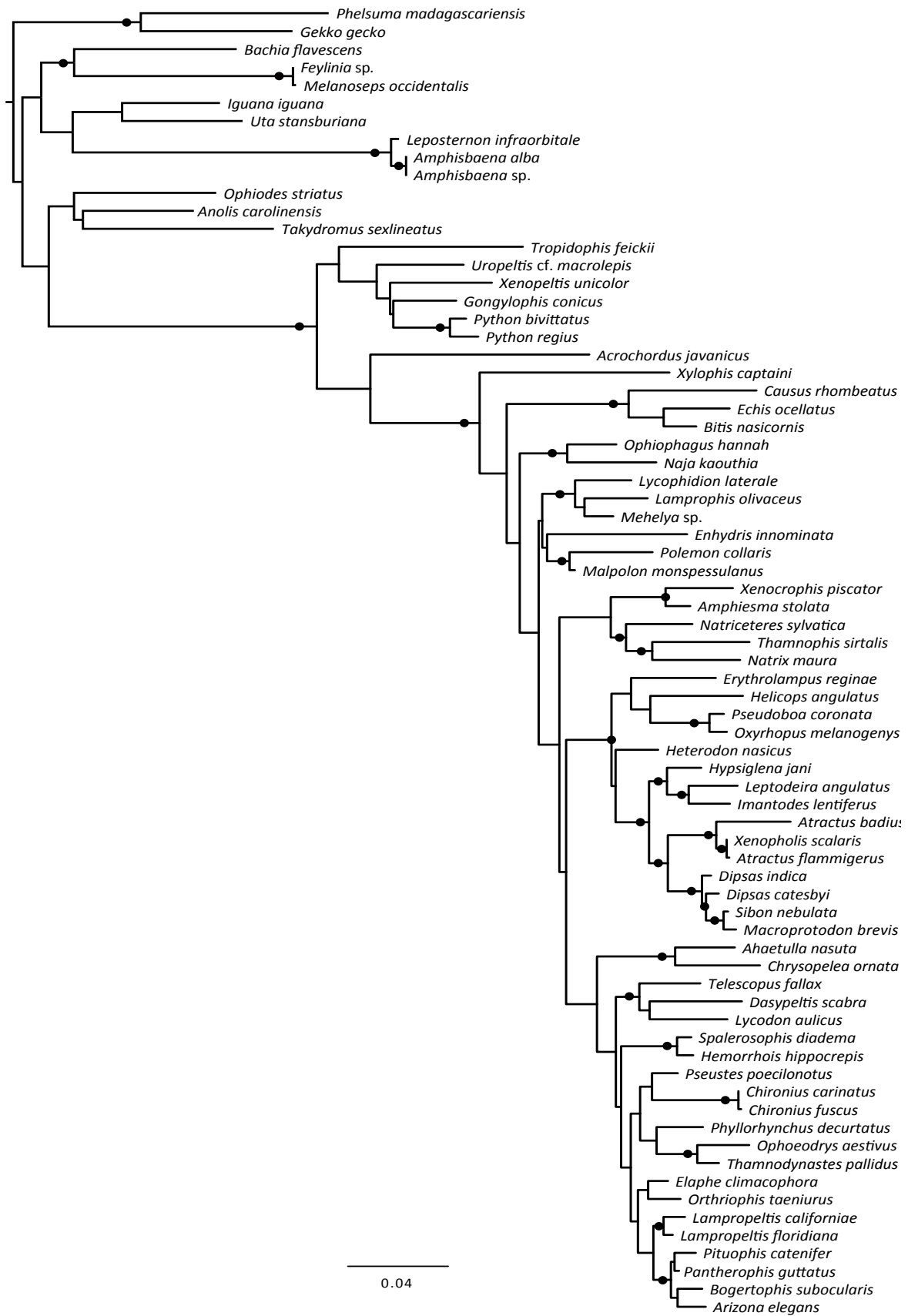


Figure S10. Maximum Likelihood short-wavelength opsin 1 (*sws1*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

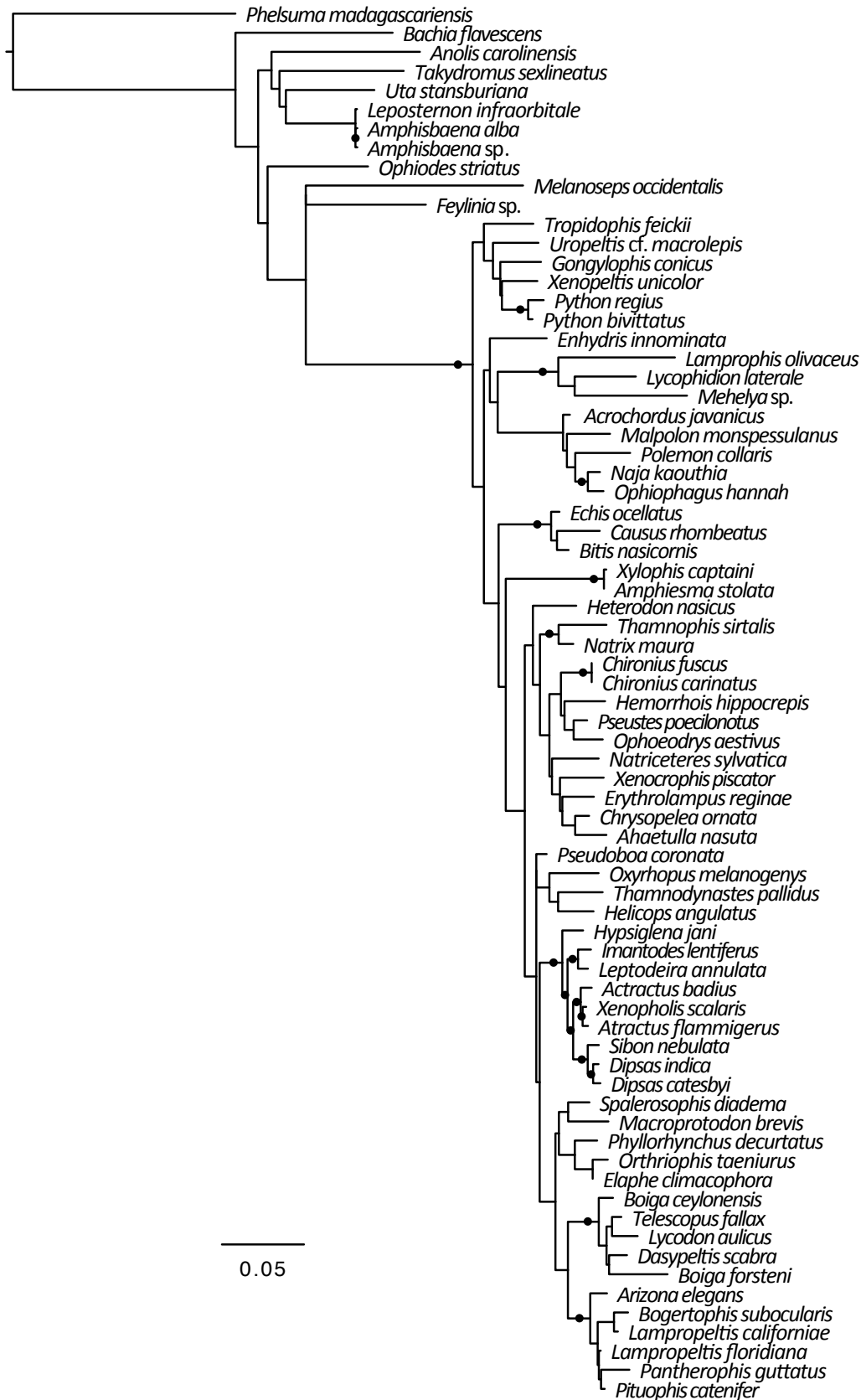


Figure S11. Maximum Likelihood medium-to-long wavelength opsin (*lws*) gene phylogenetic tree for squamates estimated by RAxML based on GTR+G model of sequence evolution. Black circles in branches represent ML bootstrap support and Bayesian posterior probabilities above or equal to 80% and 1, respectively.

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 (β & ω)	$p_0 = 0.997 (p_1 = 0.003),$ $p = 0.158, q = 0.923, \omega_s = 3.501$	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 (β & ω)	$p_0 = 0.927 (p_1 = 0.073),$ $p = 0.111, q = 0.602, \omega_s = 0.693$	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 (β & ω)	$p_0 = 0.935 (p_1 = 0.065),$ $p = 0.144, q = 0.925, \omega_s = 1.881$	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 (β & ω)	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 (β & ω)	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 (β & ω)	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	—

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