Shifts in Selective Pressures on Snake Phototransduction Genes Associated with Photoreceptor Transmutation and Dim-Light Ancestry

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Abstract

The visual systems of snakes are heavily modified relative to other squamates, a condition often thought to reflect their fossorial origins. Further modifications are seen in caenophidian snakes, where evolutionary transitions between rod and cone photoreceptors, termed photoreceptor transmutations, have occurred in many lineages. Little previous work, however, has focused on the molecular evolutionary underpinnings of these morphological changes. To address this, we sequenced seven snake eye transcriptomes and utilized new whole-genome and targeted capture sequencing data. We used these data to analyze gene loss and shifts in selection pressures in phototransduction genes that may be associated with snake evolutionary origins and photoreceptor transmutation. We identified the surprising loss of rhodopsin kinase (*GRK1*), despite a low degree of gene loss overall and a lack of relaxed selection early during snake evolution. These results provide some of the first evolutionary genomic corroboration for a dim-light ancestor that lacks strong fossorial adaptations. Our results also indicate that snakes with photoreceptor transmutation experienced significantly different selection pressures from other reptiles. Significant positive selection was found primarily in cone-specific genes, but not rod-specific genes, contrary to our expectations. These results reveal potential molecular adaptations associated with photoreceptor transmutation and also highlight unappreciated functional differences between rod- and cone-specific phototransduction proteins. This intriguing example of snake visual system evolution illustrates how the underlying molecular components of a complex system can be reshaped in response to changing selection pressures.

Key words: **evolution of vision, reptile vision, eye transcriptomes, snake origins, visual transduction, photoreceptor evolution.**

Article

Introduction

Snakes are a diverse group of squamate reptiles that are fascinating due in part to their contested evolutionary origins. Early work suggested that snakes may have had an aquatic origin based on affinities with extinct marine squamates, such as mosasaurs and dolichosaurs (Nopcsa 1908; 1923; for review see Lee and Caldwell 2000). Walls (1940), however, noted that snake eyes were heavily modified compared with other squamates such that they contain no structural features that could identify them as being squamate, or even reptilian, eyes. Walls (1940) hypothesized that these changes were due to a fossorial phase during the early evolution of snakes that led to a degeneration of the eye, followed later by recolonization of terrestrial habitats that necessitated a re-evolution of eye structure and function. Although this view was supported by later studies (Bellairs and Underwood 1951; Rieppel 1988), a quantitative morphometric analysis of eye morphology by Caprette et al. (2004) indicated that snake eyes most closely resembled those of primitively aquatic vertebrates, supporting an aquatic origin for snakes. Similarly, phylogenetic and fossil evidence has provided mixed, and often contradictory, support for both hypotheses resulting in an ongoing debate on snake origins (Caldwell and Lee 1997; Lee 2005; Longrich et al. 2012; Hsiang et al. 2015; Simões et al. 2015; Yi and Norell 2015; Lee et al. 2016).

Beyond their implications for snake origins, snake eyes are also particularly interesting due to the predominance of all-cone and all-rod retinas, a feature that is extremely rare in other vertebrate groups (Walls 1942; Underwood 1970; Schott, Muller, et al. 2016). Typical vertebrate retinas are duplex, containing both rod and cone photoreceptors, which are often identified based on their outer segment morphology (rod- or cone-shaped). Rods are much more photosensitive and less noisy (i.e., less spontaneous activation) than cones

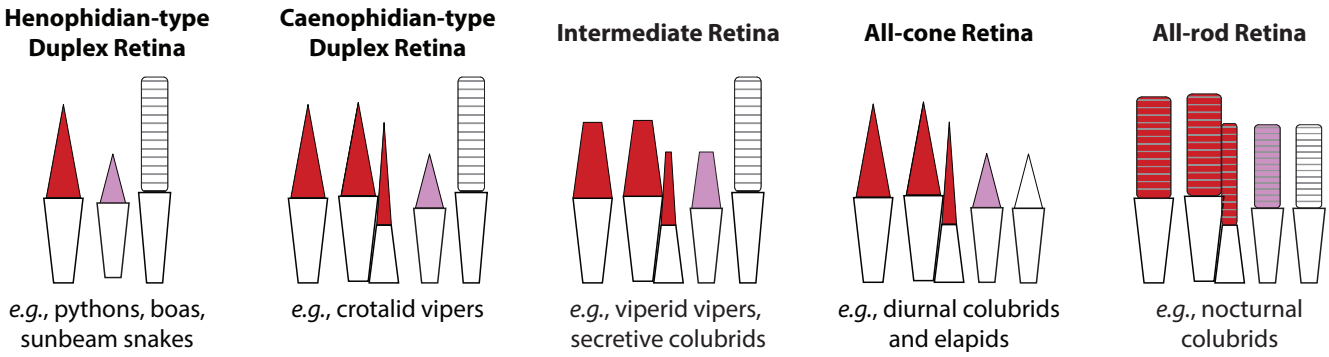

Fig. 1. Schematic illustration of major snake retina types. The henophidian-type duplex retina contains large single cones with a long-wavelength sensitive cone visual pigment (LWS; dark color), small single cones with a short-wavelength sensitive cone pigment (SWS1; light color), and rods with the rod visual pigment (RH1; white). The caenophidian-type duplex retina additionally has double cones composed of two connected cells, one of which is larger, that both contain LWS. The other retina types are variations on the caenophidian-type duplex retina and are inferred to be derived from it (and/or each other) through photoreceptor transmutation. Considerable variation in photoreceptor morphology exists within each of these major retina types. The "degenerate," all-rod retinas of scolecophidians are not shown. Generalized photoreceptor outer segment morphology is shown based on Walls (1942) and Underwood (1970). Contained visual pigments are based on MSP and sequencing data (Sillman et al. 1997, 1999, 2001; Davies et al. 2009; Simões et al. 2015; Schott, Muller, et al. 2016; Simões, Sampaio, Douglas, et al. 2016; Simões, Sampaio, Loew, et al. 2016). Photoreceptor cartoons based on those of Bowmaker (2008).

enabling vision in dim light, but have slow response and recovery kinetics causing them to saturate under bright light (Lamb 2013). Cones have much faster response and recovery times, and can respond over a wider range of intensities than rods; however, they are less sensitive and more noisy, making vision in dim light unreliable (Lamb 2010, 2013). Rod and cone photoreceptor cells differ in both their morphology and molecular components, and these contribute to their differences in physiology (for a review, see Ingram et al. 2016). Due to these differences in function, most vertebrates have both rods and cones enabling them to see under a range of natural light conditions. Only a few groups, most notably snakes and other squamate reptiles, have simplex retinas that contain only rods or only cones. Snakes in particular have a wide range of retinal compositions, including not only all-cone and all-rod retinas but also retinas with photoreceptor morphologies that are intermediate between typical vertebrate rods and cones (Walls 1942; Underwood 1970).

This diversity of retinal types and photoreceptor morphologies within snakes appears to be restricted to caenophidians, a taxonomically, ecologically, and phenotypically diverse lineage (Walls 1942; Greene 1997; Vidal et al. 2007). Although noncaenophidian snakes surveyed to date have retinas containing only reduced rods (scolecophidians), or simple duplex retinas with single cones and rods ("henophidian"-grade species, such as pythons and boas), caenophidians have retinas that are more complex and variable (fig. 1; Walls 1942; Underwood 1970). It was this variability in photoreceptor morphology that led Walls (1934, 1942) to formulate the transmutation theory, whereby he postulated that rod and cone photoreceptors could evolutionarily transition to the other cell type. As a result of photoreceptor transmutation, Walls (1934, 1942) inferred that evolutionary shifts between duplex (rod and cone) and simplex (all-cone or all-rod) retinas were possible and had occurred in a few specific vertebrate groups, most notably in geckos and caenophidian snakes.

Recently we have provided the first molecular evidence for photoreceptor transmutation in snakes (Schott, Muller, et al. 2016). We demonstrated that the diurnal garter snake, *Thamnophis proximus*, which has a morphologically all-cone retina (based on outer segment shape), expresses RH1, the rod visual pigment, in a cone-like photoreceptor that is actually evolutionarily derived from a rod, based on its ultrastructure and rod-specific molecular components (Schott, Muller, et al. 2016). This finding supports the evolution of the all-cone retina in snakes through photoreceptor transmutation, rather than the loss of rods as originally proposed by Walls (1942). Instead, the all-cone retina likely evolved from a duplex (caenophidian-type) retina similar to that seen in some vipers (fig. 1).

Despite these advances, the extent and impact of photoreceptor transmutation on the evolution and function of the visual system of snakes remains largely unknown. All previous molecular-based work in this area has focused on the visual pigments (Davies et al. 2009; Simões et al. 2015; Schott, Muller, et al. 2016; Simões, Sampaio, Douglas, et al. 2016; Simões, Sampaio, Loew, et al. 2016) and has largely ignored the numerous other proteins involved in vertebrate visual systems, including those involved in the phototransduction cascade. In vertebrates, vision begins with phototransduction, the process by which light is converted to an electrical signal in the rod and cone photoreceptors (for a review of phototransduction, see supplementary fig. S1, Supplementary Material online; for more detailed reviews, see Wensel 2008; Fain et al. 2010; Lamb 2013). The process in rods and cones is similar but involves some distinct proteins in the two photoreceptor types (table 1), including the light-sensitive visual pigments that begin the phototransduction cascade. Overall the phototransduction cascade involves over 35 proteins,

Table 1. Major Components of the Vertebrate Visual Phototransduction Cascade and Their Presence or Absence in Snakes and Other Reptile Groups.

Protein	Gene Symbol	Photoreceptor	Gene Name	Lost In
Opsin	*RH1*	Rod	Rhodopsin (RHO)	
	LWS	Cone	Long-wave sensitive cone opsin	
	RH2	Cone	Middle-wave sensitive cone opsin	Snakes
	SWS1	Cone	Short-wave sensitive cone opsin 1	
	SWS2	Cone	Short-wave sensitive cone opsin 2	Snakes
Transducin	*GNAT1*	Rod	G Protein α-subunit 1	
	GNB1	Rod	G Protein β-subunit 1	
	GNGT1	Rod	G Protein γ-subunit 1	Reptiles
	GNAT2	Cone	G Protein α-subunit 2	
	GNB3	Cone	G Protein β-subunit 3	
	GNGT2	Cone	G Protein γ-subunit 2	
Phosophodiesterase	*PDE6A*	Rod	Phosphodiesterase α-subunit 6A	Reptiles
	PDE6B	Rod	Phosphodiesterase β-subunit 6B	
	PDE6G	Rod	Phosphodiesterase γ-subunit 6G	
	PDE6C	Cone	Phosphodiesterase α'-subunit 6C	
	PDE6H	Cone	Phosphodiesterase γ-subunit 6H	
Cyclic nucleotide gated channel	*CNGA1*	Rod	CNG α-subunit 1	
	CNGB1	Rod	CNG β-subunit 1	
	CNGA3	Cone	CNG α-subunit 3	
	CNGB3	Cone	CNG β-subunit 3	
Na^{+}/Ca^{2+}-K^{+}exchanger	*SLC24A1*	Rod	Solute carrier family 24 member 1	Squamates
	SLC24A2	Cone	Solute carrier family 24 member 2	
Arrestin	*SAG*	Rod	Rod arrestin (S-antigen)	
	ARR3	Cone	Arrestin 3 (cone arrestin; X-arrestin)	
G protein-coupled receptor kinase	*GRK1*	Rod	GRK 1 (rhodopsin kinase)	Snakes
	GRK7	Cone	GRK 7 (cone opsin kinase)	
Regular of G-protein signaling complex	*RGS9*	Both	Regulator of G-protein signaling 9	
	RGS9BP	Both	RGS9 binding protein	
	GNB5	Both	G protein β-subunit 5	
Guanylate cyclase activating protein	*GUCA1A*	Both	Guanylate cyclase activator 1A	
	GUCA1B	Both	Guanylate cyclase activator 1B	
	GUCA1C	Cone	Guanylate cyclase activator 1C	
Guanylate cyclase	*GUCY2D*	Both	Guanylate cyclase 2D	
	GUCY2F	Both	Guanylate cyclase 2F	Snake eyes?
Recoverin	*RCVRN*	Both	Recoverin	

many of which are either cone or rod specific (supplementary fig. S1, Supplementary Material online; table 1).

To study the molecular evolution of phototransduction genes in snakes we sequenced whole-eye transcriptomes from seven colubrid caenophidians, including species with all-cone and all-rod retinas based on gross morphology of the outer segments. We also utilized recently sequenced snake and other reptile genomes, as well as new targeted capture sequencing data (Schott et al. 2017), and available resources from GenBank. From these sources, we extracted all known reptilian phototransduction gene coding sequences. Using these data we investigated the effect snake evolutionary origins and photoreceptor transmutation may have had on the evolution of phototransduction genes using codon-based likelihood models implemented in PAML (Yang 2007). We focused on use of the clade models (Bielawski and Yang 2004; Weadick and Chang 2012), which allow variation in selective constraint between (or among) different partitions of a phylogeny. These models have been shown to be extremely useful in testing for long-term shifts in selection pressure associated with changes in ecology and function (Schott et al. 2014; Torres-Dowdall et al. 2015; Van Nynatten et al. 2015; Baker et al. 2016; Dungan et al. 2016; Castiglione et al. 2017; Hauser et al. 2017). Evolutionary transitions between duplex, all-rod, and all-cone retinas through photoreceptor transmutation would presumably require extensive changes to the underlying molecular components of the visual system beyond the morphological changes observed by Walls (1942). These changes likely imposed distinct selection pressures on snake, and in particular caenophidian snake, visual transduction genes relative to other reptiles. Furthermore, if snakes had a fossorial origin as proposed by Walls (1940) that included a degradation in the visual system we would expect a relaxation of selective constraint on phototransduction genes early in snake evolution, as well as considerable gene loss.

Results

Loss of Rhodopsin Kinase (*GRK1*) in Snakes

A total of 35 visual transduction genes (table 1) were targeted for extraction from the de novo eye transcriptomes, as well as from NCBI GenBank, a previously published visual gene hybrid enrichment experiment (Schott et al. 2017), and publically available draft genomes (Castoe et al. 2011; Bradnam et al. 2013; Castoe et al. 2013; Vonk et al. 2013; Green et al. 2014; Georges et al. 2015; Liu et al. 2015; Song et al. 2015).

New sequences were extracted or sequenced from 22 species for a total of 577 new sequences and this was combined with sequences available on GenBank for 1,375 sequences total (supplementary files 1 and 2, Supplementary Material online). Of the 35 genes targeted, 29 were recovered in snakes. Two genes, *PDE6A* and *GNGT1*, were absent in all sampled reptiles but present in sampled mammals, amphibians, and fishes. A third gene, *SLC24A1*, was absent in all sampled squamates but is present in other vertebrates. Two visual opsins, *RH2* and *SWS2*, previously identified to have been lost in snakes (Davies et al. 2009; Castoe et al. 2013; Schott, Muller, et al. 2016; Simões, Sampaio, Loew, et al. 2016), were not recovered from any of the snake transcriptomes or genomes we analyzed, further supporting their ancestral loss in snakes. Additionally, we did not recover *GRK1* (rhodopsin kinase) in any snake eye transcriptome or genome, suggesting that this gene was also lost ancestrally in snakes. This is particularly notable because the loss of *GRK1* has not been reported in any other vertebrate group.

One gene, *GUCY2F*, was recovered from the snake genomes but was absent from the snake eye transcriptomes. *GUCY2F* sequences from the cobra and corn snake genomes were used as references for extraction of *GUCY2F* from the snake eye transcriptome, and using these references we were able to recover *GUCY2D* but not even a fragment of *GUCY2F*, suggesting that it was not expressed in the eye transcriptomes. However, it remains possible that *GUCY2F* is only expressed in the eye under specific conditions (e.g., in juveniles) or may be expressed outside of the retina.

Distinct Selection Pressures on Snake Phototransduction Genes

The 29 phototransduction genes recovered in snakes and other reptiles were analyzed with codon-based likelihood models implemented in PAML (Yang 2007). The genes were broken into three groups: 8 rod-specific genes, 13 cone-specific, and 8 nonspecific genes found in both photoreceptor types. A single species phylogeny was used to maintain an even comparison between all genes (supplementary figs. S2 and S3, Supplementary Material online), although a subset of genes were additionally analyzed using maximum likelihood (ML) gene trees to ensure the results were robust to differences in topology.

Random-sites models were used to determine overall selective constraint acting on each gene in reptiles and snakes (M0 and M3), and to test for positive selection (M2a vs. M1a, and M8 vs. M8a/M7). Overall constraint was highly variable for the phototransduction genes in reptiles ranging from 0.005 for *GNB1* to 0.250 for *GUCA1C*, with an average ω (d_N/d_S) of 0.122 (supplementary table S1, Supplementary Material online, Schott et al. 2018); these values span the range expected for functional protein coding genes (Fay and Wu 2003). Positive selection across reptiles was somewhat rare, with significant evidence from the M8 or M2a models occurring in ten of the genes (supplementary tables S2 and S3, Supplementary Material online, Schott et al. 2018). Relative to reptiles, snakes had significantly higher ω (average of 0.233, $P < 0.0001$, paired samples *t*-test), ranging from 0.010 for *GNB1* to 0.498 for *CNGB3* (supplementary table S1, Supplementary Material online, Schott et al. 2018). Concordantly, pervasive positive selection was more widespread in snakes with significant evidence occurring in 17 genes (supplementary tables S2 and S4, Supplementary Material online, Schott et al. 2018). In most cases, the positive selection within snakes seems to account for the positive selection seen in reptiles generally; however for two genes (*CNGB1*, *GUCA1B*), we found significant positive selection in the reptile data set but not in the snake-only data set. This could be a result of positive selection elsewhere in the reptile tree or may be a result of a lack of power to detect the weak signal of positive selection found in these genes with the smaller number of taxa present in the snake-only data set.

Within snakes, cone-specific genes had significantly higher ω than rod-specific genes (average ω 0.301 vs. 0.161, unpaired *t*-test, two-tailed $P = 0.013$; supplementary table S1, Supplementary Material online). Nonspecific genes had an intermediate ω (0.192), and a one-way ANOVA comparing all three groups was significant ($P = 0.019$). The elevated ω of cone-specific genes was reflected in the presence of positive selection, which was found to be significant for 10 of the 13 cone-specific genes with either the M2a or M8 models, with an additional two genes showing positive selection under M3 (supplementary tables S2 and S4, Supplementary Material online). The difference between cone- and rod-specific genes was not maintained across nonsnake reptiles (average ω 0.09 vs. 0.11, unpaired *t*-test, two-tailed $P = 0.565$; supplementary table S1, Supplementary Material online), suggesting that the elevated ω of cone-specific genes is particular to snakes. We also compared average ω for genes involved in phototransduction activation with those involved in recovery but found no significant difference between them in snakes (average ω 0.22 vs. 0.25, unpaired *t*-test, two-tailed $P = 0.504$). Ion channels, which were found to have some of the highest ω values among visual genes in mammals (Invergo et al. 2013), were not found to have significantly higher ω than other phototransduction genes in snakes (average ω 0.28 vs. 0.22, unpaired *t*-test, two-tailed $P = 0.337$). Overall patterns of ω appear to be largely driven by differences between reptiles and snakes and, within snakes, between cone-specific and other gene types. Previous analyses of visual gene molecular evolution in snakes have focused solely on the visual opsins (Simões et al. 2015; Schott, Muller, et al. 2016; Simões, Sampaio, Douglas, et al. 2016). Simões, Sampaio, Douglas, et al. (2016) found significant positive selection in all three opsin genes using random-sites models, which differs from our current results that did not recover significant positive selection in *SWS1* (supplementary tables S2 and S3, Supplementary Material online).

Of the genes we analyzed, *RH1* stands out in our data set as being the only rod-specific gene with pervasive positive selection in snakes. This appears to be due to the larger sample sizes for this gene, and the other visual opsins, thanks to the sequencing efforts of Simões et al. (2015), Simões, Sampaio, Douglas, et al. (2016), and Simões, Sampaio, Loew, et al. (2016). When the *RH1* data are restricted to the same taxon sampling as the other genes, evidence for a positively selected

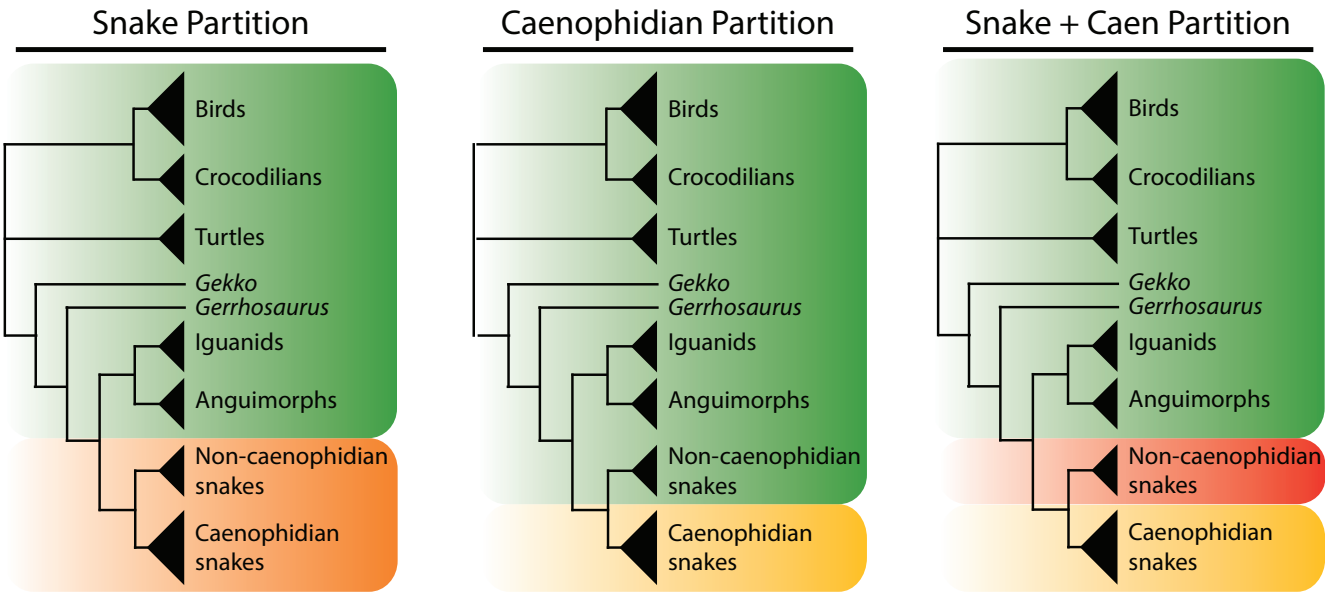

Fig. 2. Partitions used to analyze shifts in selective constraint in snakes relative to other reptiles. The snake partition compares snakes with all other reptiles. We additionally compared the branch leading to snakes with all other branches (supplementary fig. S5, Supplementary Material online). The caenophidian partition compares caenophidian snakes with all other reptiles, and was additionally tested within only snakes by comparing caenophidian snakes with other snakes (supplementary fig. S5, Supplementary Material online). Because the two-partition tests are not mutually exclusive we tested them simultaneously by comparing caenophidian snakes, noncaenophidians snakes, and nonsnake reptiles using a three-partition test (Snake + Caen partition). Topology of trees is based on the species tree shown in supplementary figure S2, Supplementary Material online.

class of sites was not found. This suggests a more subtle effect that may be restricted to particular taxa. When sampling was restricted for the other opsins the results were qualitatively the same, although the strength of positive selection in *LWS* was even higher, again suggesting taxon-specific differences. These results suggest that additional sampling may be needed to detect more subtle and taxon-specific effects, but more broad scale patterns are readily captured by our data.

Long-Term Shifts in Selection Pressures on Caenophidian Snake Phototransduction Genes

To further explore the selective pressures acting on the snake visual system relative to other reptiles, we analyzed the reptile data sets using Clade Model C (CmC) and Clade Model D (CmD) (Bielawski and Yang 2004). These models allow selective constraint on a proportion of sites to vary between two or more partitions of the phylogeny. Through a comparison to a null model that does not allow different partitions (M2a_rel; Weadick and Chang 2012), these models test for a long-term shift in the intensity of selection (i.e., divergent selective pressures; Bielawski and Yang 2004; Schott et al. 2014; Baker et al. 2016). We used three different partitions in order to test whether caenophidian snakes, where photoreceptor transmutation has occurred, have experienced a shift in selective pressures relative to other reptiles and snakes (fig. 2). We first tested for a difference between snakes and all other reptiles (snake partition), which could be due to general differences in snakes, perhaps as a result of their evolutionary origins. Next, we tested for a difference between caenophidian snakes and other reptiles (caenophidian partition) to examine the potential influence of photoreceptor transmutation. Finally, because the first two partitions overlap we also use a three-partition model that compared caenophidians, noncaenophidian snakes, and nonsnake reptiles (snake + caenophidian partition), allowing us to differentiate between shifts in selective pressures that may be present in all snakes and those specific to caenophidians.

We found significant evidence for a shift in selection pressure in snakes relative to other reptiles in 26 of the 29 phototransduction genes (fig. 3, supplementary fig. S4 and tables S2 and S3, Supplementary Material online). One of the genes that did not have any evidence of divergent selection (*GNB1*) was under extremely high constraint, but the other (*SAG*) was under low constraint, and it appears there may have been elevated ω in one or more of the background (nonsnake) lineages. Two of the genes showed a shift in selection in the opposite direction to the other genes, with elevated ω in the background (nonsnake) partition (*CNGA1*, *GUCY2F*). We also found significant shifts in selection pressures in the same 26 genes when the caenophidian partition was used, with one additional gene (*PDE6G*) also showing significant differences (fig. 3, supplementary tables S2 and S3, Supplementary Material online). The other two genes (*GNB1* and *SAG*) lacked significant differences, and *CNGA1* similarly had elevated rates in the background rather than the foreground. This similarity is not unexpected as the snake partition contains all the taxa present in the caenophidian partition (fig. 2).

To differentiate between the snake and caenophidian two-partition models, we conducted a third set of tests with three partitions: Nonsnakes, noncaenophidian snakes, and caenophidian snakes (fig. 2). The combined tests using the three-

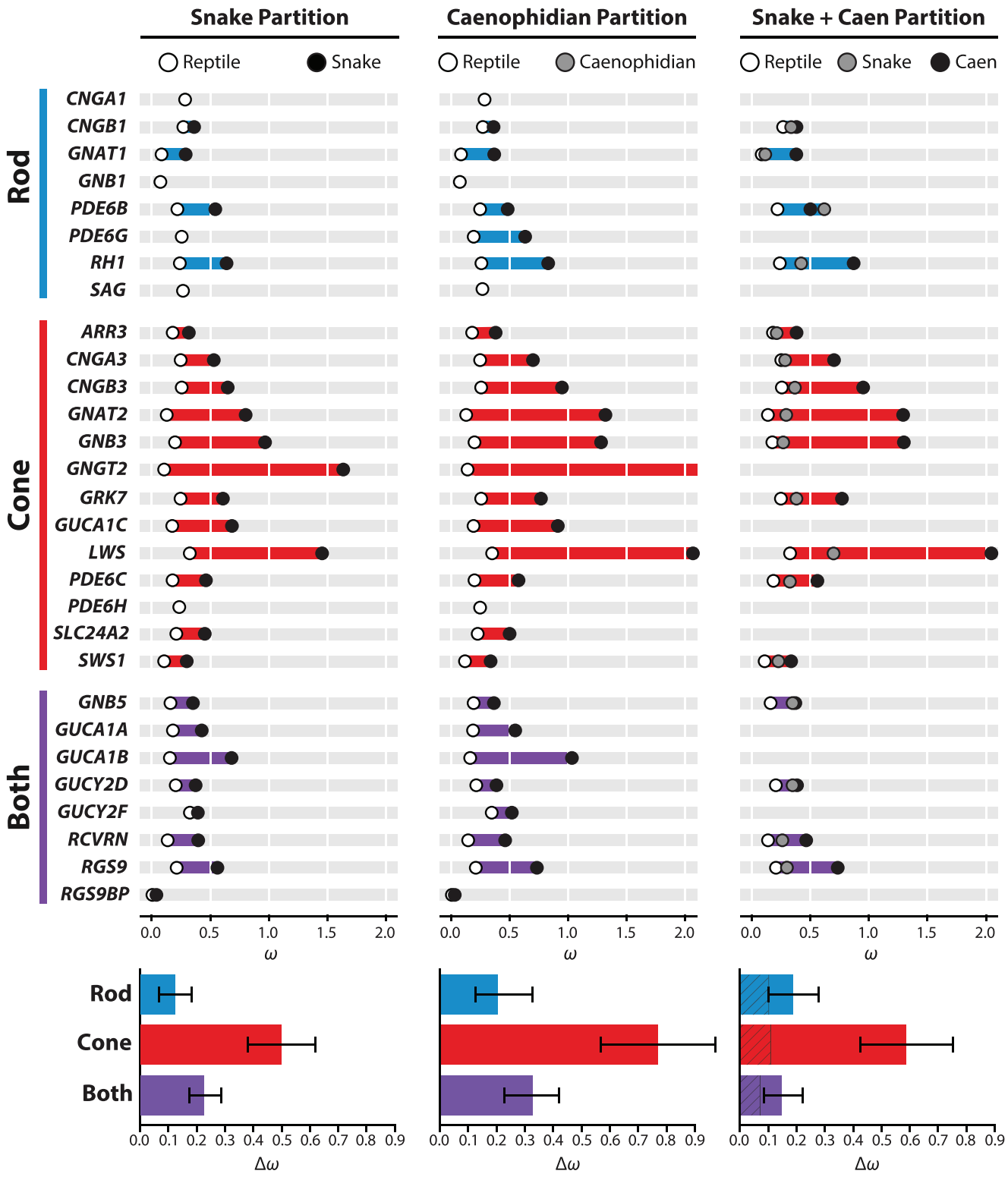

FIG. 3. Tests for shifts in selective pressures on phototransduction genes between snakes and other reptiles (Snake Partition), caenophidian snakes and other reptiles (Caenophidian Partition), and between caenophidians, other snakes, and other reptiles (Snake + Caen Partition) as shown in figure 2. The ω (d_N/d_S) values of the divergent site class using CmC are shown highlighting the difference between the background (open circle) and foreground (closed circle) partitions for each gene. When only an open circle is shown the difference was not significant and instead the equivalent value from the null model (M2a_rel) is shown. Differences in ω were averaged for rod-, cone-, and nonrod/cone-specific genes demonstrating the relative strength of divergent selection. For the Snake + Caen partition, the hatched area represents the difference in ω between snakes and other reptiles, whereas the full bar represents the difference between caenophidian and other reptiles. Error bars are standard error. A summary of *P*-values is given in supplementary table S3, Supplementary Material online, and full results tables are available on Dryad (Schott et al. 2018).

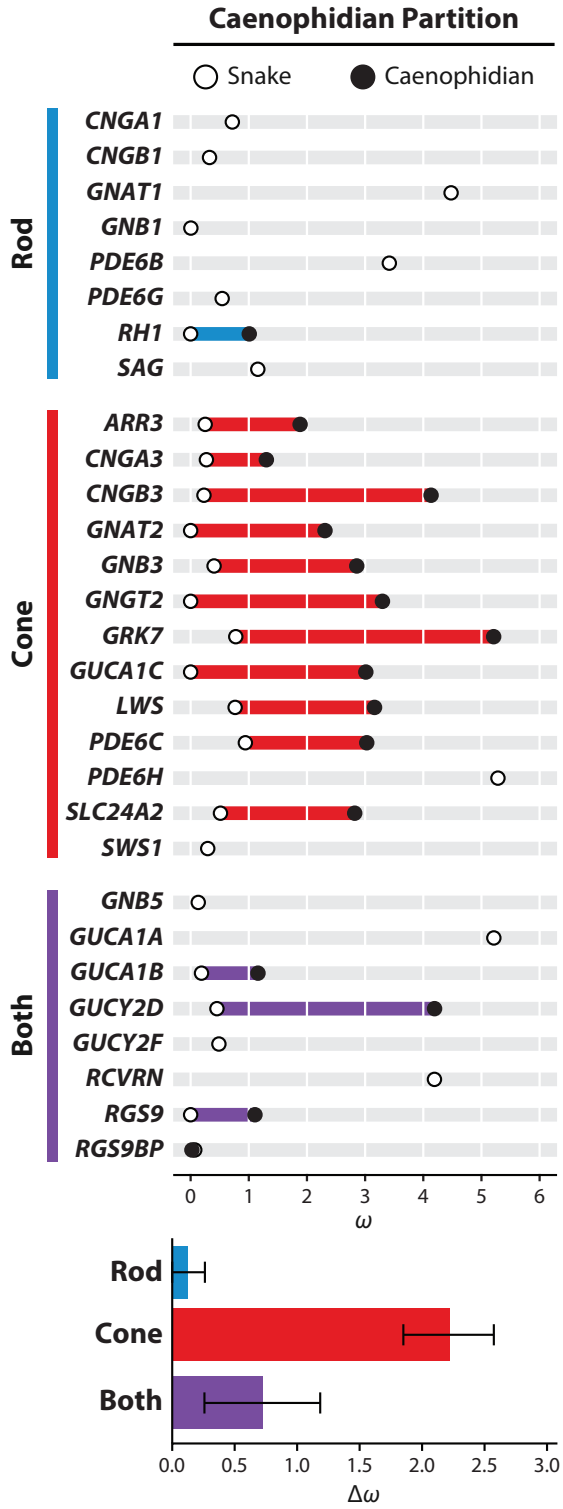

Fig. 4. Tests for shifts in selective pressures on phototransduction genes between caenophidian and noncaenophidian snakes (supplementary fig. S5, Supplementary Material online). The ω (d_N/d_S) values of the divergent site class using CmC are shown highlighting the difference between the background (open circle) and foreground (closed circle) partitions for each gene. Where only an open circle is shown the differences were not significant and instead the equivalent value from the null model (M2a_rel) is shown. Differences in ω were averaged for rod-, cone-, and nonrod/cone-specific genes demonstrating the relative strength of divergent selection. Error bars are standard error. A summary of *P*-values is given in supplementary table S4, Supplementary Material online, and full results tables are available on Dryad (Schott et al. 2018).

partition model were significant for 19 genes, and in seven cases the three-partition model was the overall best-fitting model as determined by Akaike Information Criterion (supplementary table S2, Supplementary Material online; Schott et al. 2018). For several genes the three-partition model failed to converge, likely due to the low sample size for noncaenophidian snakes and these results were also reported as nonsignificant. The overall best-fitting partition varied considerably among the genes with the best-fit being the snake partition 8 times, caenophidian 12 times, and the 3-partition model 7 times, with no discernible pattern among rod-specific, cone-specific, or nonspecific genes. These findings are likely influenced by the fact that only two noncaenophidian snakes were present for the majority of the gene data sets (supplementary files 1 and 2, Supplementary Material online). Only for the three opsin genes was a larger sampling of noncaenophidians snakes possible. For these genes, the snake partition was best-fitting for *RH1* and *SWS1*, whereas the three-partition model was the best fit for *LWS* (supplementary table S2, Supplementary Material online, Schott et al. 2018).

Overall, we found support for a shift in selective pressure specific to snakes for 15 genes, whereas we found support for a shift specific to caenophidians for 19 genes. The magnitude of the shift was larger for caenophidian snakes than noncaenophidian snakes, and, in line with the random site results, was larger for cone-specific genes than for other genes (fig. 3). Together, these findings represent a high number of positive outcomes and are consistent with the expectation that photoreceptor transmutation would have resulted in substantial changes to the phototransduction machinery.

To account for the fact that each test was performed on 29 different alignments we followed Baker et al. (2016) in implementing the false discovery rate (FDR) control method of Storey (2002). After FDR control, the clade model results all remained significant (supplementary results and file 3, Supplementary Material online). We further tested the robustness of the data to differences in tree topology by reanalyzing the data using ML gene trees and found very similar results (Supplementary Results; Schott et al. 2018).

Positive Selection in Caenophidians Primarily in Cone-Specific Phototransduction Genes

To examine the effect that photoreceptor transmutation may have had on phototransduction genes more specifically, we utilized a snake-only data set to test for shifts in selective pressure, and positive selection, between caenophidian and noncaenophidian snakes directly (supplementary fig. S5, Supplementary Material online). Among the rod-specific genes, only *RH1* showed significant evidence for different selection pressures between caenophidians and other snakes (fig. 4, supplementary tables S2 and S4, Supplementary Material online). The cone-specific genes showed a strong

pattern where all genes expect *PDE6H* were found to have significant shifts in selection between caenophidians and other snakes, with positive selection in the caenophidians snakes (fig. 4, supplementary table S2, Supplementary Material online; Schott et al. 2018). This includes *SWS1*, which did not have evidence of pervasive positive selection in snakes in general (when using the random-sites models) but did show positive selection in caenophidian snakes with the CmD model (supplementary table S2, Supplementary Material online). Nonrod/cone-specific genes showed a somewhat intermediate pattern with five of the eight genes having significant evidence for a shift in selective pressures between caenophidians and other snakes. For one nonrod/cone-specific gene (*GUCY2D*), we also found evidence of positive selection in caenophidians. In two other nonrod/cone-specific genes (*GUCY2F*, *RGS9BP*), the elevated ω was found to be in non-caenophidians, rather than caenophidians. These results support our hypothesis that caenophidians have experienced positive selection in phototransduction genes that may be associated with photoreceptor transmutation, although this is surprisingly limited primarily to cone-specific genes.

No Evidence for a Relaxation of Constraint on the Branch Leading to Snakes

A fossorial origin of snakes, as hypothesized by Walls (1940), would predict that relaxed selection along the branch leading to snakes may have occurred (supplementary fig. S5, Supplementary Material online). To test for this, we employed the branch and clade models. We also applied the branch-site model to this branch to test for positive selection.

We found sporadic evidence for elevated ω along the branch leading to snakes using the branch model and CmC in each of the three categories of visual transduction genes; however, consistent support for a relaxation of selection on the branch leading to snakes was not found (supplementary table S5, Supplementary Material online; Schott et al. 2018). Four of the cone-specific genes (*ARR3*, *GNAT2*, *GNGT2*, *SLC24A2*) showed significant evidence of positive episodic selection with the branch-site model (supplementary table S5, Supplementary Material online), indicating the potential for adaptive evolution along the snake branch in these genes. Surprisingly, no evidence for positive selection, or a shift in selection pressure, was found on *GRK7*, which may have been expected alongside the loss of *GRK1*. Genes that lacked a shift in selective pressure in snakes and caenophidians (*SAG* and *GNB1*) also had no evidence for shifts along the branch leading to snakes.

Evidence for divergent and episodic positive selection on the branch leading to caenophidians was much more prevalent (supplementary table S5, Supplementary Material online). This is likely due, at least in part, to the long-term shifts in selection pressures found in snakes and caenophidian snakes, rather than selection specifically along the branch leading to caenophidians. However, this may also indicate potential changes associated re-evolution of double cones inferred to have occurred ancestrally in caenophidians (fig. 1).

Discussion

We used new whole-eye transcriptome data, combined with data derived from recent whole genomes and targeted capture, to compile a large data set of reptilian visual transduction genes. This data set was analyzed with a suite of codon-based likelihood models to examine changes in selective pressures on phototransduction genes in snakes that may be associated with snake evolutionary origins and photoreceptor transmutation. Within the set of 29 visual transduction genes analyzed we found strong support for elevated ω in snakes relative to other reptiles in 26 genes. Surprisingly, we found very little evidence for relaxed selection on the branch leading to snakes. However, we did find significant evidence for a long-term shift in selection between caenophidians and other reptiles in 27 of the genes. Within caenophidian snakes, we found the strongest evidence for positive selection in cone-specific genes. We also confirmed the loss of two cone opsins in snakes with transcriptomic and genomic data, and further identified the apparent loss of expression of *GUCY2F* within the snake eye, as well as the unique, snake-specific loss of *GRK1*.

The loss of *GRK1* in snakes is surprising because this gene encodes rhodopsin kinase, which is expressed in rods (and in some species also cones; Osawa and Weiss 2012). Regardless of the precise nature of the evolutionary origin of snakes, a nocturnal or otherwise dim-light ancestry for extant snakes is well supported (Walls 1942; Hsiang et al. 2015; Simões et al. 2015; Lee et al. 2016; Anderson and Wiens 2017). Rather than the loss of *GRK1*, the loss of *GRK7* (cone opsin kinase), for example, would be more consistent with a dim-light lifestyle, and such a loss has occurred in nocturnal rodents, which express *GRK1* in both cones and rods (Weiss et al. 2001). As extant snakes generally have rods and dim-light vision, it is likely that *GRK7* has been co-opted to also be expressed in rods. This suggests that *GRK7* was already expressed in rods prior to its loss in snakes and raises the possibility that other squamates may also have co-opted expression of *GRK7* in both rods and cones. *GRK7* was shown to have a 10- to 30-fold higher specific activity than *GRK1* in fishes and has been implicated as contributing to the much faster photoresponse recovery times characteristic of cones (Wada et al. 2006; Tachibanaki et al. 2012; but see also Horner et al. 2005). We did not, however, find evidence of episodic positive selection on the branch leading to snakes in *GRK7* but did find evidence for positive selection on *GRK7* within caenophidian snakes. This implies that there may be adaptive shifts in GRK7 activity associated with the evolution of all-cone and all-rod retinas through transmutation in caenophidians. Further work to clarify these functional differences in diurnal and nocturnal caenophidian snakes would provide valuable insight into the evolution of snake visual systems.

We found evidence for a long-term shift in selection pressure in snakes versus other reptiles in nearly all visual transduction genes, with significantly elevated ω (d_N/d_S) in snakes, which for some genes included evidence of positive selection. Compared with recent studies of phototransduction gene evolution in mammals and raptorial birds (Invergo et al.

2013; Wu et al. 2016), snakes had a much higher incidence of positive selection across genes, and the only evidence of positive selection detected across an entire clade. Furthermore, the elevated ω in cone-specific genes relative to rod-specific genes that we found in snakes, but not reptiles, has not been found in either mammals or birds (Invergo et al. 2013; Wu et al. 2016). Overall, striking differences in patterns of molecular evolution, and the scope of shifts in evolution, between snakes and other vertebrates are indicative of the distinct nature of snake visual system, as well as their unique and complex evolutionary origins.

Snakes are thought to have originated from either fossorial or aquatic lizards, but distinguishing between these hypotheses has been difficult, with contradictory evidence presented on both sides (Walls 1942; Caldwell and Lee 1997; Caprette et al. 2004; Lee 2005; Longrich et al. 2012; Hsiang et al. 2015; Simões et al. 2015; Yi and Norell 2015; Lee et al. 2016). We postulated that an extended fossorial phase during snake evolution may be detectable via a relaxation of selective constraint, as well as in the loss of visual transduction genes. Although we found no consistent evidence for relaxation in selective constraint along the branch leading to snakes, we did find that snakes have lost three visual transduction genes (two opsins and one kinase). These findings are remarkably similar to patterns observed in nocturnal, burrowing rodents such as mice that have also lost two opsins and one kinase, rather than the more extreme patterns observed in fossorially adapted mammals that have lost 5–16 phototransduction genes, depending on the degree of fossorial adaptation (Emerling and Springer 2014). The relatively low degree of gene loss in snakes and lack of evidence for relaxed evolutionary constraint early in their evolutionary history are most consistent with a dim-light activity phase during early snake evolution that may have included nocturnal, burrowing, and/or aquatic habits, but did not entail strong adaptation to fossoriality. These findings are exciting because they provide new genomic insight into long-standing debates on snake origins and are further consistent with the conclusions of other recent studies based on visual pigment complement (Simões et al. 2015), phylogenetics (Hsiang et al. 2015), and the morphology of the candidate stem-snake *Tetrapodophis* (Lee et al. 2016). Additionally, these findings also agree, at least in part, with the early views of Rochon-Duvigneaud (1943) and Underwood (1977) that nocturnality played a key role in the evolution of the snake eye (see Simões et al. 2015). Unfortunately we were unable to obtain a complete set of phototransduction genes from the early diverging and highly fossorial scolecophidian snakes, but data from scolecophidian visual opsin genes are available (Simões et al. 2015). Scolecophidians have lost the *SWS1* and *LWS* genes (Simões et al. 2015) consistent with strong adaptations to fossorial habits in this lineage. Analysis of relaxed selection on *RH1* along the branch leading to snakes did not show a different pattern from the other genes, despite the inclusion of four scolecophidian species. However, further analysis of phototransduction gene evolution in scolecophidian snakes is needed and will likely provide additional insight.

In addition to the extensive changes to the eye that occurred during the evolutionary origin of snakes, major evolutionary transitions between retina types in caenophidian snakes are thought to have occurred through photoreceptor transmutation. This likely required extensive changes to the underlying molecular components of the phototransduction cascade. Consistent with this idea, we found 14 visual transduction genes under positive selection in caenophidian snakes. Somewhat surprisingly, the strongest positive selection was found on cone-specific genes, whereas rod-specific genes showed very little difference in selection between caenophidians and other snakes. This pattern might be explained by many evolutionary transitions from diurnality to nocturnality, with much fewer transitions in the opposite direction. Under these conditions, we could expect positive selection in cone-specific genes as they adapted to function under dim-light conditions in the transmuted all-rod retinas of nocturnal species. These adaptations may have acted to enable greater spectral sensitivity and even nocturnal color vision, as has been demonstrated in nocturnal geckos (Roth and Kelber 2004). However, we might lack the power to detect positive selection on rod-specific genes as the nocturnal to diurnal transitions would have occurred much less frequently. It may also be that adaptive change during cone-to-rod transmutation tended toward shifts in function of phototransduction proteins, whereas rod to cone transitions instead involved changes in gene expression and/or other retinal pathways. In general, we might expect less change in rod-specific transduction genes as rods are already thought to be operating near their biophysical limits (Gozem et al. 2012).

Despite having been proposed over 80 years ago, the molecular mechanisms underlying photoreceptor transmutation in snakes have only recently begun to be revealed. Schott, Muller, et al. (2016) demonstrated that the morphologically "all-cone" retina of the diurnal natricine garter snake *Thamnophis proximus* in fact contains a photoreceptor class with rod ultrastructural features that expresses rod-specific proteins, such as RH1 and rod transducin, strongly suggesting that it is actually a transmuted, cone-like, rod. We also recently confirmed this in a second species, the colubrine pine snake *Pituophis melanoleucus*, which is not closely related to garter snakes (Bhattacharyya et al. 2017). In both species, *RH1* was evolutionarily highly conserved, functional when expressed in vitro, and possessed cone-like functional properties, such as a blue-shifted absorption spectrum, decreased stability, and a cone-like retinal binding pocket (Schott, Muller, et al. 2016; Bhattacharyya et al. 2017). Our inference of positive selection in caenophidian snake *RH1* is consistent with these results and may reflect adaptation toward a more cone-like function of RH1 in species that evolved morphologically "all-cone" retinas. Furthermore, these results agree with the only electrophysiological study in diurnal colubrids, which found no evidence of a separate scotopic (dim-light) visual response (Jacobs et al. 1992). We proposed that the transmuted cone-like rods of diurnal colubrids may contribute to an increased range of spectral sensitivity and lay the basis for trichromatic color vision under mesopic (when both the rods and cones are typically active), and potentially even

photopic, conditions (Schott, Muller, et al. 2016). Although this has not been investigated in snakes, increasing evidence from mammals suggests that rods can contribute to color vision (Cao et al. 2008; Joesch and Meister 2016) under both mesopic (McKee et al. 1977; Reitner et al. 1991) and photopic conditions (Oppermann et al. 2016). Additional studies, including behavioral experiments, to further evaluate the functional consequences of rod-to-cone transmutation in snakes would be ideal for corroborating these hypotheses.

In contrast to the morphologically "all-cone" retinas of typical of diurnal colubrids and other caenophidian snakes, some highly nocturnal species have retinas that appear to contain only rods (Walls 1942; Underwood 1970). Although the morphological changes to the photoreceptor cells in these "all-rod" retinas, and inferences of strong positive selection in cone-specific visual transduction genes, are suggestive of transmuted rod-like cones that function under scotopic conditions (similar to the recently discovered transmuted cones of deep-sea pearlside fishes; de Busserolles et al. 2017), the visual capabilities of nocturnal snakes with "all-rod" retinas have not been studied. An analogous process, however, may have occurred in gecko retinas. Geckos have true all-cone retinas that in nocturnal species resemble all-rod retinas in both their appearance and function (Walls 1942; Tansley 1964; Underwood 1970; Röll 2000; Zhang et al. 2006). With their rod-like cones, nocturnal geckos are able to discriminate colors at dim-light levels at which humans are color blind (Roth and Kelber 2004). Nocturnal caenophidian snakes with "all-rod" retinas may have similar nocturnal visual capabilities. However, a key difference between nocturnal geckos and caenophidians is that geckos have lost true rods and RH1, whereas caenophidian snakes have not (Simões, Sampaio, Douglas, et al. 2016; Simões, Sampaio, Loew, et al. 2016). What difference this makes, and how true rods interact with rod-like cones remain open questions for future studies.

Conclusions

Our results indicate that snake phototransduction genes are under different selective constraints compared with reptiles and have experienced positive selection to a degree not generally found in other vertebrate groups. These exceptional selective patterns appear to be linked to both the evolutionary origins of snakes and the evolutionary process of photoreceptor transmutation in caenophidian snakes. The degree of gene loss and divergent selection further supports a dim-light early snake ancestor that was not highly adapted for fossoriality. Our data provide genomic support for a nocturnal origin of snakes but unfortunately provide limited insight into the terrestrial/fossorial versus aquatic debate that is ongoing based on controversial fossil data (Caldwell and Lee 1997; Lee 2005; Longrich et al. 2012; Yi and Norell 2015; Lee et al. 2016). Further analyses of the evolution of visual genes in fossorial squamates (both snakes and nonsnakes) and aquatic reptiles are needed to address these issues. Within caenophidian snakes, high levels of positive selection in cone-specific genes likely reflect adaptive evolution toward a more rod-like function that occurred on multiple branches within the caenophidian clade to facilitate the development of all-rod retinas. Our findings further suggest considerable differences in the function of rod and cone visual transduction proteins that warrants further study. For instance, studies have repeatedly found that rod and cone transducins are functionally similar or even equivalent (Deng et al. 2009; Gopalakrishna et al. 2012; Tachibanaki et al. 2012; Mao et al. 2013); however, we have found strong evidence for positive selection in snake rod and cone transducins (*GNAT1*, *GNAT2*, *GNB3*) that suggests adaptation and functional divergence. Differences between rod- and cone-specific copies of phototransduction proteins are currently an area of active research (Kawamura and Tachibanaki 2008; Renninger et al. 2011; Tachibanaki et al. 2012; Mao et al. 2013; Majumder et al. 2015; Orban and Palczewski 2016; Sakurai et al. 2016). Our results are important for understanding how visual systems evolve and adapt in response to gene loss and changes to activity patterns. Further work will need to be done to elucidate the functional consequences of the changes that occurred in visual genes and to expand sampling in order to better understand the evolutionary history of those changes.

The evolution of phototransduction in the snake visual system provides an extreme and illustrative example of how powerful selective forces can be in fundamentally reshaping and repurposing genetic components of such a complex system as the vertebrate eye. In addition to evidence for broad selection and substantial functional shifts in snake visual systems, evidence that snake evolution has also involved major adaptive shifts in genes underlying metabolism, physiology, and development (Castoe et al. 2013; Vonk et al. 2013) suggests that snakes are a unique and outstanding vertebrate model for studying large-scale adaptation and functional innovation. These findings broadly raise the question of why snakes show extreme adaptation on so many levels, and whether these apparently diverse adaptive shifts may have somehow interacted or been functionally linked.

Materials and Methods

Animals

Colubrid snake samples were obtained from specimens accessioned at the University of Texas at Arlington Amphibian and Reptile Diversity Research Center and from animals euthanized under approved protocols at the University of Toronto (supplementary table S6, Supplementary Material online). Eyes were preserved in either liquid nitrogen or RNAlater (Ambion) and stored at −80 °C.

Transcriptome Sequencing

Whole eyes were homogenized in Trizol (Invitrogen) using a BeadBug (Benchmark Scientific). Total RNA was extracted following a combined Trizol/RNeasy (Qiagen) protocol according to the manufacturer's instructions. Library construction and sequencing on the Illumina HiSeq pipeline were performed according to standard protocols at The Centre for Applied Genomics, the Hospital for Sick Children (Toronto). Resulting 150-bp paired-end reads were trimmed with Trimmomatic v0.33 (Bolger et al. 2014) using default

settings. Trimmed reads were assembled de novo using Trinity (Grabherr et al. 2011) under default settings. Total number of read pairs after quality control varied from ~6 million to ~30 million (supplementary table S6, Supplementary Material online). Visual transduction gene transcripts were identified and extracted using BLAST (discontinuous megablast, e-value cutoff of 1e-10). Transcript identities (i.e., orthology to annotated genes) were confirmed through phylogenetic analysis.

Visual Transduction Gene Data Sets

Genes encoding each of the major, known components of the visual transduction cascade (Lamb 2013) were targeted, comprising a total of 35 genes (table 1). The NCBI GenBank database was searched for these genes and coding regions extracted using BlastPhyMe (Schott, Gow, et al. 2016). All nonavian reptile sequences were retained, with a representative sample of ~17 avian sequences selected that span avian diversity (Jarvis et al. 2014) in order to not bias the data sets heavily toward birds. Coding regions from GenBank were used as references to extract those genes from the de novo eye transcriptomes, a previous visual gene hybrid enrichment experiment (Schott et al. 2017), and publically available unannotated draft genomes (Castoe et al. 2011; Bradnam et al. 2013; Castoe et al. 2013; Vonk et al. 2013; Green et al. 2014; Georges et al. 2015; Liu et al. 2015; Song et al. 2015). Coding regions for each gene data set were aligned using MUSCLE codon alignment as implemented in MEGA (Edgar 2004; Tamura et al. 2011). Alignment with other programs (e.g., PRANK) produced very similar results. Areas of nonhomology and poor alignment were removed in order to improve the accuracy of inferences of positive selection (Privman et al. 2012). This often included trimming the ends of the sequences, as well as removing sequence that was nonhomologous either due to being from a transcript variant or incorrectly included in the coding sequence due to errors in automated prediction. ML trees were estimated in MEGA using the GTR + G model in order to ensure that all genes were correctly identified, free of contaminants, and properly aligned prior to downstream analyses.

To maintain an even comparison among genes, and to avoid potential issues of convergence and homoplasy in individual genes, a single species tree was used for most analyses. The topology was based on Pyron et al. (2013) for the squamate relationships, Jarvis et al. (2014) for the avian relationships, and Chiari et al. (2012) and Crawford et al. (2012) for the higher order relationships with the basal trichotomy required by PAML formed by turtles, archosaurs, and squamates (supplementary figs. S2 and S3, Supplementary Material online). The species tree was trimmed or added to as needed to match the sampling available for each gene (Schott et al. 2018). In addition to the full reptile data set and tree, each gene data set and tree was trimmed to contain only snakes. To ensure robustness of the results to changes in tree topology, we additionally analyzed a subset of six genes (*GNAT2*, *GNB1*, *GRK7*, *PDE6B*, *RCVRN*, and *SWS1*) using ML gene tree topologies for the reptile data set. Gene trees were estimated in PhyML 3 (Guindon et al. 2010) under the GTR + G+I model with a BioNJ starting tree, the best of NNI and SPR tree improvement, and aLRT SH-like branch support (Anisimova and Gascuel 2006).

Molecular Evolutionary Analyses

To estimate the strength and form of selection acting on visual transduction genes of reptiles and snakes, each data set was analyzed using codon-based likelihood models from the codeml program of the PAML 4 software package (Yang 2007). Specifically, the random sites (M0, M1a, M2a, M2a_rel, M3, M7, M8a, and M8), branch (Br), branch-site (BrS), and clade models (CmC, CmD) were used (Bielawski and Yang 2004; Zhang et al. 2005; Yang 2007). All analyses were run with varying starting values to avoid potential local optima. To determine significance, model pairs were compared using a likelihood ratio test with a $\chi 2$ distribution, whereas nonnested models were evaluated using Akaike Information Criterion.

Random-sites models were used to determine overall selective patterns and to test for gene-wide positive selection in reptiles and in snakes. The M3 versus M0 comparison tests for variation among sites, whereas the M2a versus M1a and M8 versus M7/M8a comparisons test for a proportion of positively selected sites. M0, M2a_rel, and M3 are also the null models for the Br, CmC, and CmD models, respectively. To test for long-term shifts in selection pressures (i.e., divergent selection) in phototransduction gene we utilized the clade models (CmC and CmD). CmC assumes that some sites evolve conservatively across the phylogeny (two classes of sites where $0 < \omega_0 < 1$ and $\omega_1 = 1$), whereas a class of sites is free to evolve differently among two or more partitions (e.g., $\omega_{D1} > 0$ and $\omega_{D1} \neq \omega_{D2} > 0$). Despite the name, partitions can be any combination of branches and entire clades. CmD is similar, but all three site classes (ω_0, ω_1, ω_D) are unconstrained (meaning they can assume any value). This can be useful when there is little support for a neutral class of sites. A number of different partitions were tested, using both the reptile and snake-only data sets as shown in figure 2 and supplementary figure S5, Supplementary Material online.

We also tested for relaxed selection, and episodic positive selection, on the branch leading to snakes, and to caenophidians (supplementary fig. S5, Supplementary Material online). The branch leading to snakes or caenophidians was placed in the foreground partition and tested using the Br, BrS, and CmC. The Br model is similar to the clade models but contains only a single class of sites, and thus tests for average differences between partitions. This results in a less sensitive test, but it is useful for detection of relaxed selective constraints. The BrS model was designed to test for episodes of positive selection on specific branches (although it can be applied to clade or mixed partitions as well). Unlike the branch and clade model, the BrS model explicitly differentiates between the background and foreground partitions. It has four site classes: 0) $0 < \omega_0 < 1$ for all branches, 1) $\omega_1 = 1$ for all branches, 2a) $\omega_{2a} = \omega_{2b} \geq 1$ in the foreground and $0 < \omega_{2a} = \omega_0 < 1$ in the background, and 2b) $\omega_{2b} = \omega_{2a} \geq 1$ in the foreground and $\omega_{2b} = \omega_1 = 1$ in the background. Positive selection is only allowed in the foreground, which

results in a powerful test for episodic positive selection, but can result in false positives when positive selection is also present in the background (Schott et al. 2014). Additional details on these models and their use to test for long-term selective shifts, episodic selection, and positive selection can be found in Schott et al. (2014) and Baker et al. (2016). Because we perform each test for 26 different alignments, we followed Baker et al. (2016) in implementing the FDR control method of Storey (2002). FDR control was performed using the Shiny implementation of the qvalue R package (http://qvalue.princeton.edu/). As a conservative comparison, we also performed a simple Bonferroni correction.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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