

Transcriptomic data support a nocturnal bottleneck in the ancestor of gecko lizards

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ABSTRACT

Gecko lizards are a species-rich clade of primarily-nocturnal squamate reptiles. In geckos, adaptations to nocturnality have dramatically reshaped the eye. Perhaps the most notable change is the loss of rod cells in the retina and subsequent “transmutation” of cones into a rod-like morphology and physiology. While many studies have noted the absence of some rod-specific genes, such as the visual pigment Rhodopsin (RH1), these studies have focused on just a handful of species that are nested deep in the gecko phylogeny. Thus, it is not clear whether these changes arose through convergence, are homologous and ubiquitous across geckos, or restricted to a subset of species. Here, we used *de novo* eye transcriptomes from five gecko species, and genomes from two additional gecko species, representing the breadth of extant gecko diversity (i.e. 4 of the 7 gecko families, spanning the deepest divergence of crown Gekkota), to show that geckos lost expression of almost the entire suite of necessary rod-cell phototransduction genes in the eye, distinct from all other squamate reptiles. Geckos are the first vertebrate group to have lost their complete rod-cell expression pathway, not just the visual pigment. In addition, all sampled species have also lost expression of the cone-opsin SWS2 visual pigment. These results strongly suggest a single loss of rod cells and subsequent cone-to-rod transmutation that occurred prior to the diversification of extant geckos.

1. Introduction

Behavioral shifts between different photic environments, such as changes from diurnal (day-time) to nocturnal (night-time) activity patterns, have led to major modifications to the vertebrate eye over evolutionary time (Walls, 1934; Walls, 1942; Davies et al., 2012). These modifications involve changes to the types of photoreceptor cells present in the retina, changes in cell morphology, and alterations to the ancestral gene complement used by these cells to transmit light into a biochemical signal (Walls, 1942; Simões et al., 2015; Lamb and Hunt, 2017). Two types of photoreceptors are present in most vertebrate retinas, rods and cones, used for low-light vision and bright-light vision, respectively (Kojima et al., 1992; Lamb, 2013). Rods and cones possess significant differences in their sensitivities to light (rods being more light-sensitive than cones) and phototransduction speed (cones transmit biochemical signals faster than rods) (Li et al., 2010), providing tradeoffs in the selective forces driving adaptation to differing light environments (Simões et al., 2015; Simões et al., 2016; Schott et al.,

2016). Indeed, lineages that have experienced dramatic evolutionary shifts in light environment during their evolution, such as snakes and geckos, have seen concomitant changes in photoreceptor cells, resulting in ‘transmutations’, a process where cone cells take on a rod-cell-like morphology, or rod cells take on a cone-like morphology (Walls, 1934; Walls, 1942; Pedler and Tilly, 1964; Tansley, 1964; Underwood, 1970; Goldsmith, 1990; Zhang et al., 2006; Schott et al., 2016).

The ancestral tetrapod eye utilized five photopigments: four opsins in cone cells: RH2 (Middle-wave sensitive cone opsin), LWS (Opsin 1 – Long-wave sensitive cone or *Opn1lw*), SWS1 (Opsin 1 – Short-wave sensitive cone or *Opn1sw*), and SWS2 (Opsin 2 – Short-wave sensitive cone or *Opn2sw*); and one opsin in rod cells: RH1 (Rhodopsin or *Rho*) (Okano et al., 1992; Davies et al., 2012; Lamb and Hunt, 2017). However, many vertebrate groups lack this ancestral visual opsin complement due to subsequent adaptations to nocturnal, fossorial, and other low-light environments (colloquially referred to as a “nocturnal bottlenecks”), groups including: crocodilians (Emerling, 2017a), burrowing mammals (Emerling and Springer, 2014), snakes (Davies et al.,

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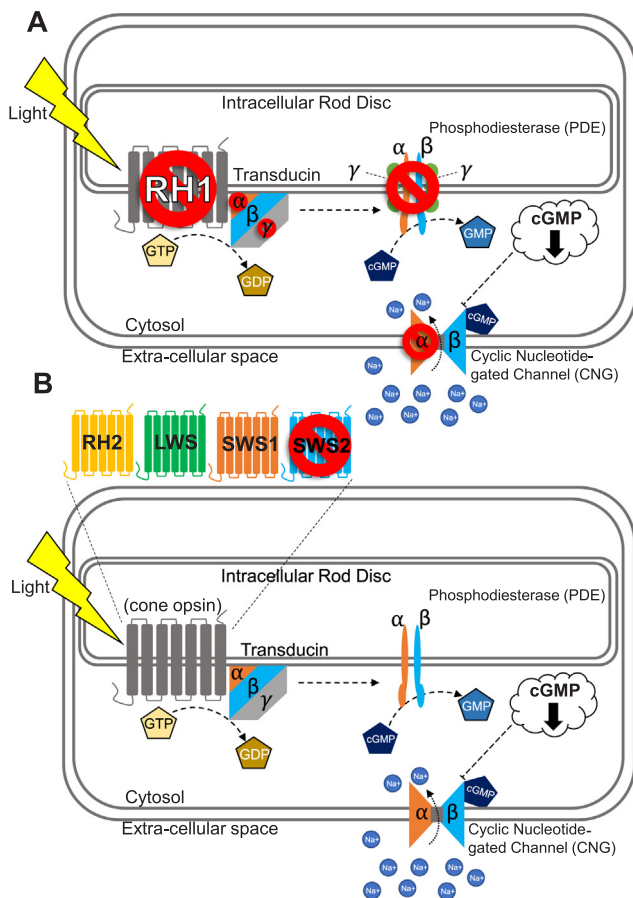


Fig. 1. Cartoon depictions of the hypothetical gecko phototransduction pathways for (A) rod and (B) cone cells, showing the absent members of rod-cell phototransduction alongside the functional cone-to-rod cell phototransduction (also shown in Fig. 3). (A) Rod cells in the gecko retina would be unable to detect light or transmit a biochemical signal. Indeed, further corroborating the absence of rod cells in geckos: downstream from the missing photopigment (RH1), 2/3 subunits of the rod-specific transducin (GNAT1 [α] & GNGT1 [γ]), 3/3 subunits of the rod-specific phosphodiesterase (PDE6A [α], PDE6B [β], & PDE6G [γ]), and 1/2 subunits of the cyclic nucleotide-gated channel (CNGA1 [α]) are missing from the retina. By contrast, (B) all members of the cone cell-specific phototransduction cascade are present, except for a single photopigment (SWS2), in the retina of geckos. Thus, we hypothesize that this pathway has remained unchanged even in the face of the cone-to-rod transmutation in the shared ancestral lineage of extant geckos. Missing transcripts of genes denoted by (denoted by the “Ghostbusters” symbol (circle with slash)), color changes simply denote different protein subunits, and cone opsin color scheme doesn’t correspond with light-sensitivity.

2009; Simões et al., 2015), cetaceans (Levenson and Dizon, 2003), and geckos (Crescitelli et al., 1977; Kojima et al., 1992; Yokoyama and Blow, 2001). Characterizing the presence and absence of components of the phototransduction signaling pathway – particularly photopigments and key members of the phototransduction cascade – among extant species in a phylogenetic context, facilitates investigation of visual adaptation to a particular light environment (Serb and Oakley, 2005).

Photopigments (opsins), which initiate phototransduction, are derived G-protein coupled receptors (GPCRs) that intercept a photic signal. In the ‘canonical’ vertebrate phototransduction pathway (Fig. 1), (1) a photon strikes the opsin protein (rods: RH1; cones: RH2, LWS, SWS1, or SWS2), (2) stimulating the heterotrimeric G-protein transducins (rods: GNAT1, GNB1, & GNGT1; cones: GNAT2, GNB3, & GNGT2), (3) activating a nearby phosphodiesterase (rods: PDE6A, PDE6B, & PDE6G; cones PDE6C & PDE6H) that reduces levels of cellular cGMP, (4) causing the closing of cyclic nucleotide-gated channels

(rods: CNGA1 & CNGB1; cones: CNGA3 & CNGB3), and (5) leading to the hyperpolarization of the cell (Lamb, 2013). The multitude of other genes in this pathway are involved in post-excitation recovery, many of which are shared between cell types. Thus, many proteins within the phototransduction signaling cascade are specific to a respective cell type, either rods or cones, and are necessary for correct signaling (Emerling et al., 2017). To this effect, only a small number of genes appear to have been missing in the most-recent common ancestor of squamates, the lizards and snakes. This includes the γ -subunit of the rod-specific transducin (GNGT1), the α -subunit of the rod-specific phosphodiesterase (PDE6A), and the rod-specific ion exchanger involved in recovery (Solute carrier family 24 member 1; SLC24A1) (Schott et al., 2018); it is currently unknown how, or whether, the absence of these genes is compensated in squamates. However complex the pathway, to-date most characterizations of transmutation and loss of cell morphologies relied solely upon the presence/absence of the cone (LWS, RH2, SWS1, & SWS2) or rod (RH1) photopigments and cellular morphology. For instance, the loss of cone photopigments (SWS2 and RH2) in mammals provided the first evidence of a “nocturnal bottleneck”, a period of dim-light adaptation early in our evolutionary history (Menaker et al., 1997; Gerkema et al., 2013). However, loss of a number of the downstream, cell-type specific signaling genes could reduce signaling efficiency or shut down phototransduction altogether, such is the case in some cetaceans and burrowing mammals, and humans with complete color blindness (Emerling and Springer, 2015; Emerling et al., 2017).

Geckos are thought to be ancestrally nocturnal—with multiple, independent transitions to diurnality throughout their evolutionary history—making them an important model for investigating how changes in light environment impact vision (Walls, 1934; Walls, 1942; Kojima et al., 1992; Röhl, 2000; Roth and Kelber, 2004; Gamble et al., 2015). Nocturnal geckos are, superficially, rod monochromats (possessing a rod-cell gross morphology), contrastingly diurnal species are cone monochromats – with retinas composed of a single rod-like photoreceptor type (Walls, 1942; Underwood, 1951; Underwood, 1954; Röhl, 2000). Examination of opsins and other phototransduction genes of the tokay gecko (*Gekko gekko*) have shown that, despite their rod-like morphology, they produce solely cone-opsin proteins, consistent with the ‘transmutation’ hypothesis (Walls, 1942; Crescitelli et al., 1977; Kojima et al., 1992; Yokoyama and Blow, 2001; Zhang et al., 2006). Indeed, detailed examination of the cellular ultrastructure reveals many characteristics unique to cones and the cellular morphology are only superficially rod-like (Fig. 1; Goldsmith, 1990; Röhl, 2000). Thus, the rod-like cells in the retinas of *Gekko gekko* are transmuted cone cells and the ancestral “true rods” are absent.

Despite their historic importance for studying visual system evolution, nearly all studies of the molecular components of the gecko visual system to-date have been conducted within the genus *Gekko* (mostly *Gekko gekko*) and a few species of Malagasy day geckos (*Phelsuma* spp.) (Crescitelli et al., 1977; Kojima et al., 1992; Loew, 1994; Taniguchi et al., 1999; Taniguchi et al., 2001; Yokoyama and Blow, 2001; Roth et al., 2009; Liu et al., 2015; Schott et al., 2019). Both genera are within the family Gekkonidae, an ancestrally nocturnal family nested deep within the infraorder Gekkota (composed of seven families), and, thus, fail to show whether these changes are gecko-wide or specific to the family Gekkonidae (Gamble et al., 2015; Fig. 2). Indeed, Underwood (1954) suggested that transmutation may have occurred up to three times in geckos and gecko rod-like retinas evolved repeatedly through convergent evolution. In line with these observations, more recently, the examination of pseudogenes in the *Gekko japonicus* genome suggested a possible step-wise loss of visual opsins (Liu et al., 2015), with loss of SWS2 occurring approximately ~202 million years ago (mya), preceding the loss of the rod opsin, RH1, ~81mya (Emerling, 2017b), well-after the hypothesized divergence of extant gekkotan families ~120 mya (Gamble et al., 2011; Gamble et al., 2015). Therefore, it remains unclear whether the cone-to-rod transmutation and loss of rod

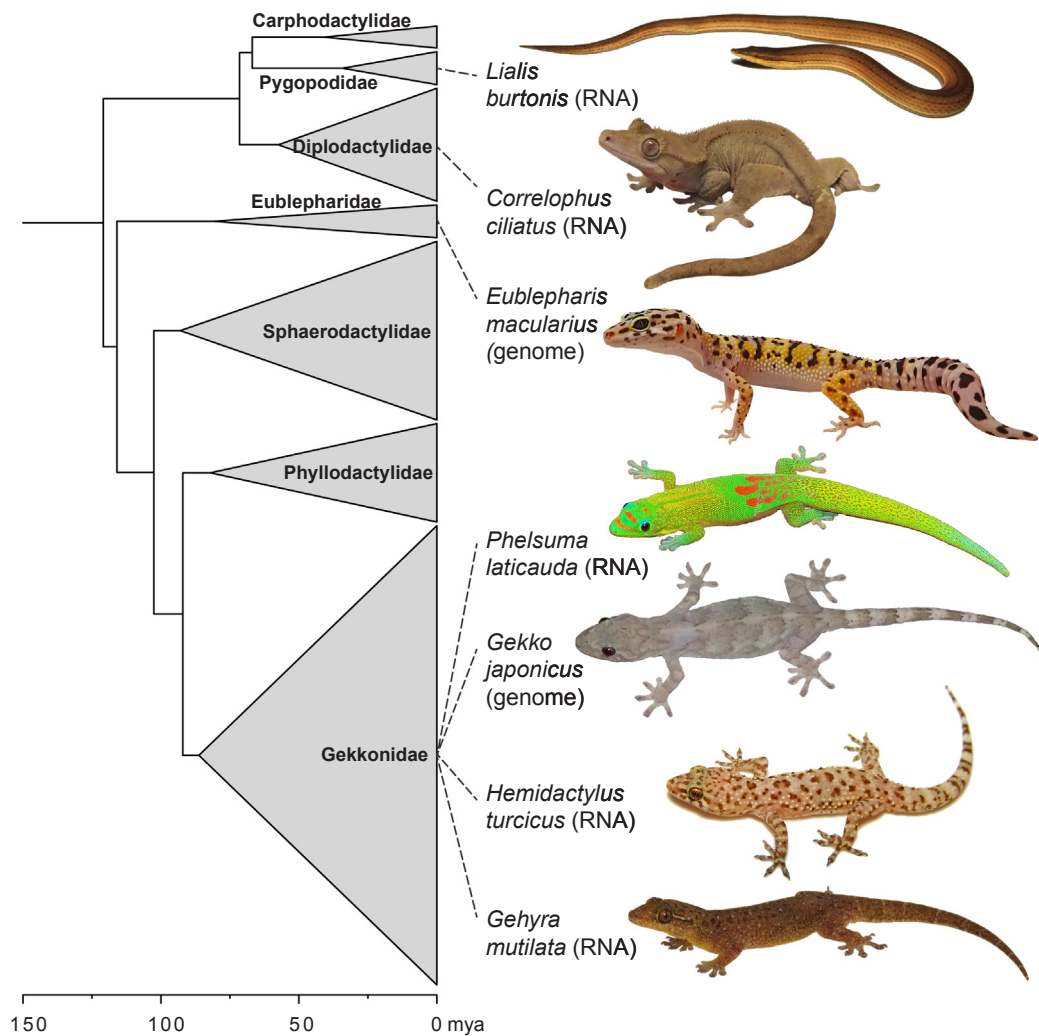


Fig. 2. Phylogeny, pruned from Gamble et al. (2015), depicting relationships among seven gecko families (> 1800 described species). Size of the triangle representing each family is roughly proportional to the number of species in that clade. Sampled gecko species are illustrated indicating whether data came from transcriptomes or genomes. Time scale is millions of years ago (mya).

photoreceptor cells occurred multiple times independently in geckos or prior to the diversification of extant geckos.

Here, we combine six *de novo* eye transcriptomes (5 geckos + 1 outgroup), with data from two previously-published gecko genomes to investigate the early evolution of gecko vision across the entire phylogenetic breadth of extant geckos for the first time (Fig. 2). We find that a suite of genes, necessary for rod-cell phototransduction, as well as cone opsin (SWS2), are not expressed in any sampled gecko species, consistent with (i) a complete loss of ancestral rod cells and (ii) a cone-to-rod transmutation during an ancestral transition to nocturnality in the shared ancestral lineage leading to extant geckos, resulting in the simplex rod-only (or cone-only) retinas observed in past studies of gecko vision.

2. Materials and methods

2.1. RNAseq and transcriptome assembly

During daytime hours, following a 12-hour dark cycle, we euthanized and removed whole eyes from 5 geckos, representing the breadth of extant gecko diversity (Fig. 2; *Correlophus ciliatus*, *Gehyra mutilata*, *Hemidactylus turcicus*, *Lialis burtonis*, and *Phelsuma laticauda*) and an outgroup (Pinto et al., 2019). All species are nocturnal or crepuscular except *P. laticauda*, which is diurnal. Tissues were flash frozen

at -80°C in TRIzol™ reagent. RNA extraction, library prep, and transcriptome assembly processes are identical to those described by Pinto et al. (2019). Briefly, we extracted RNA using the Qiagen RNeasy™ Mini Kit and prepared RNAseq libraries with KAPA® Stranded mRNA-Seq Kit (KR0960 [v5.17]). Libraries were sequenced on an Illumina® HiSeq 2500 (paired-end 125 bp reads). We assembled *de novo* transcriptomes for each species using the *De novo* RNA-Seq Assembly Pipeline (DRAP) [v1.91] (Cabau et al., 2017), which is a compilation of automated assembly (Trinity [v2.4.0]; Grabherr et al., 2011) and quality-control tools to reduce transcript redundancy.

2.2. Ortholog identification and phylogenetic analyses

We downloaded a set of 35 key phototransduction genes, including visual opsins, assumed to be present in the ancestor to all tetrapods (Schott et al., 2018), for nine species (Supplemental Table 3), originally from Ensembl [v91.0]. We used BLAST, implemented in Geneious® [v11.1.2] (Altschul et al., 1990; Kearse et al., 2012) to identify putative orthologs to these genes from annotated CDS's from the published genomes of nine additional species, including two additional geckos (*Eublepharis macularius*; Xiong et al., 2016, and *Gekko japonicus*; Liu et al., 2015), and eye transcriptomes from a chameleon (*Chamaeleo calyptratus*; Pinto et al., 2019) and the five gecko species described above (Supplemental Table 3). Sequences were translation-aligned

using MAFFT [v7.388] implemented in Geneious® [v11.1.2] (Katoh et al., 2002; Kearse et al., 2012). We used phylogenies to confirm sequence orthology for several gene families. Thus, we generated gene trees of paralogous gene alignments (a subset of which are presented in Supplemental Figs. 1–4) on the CIPRES portal (Miller et al., 2010) using the RAXML Blackbox [v8.2.10] (Stamatakis, 2014), under the GTR + Γ model and RAXML's automatic bootstopping function. CNGA3 sequences were constrained as monophyletic – following Lamb and Hunt (2017).

Four transcripts were not found in the assembled transcriptomes (GNAT2 in *Correlophus*, NGT2 and GUCY2D in *Lialis*, and SAG in *Hemidactylus*). However, since the numbers of assembled *de novo* transcripts can vary greatly when assembled from short reads (Zhao et al., 2011), we suspected that these ‘rogue’ transcripts were present, just not assembled. We confirmed their presence, or absence, by mapping quality-filtered RNAseq reads to the respective transcript of their closest sampled relative using Geneious® [v11.1.2]. Similarly, two genes (GNAT1 and GUCY2F) had functional copies present in the two gecko genomes but were not assembled in any of the five gecko transcriptomes. We mapped RNAseq reads to GNAT1 and GUCY2F in the *G. japonicus* genome but recovered no orthologous transcripts for either gene in any of the sampled geckos. Interestingly, GUCY2F expression was also absent in *C. calyptatus* and has been identified previously to be absent in snakes (Schott et al., 2018). We performed this read-mapping strategy to all rod-specific transcripts that were consistently missing from all gecko transcriptomes (mapped to assembled *C. calyptatus* transcripts). To visualize these data: (1) in a phylogenetic context, we produced a character matrix indicating presence/absence of each phototransduction gene from the genome or transcriptome for every sampled species using *phytools* [v0.6–60] (Revell, 2012) in R (R Core Team, 2008), and (2) in a molecular pathway, we generated a simplified phototransduction cascade for the ancestral rod (Fig. 1a) and cone (Fig. 1b) cells.

3. Results and discussion

We assembled *de novo* eye transcriptomes for five gecko species and one chameleon, *Chamaeleo calyptatus* (assembly statistics and benchmarking information are in Supplemental Table 1). We recovered the same 25 (out of 35) phototransduction genes in the RNAseq data from the five gecko eyes. In contrast, we recovered 30 (out of 35) phototransduction genes from the chameleon transcriptome (Fig. 3). Eight rod-specific genes, including the visual opsin RH1, were missing in all the gecko transcriptomes, which supports the hypothesis that rod cells were lost in the shared ancestral lineage of extant geckos (Fig. 3). Similarly, the cone-specific opsin, SWS2, was missing from all sampled geckos but present in chameleon. Maximum-likelihood phylogenies from visual opsins and several other phototransduction genes (Supplemental Figs. 1–4) were largely concordant with previously published gene trees (Lamb and Hunt, 2017).

While there was broad concordance between our transcriptomic data and previously-published genomes of *Eublepharis* and *Gekko*, there were two genes (GNAT1 and GUCY2F) that were not expressed in the eye but still had functional copies in the genomes. Interestingly, in parallel, the loss of GNAT1 expression in the eye was confirmed with immunohistochemistry of retinal slices in *Gekko gekko* (Schott et al., 2019). We also failed to recover GUCY2F in *C. calyptatus*. However, functional copies of both genes in the *Gekko* and *Eublepharis* genomes suggest an additional function for these genes outside of the eye. For instance, the non-visual opsin, OPNP (pineal opsin or *Opn1lw*), is intact in gecko genomes (Emerling, 2017b) and may be able to activate GNAT1 (Max et al., 1998). Thus, although loss of ocular GNAT1 expression appears to be gecko-specific, GUCY2F is not expressed in the eye of snakes (Schott et al., 2018) or acrodonts (*C. calyptatus*), indicating this loss of ocular expression may be squamate-wide.

Within geckos, we observed no differences in the occurrence of

phototransduction transcripts amongst the samples, whether or not the geckos were nocturnal or diurnal (Fig. 3). The overall pattern of expression loss of phototransduction genes appear most closely analogous to convergent cone-cell inactivation observed in baleen whales and burrowing mammals (Emerling and Springer, 2015; Springer et al., 2016). Indeed, within the rod-cell signaling pathway, geckos have lost expression of the rod photopigment (RH1), 2/3 subunits of the rod transducin (GNAT1 & NGT1), 3/3 subunits of the rod-cell associated phosphodiesterase (PDE6A, PDE6B, & PDE6G), and 1/2 subunit of the heterodimer ligand-gated channel (CNGA1) (Fig. 1a). These results are consistent with the gekkotan ancestor undergoing a shift to nocturnality, resulting in the transmutation of cones into a rod-like morphology and complete loss of the ancestral rod cell type (Fig. 2).

This is the only known example of a near complete loss of rod phototransduction genes (downstream of RH1) in a vertebrate. Paradoxically, it seems almost ludicrous that rod cells would be lost during a nocturnal bottleneck as the light sensitivity of rods makes them better suited to low light vision than cones. Geckos seem to have overcome this apparent paradox with cones that are rod-like in both their morphology and physiology (Walls, 1934; Goldsmith, 1990; Zhang et al., 2006; Kolesnikov et al., 2007). Thus, we can posit that the selective forces to retain color vision, even in dark environments, must have been exceedingly strong – possibly coupled with a need for the faster signal transmission that cone cells provide (Li et al., 2010). This change in diel activity, however severe, did not diminish the need for a keen visual system in ancestral geckos. One likely selective force is foraging behavior, as geckos are reliant on visual cues for foraging and locomotion (Bauer, 2007; Vitt and Pianka, 2005; Birn-Jeffery and Higham, 2016). Intraspecific visual communication is another potential reason that geckos may see so well in low light environments and some species utilize visual communication and/or are sexually dichromatic (Marcellini, 1977; Regalado, 2012). However, this seems less-likely as many gecko species are more reliant upon vocal and olfactory communication than for visual communication (Gans and Maderson, 1973; Marcellini, 1977; Brillet, 1993; Zozaya et al., 2019). Further investigations into the signaling pathways and electrophysiology utilized in the rod-like cone cells in the gecko retina could provide insight into the many adaptations required for transmutation (assuming a rod-like cone cell could provide a selective advantage) and possible phylomedical implications of retina-related disease states (Emerling et al., 2017).

These data provide the first molecular evidence that extant geckos share a visual system principally shaped by adaptation to a low-light environment, and nocturnality, in their shared ancestral lineage. Gecko eyes are broadly characterized by the loss of cone-opsin SWS2 and rod-opsin RH1, as well as the majority of rod-specific phototransduction genes (Fig. 1a). Prior characterizations of the gekkonid visual system using *Gekko gekko* (nocturnal) and *Phelsuma* (diurnal) are, thus, likely representative of gekkotan eyes overall, at least at the molecular level (Crescitelli et al., 1977; Kojima et al., 1992; Loew, 1994; Taniguchi et al., 1999, 2001; Yokoyama and Blow, 2001; Zhang et al., 2006; Roth et al., 2009; Liu et al., 2015). While most rod-specific phototransduction genes that we searched for were no longer expressed in the eye, we did find a few functional rod-specific genes. These may be genes that retain some function elsewhere in the retina or may have been co-opted in the transmutation of cone cells into their rod-like morphology. For example, they may be used in the signal transduction of non-visual opsins, opsin-3 (OPN3) and opsin-4 (OPN4), which we found in all gecko and chameleon eyes, and are known to be expressed in the eyes of other vertebrate species (Halford et al., 2001; Peirson et al., 2004). Further research that localizes expression of these remaining rod-specific genes in the gekkotan retina, possibly with regard to the expression of non-visual opsins, would help resolve this.

Additional lines of evidence also support a nocturnal ancestor in geckos. These traits are widespread in geckos and include numerous adaptations to a low-light lifestyle, such as: sustained locomotion at

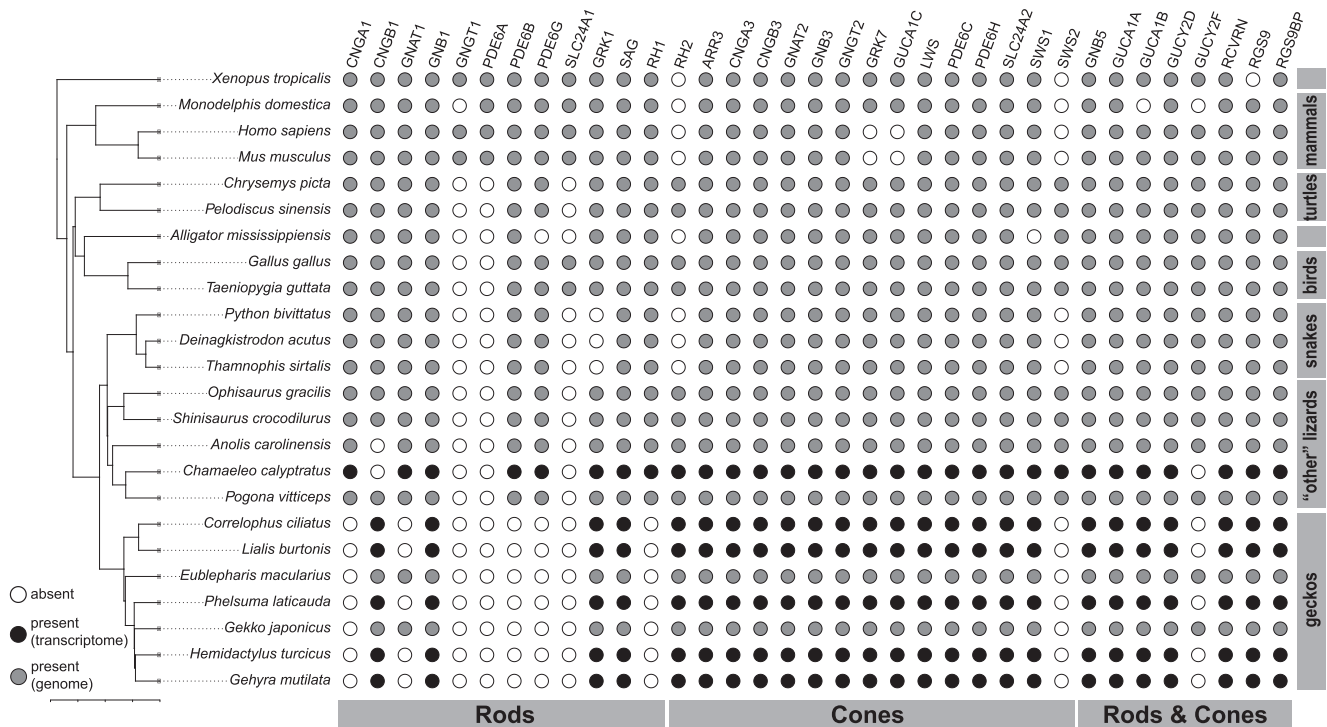


Fig. 3. Presence/absence of 35 ancestral phototransduction genes for sampled tetrapod species. Filled gray circles indicate presence in the sampled genome, filled black circles indicate presence in the sampled transcriptome, white circles indicate absence. Expression in rods, cones, or both cell types is indicated along the bottom of the matrix. Phylogeny is a composite from Gamble et al. (2015) – geckos – and Irisarri et al. (2017) – other vertebrates.

lower temperatures than other squamate species (Autumn et al., 1999); olfactory specialization (Schwenk, 1993); widespread acoustic communication (Gans and Maderson, 1973; Marcellini, 1977); identification of pseudogene remnants of phototransduction-related genes (Schott et al., 2019); and additional eye modifications such as increased size, pupils capable of extreme constriction and dilation, and retinas lacking foveae (Röll, 2001; Schmitz and Higham, 2018). Finally, comparative phylogenetic analyses of diel activity patterns of extant geckos also indicate the most-recent common ancestor of extant geckos was nocturnal (Gamble et al., 2015). Thus, when combined with the molecular data presented here, these multiple lines of evidence overwhelmingly support a “nocturnal bottleneck” of the shared ancestral lineage of extant gecko lizards, which not only led to a dramatic restructuring of the eye to adapt to low-light vision, but also to changes in nearly all aspects of gekkotan morphology, physiology, and behavior. While the ancestor to extant geckos was ancestrally nocturnal, these data do not exclude the possibility that geckos are secondarily nocturnal (i.e. the most-recent common ancestor to squamates, prior to the divergence of geckos, was diurnal; Anderson and Wiens, 2017). Indeed, this seems likely given the unbiased representation of phototransduction genes expressed in chameleons and most other non-gekkotan and non-serpentine squamates. Nevertheless, it is clear that many gecko lineages have subsequently adapted to a diurnal lifestyle and future work should focus on elucidating the molecular evolution of visual opsins and other phototransduction genes as they adapt from ancestral nocturnality to diurnality across the breadth of convergent shifts in diel activity patterns.

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Data Availability

SRA project for data generated in this study is available in Supplemental Table 1. Assembled transcriptomes and alignments are available via Figshare (doi:10.6084/m9.figshare.7365005).

Author Contributions

TG conceived the project. BJP and TG developed project aims, assessed gene orthology, and wrote the manuscript. BJP assembled and annotated transcriptomes. SVN extracted RNA, made and sequenced eye RNAseq libraries. All authors conducted fieldwork, read, and approved the final manuscript. This work was supported by Marquette University startup funds [to TG].

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2019.106639>.

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