



EVIDENCE FOR REPEATED LOSS OF SELECTIVE CONSTRAINT IN RHODOPSIN OF AMBLYOPSID CAVEFISHES (TELEOSTEI: AMBLYOPSIDAE)

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Received June 2, 2012

Accepted September 18, 2012

Data Archived: Dryad doi:10.5061/dryad.bv7tv

The genetic mechanisms underlying regressive evolution—the degeneration or loss of a derived trait—are largely unknown, particularly for complex structures such as eyes in cave organisms. In several eyeless animals, the visual photoreceptor *rhodopsin* appears to have retained functional amino acid sequences. Hypotheses to explain apparent maintenance of function include weak selection for retention of light-sensing abilities and its pleiotropic roles in circadian rhythms and thermotaxis. In contrast, we show that there has been repeated loss of functional constraint of *rhodopsin* in amblyopsid cavefishes, as at least three cave lineages have independently accumulated unique loss-of-function mutations over the last 10.3 Mya. Although several cave lineages still possess functional *rhodopsin*, they exhibit increased rates of nonsynonymous mutations that have greater effect on the structure and function of *rhodopsin* compared to those in surface lineages. These results indicate that functionality of *rhodopsin* has been repeatedly lost in amblyopsid cavefishes. The presence of a functional copy of *rhodopsin* in some cave lineages is likely explained by stochastic accumulation of mutations following recent subterranean colonization.

KEY WORDS: Loss of function, molecular clock, regressive evolution, species tree, subterranean.

Similar phenotypes often evolve repeatedly when independent lineages are exposed to similar ecological conditions, but the likelihoods of convergent, parallel, and reverse evolution are thought to differ among traits, depending on their complexity and genetic basis (Schluter 2004; Futuyma 2009; Losos 2011; Wake et al. 2011). The repeated degeneration or loss of derived traits, regressive evolution, has long been of particular interest to evolutionary biologists (Darwin 1859; Protas et al. 2007; Jeffery 2009; Futuyma 2010), because it highlights the importance of stabilizing selection for maintenance of adaptive traits (Porter and Crandall 2003; Dorken et al. 2004; Wiens 2001), illustrates the role of genetic drift in morphological evolution (Haldane 1933; Poulson

and White 1969; Wilkens 1988), and raises questions about the repeatability and reversibility of evolutionary change (Dollo 1893, 1922; Gould 1970; Collin and Miglietta 2008). Despite the broad recognition of regressive evolution, the developmental and genetic mechanisms underlying character loss remain poorly understood. Important questions remain, including how character loss affects the molecular evolution of genes involved in its development and function, and whether degeneration is driven by the accumulation of neutral mutations or selection.

Obligate cave-dwelling animals are well-known examples of regressive evolution. Many independent lineages have either lost their eyes altogether or possess functionally degenerate eyes

and pigmentation in the challenging environmental conditions of subterranean habitats, which is characterized by the absence of light and generally low food resources (Culver and Pipan 2009; Hüpopp 2012). Theory predicts that genes freed of selective constraint should evolve like pseudogenes (Yokoyama et al. 1995; Culver and Wilkens 2000), evolving under mutation and drift and accumulating loss-of-function (LOF) mutations. Studies demonstrating that regressive evolution of eyes in subterranean organisms is associated with loss of gene function at the molecular level are limited to subterranean diving beetles (Leys et al. 2005), Mexican cavefish (Yokoyama et al. 1995), marsupial moles (Springer et al. 1997), naked mole rats (Kim et al. 2011), and cave-roosting bats (Zhao et al. 2009a). Several additional studies found no support for LOF of vision-related genes in a variety of subterranean organisms (Yokoyama and Yokoyama 1990a,b; Crandall and Hillis 1997; Janssen et al. 2000; Smulders et al. 2002; Jeffery 2005, 2009; Li and He 2009; Li et al. 2009b).

The visual photoreceptor gene *rhodopsin*, a G-coupled receptor that is critical for vision in low light conditions (Stenkamp et al. 2002; Palezewski 2006), has been a candidate eye gene for examining regressive evolution. This well-known protein has been the subject of numerous studies from structural, biochemical, and phylogenetic perspectives and has been an important model for ligand-activated G protein-coupled receptors (GPCRs) (Stenkamp et al. 2002; Palezewski 2006; Hofmann et al. 2009; Smith 2010) and for studies examining the evolutionary mechanisms involved in the adaptation of organisms to different photic environments (Archer et al. 1995; Hunt et al. 1996, 2001; Fasic and Robinson 2000; Sugawara et al. 2002, 2005, 2010; Sivasundar and Palumbi 2010). Because *rhodopsin* is located downstream in eye developmental and regulatory cascades (Lamb et al. 2007 and references therein), it may become nonfunctional in aphotic environments, as it is released from purifying selection for visual function. However, previous studies examining selective constraint of *rhodopsin* in subterranean organisms have yet to uncover compelling evidence of loss of functionality at the molecular level (Crandall and Hillis 1997; Janssen et al. 2000; Li and He 2009; Zhao et al. 2009b; Garcia-Machado et al. 2011; Kim et al. 2011). At least 19 genes associated with visual perception were discovered to be lost or showed evidence of LOF constraint in a genome analysis of the naked mole rat (Kim et al. 2011); however, *rhodopsin* was not one of these loci. Apparent maintenance of functionality has been attributed to weak selection for retention of light-sensing abilities (Culver and Pipan 2009; Kim et al. 2011) or potential pleiotropic roles, such as in circadian rhythms (Crandall and Hillis 1997; Janssen et al. 2000; Li and He 2009), as has been implicated for other opsin genes in subterranean organisms (Janssen et al. 2000; Cavallari et al. 2011; Mejia 2011). A recent study has discovered that *rhodopsin* is important in initiating thermosensory-signaling cascades in *Drosophila* (Shen et al. 2011), confirming the poten-

tial for nonvisual function. An alternative hypothesis to explain fewer LOF mutations is that insufficient time has occurred for the accumulation of LOF mutations that render the gene nonfunctional, if selection is not the driving force behind degeneration (Leys et al. 2005). Consequently, the integrity of a gene may persist for long periods of time due to chance alone (Marshall et al. 1994) and statistical tests may not have sufficient power to reject the null hypothesis of no change in selective constraint in genes that are actually no longer under selection (Leebens-Mack and dePamphilis 2002). Inferred maintenance of a visual gene might be the result of recent subterranean colonization rather than invoking hypotheses of retained functionality.

Here we investigate whether *rhodopsin* shows evidence of loss of selective constraint in amblyopsid cavefishes (Teleostei: Amblyopsidae), which includes surface, facultative-cave, and at least five obligate cave-dwelling species in eastern North America (Woods and Inger 1957; Niemiller and Poulson 2010; Niemiller et al. 2012). Morphological, physiological, behavioral, and ecological studies support a gradient of subterranean specialization resulting from varying durations of subterranean inhabitation over a long evolutionary timescale (reviewed in Niemiller and Poulson 2010), making this a potential system to examine molecular mechanisms of adaptation and potential loss of functional constraints in aphotic environments. First, we generate the first fossil-calibrated, multilocus molecular phylogeny with complete taxon sampling of the amblyopsid cavefishes and related percopsiform teleost fishes to provide a temporal and phylogenetic context for elucidating the evolutionary history and potential loss of functionality of *rhodopsin* and to clarify the phylogenetic relationships in this lineage. We then investigate patterns of molecular evolution in *rhodopsin* to determine if evidence exists for loss of selective constraint and functionality in this well-studied retinal protein. Our study provides compelling molecular evidence that functionality of *rhodopsin* has been repeatedly lost in several amblyopsid cavefishes over the last 10 Mya; while other cave lineages still possess a functional copy despite living in an aphotic environment, likely reflecting more recent colonization of subterranean aquatic habitats.

Materials and Methods

TAXON SAMPLING

Specimens and tissue samples (fin clips) were collected from 361 individuals for all eight currently recognized amblyopsid species, including morphologically cryptic lineages of *Typhlichthys* identified by Niemiller et al. (2012), and four surface-dwelling species of *Percopsis* and *Aphredoderus* that are classified along with Amblyopsidae in Percopsiformes (Nelson 2006). Fin clips were stored in 95% or 100% ethanol or were frozen at -80°C .

Table 1. Loci and selected best-fit molecular evolutionary models for data partitions implemented in phylogenetic analyses.

Locus	Abbreviation	Length (bp)	Ploidy	Model of first codon	Model of second codon	Model of third codon	Model of intron
NADH dehydrogenase 2	<i>nd2</i>	1044	<i>n</i>	TVM+I+G	GTR+I+G	GTR+I+G	NA
Intron 1 of ribosomal protein S7	<i>s7</i>	841	<i>2n</i>	NA	NA	NA	HKY+G
Exon 3 of recombination activating gene 1	<i>rag1</i>	1446	<i>2n</i>	HKY+I	TVM+I	TVM+G	NA
Rhodopsin	<i>rh1</i>	798	<i>2n</i>	HKY+G	TIM+I+G	HKY+G	NA
Zic family member 1	<i>zic1</i>	855	<i>2n</i>	F81	F81	TVM	NA
Myosin heavy polypeptide 6	<i>myh6</i>	786	<i>2n</i>	HKY+I	HKY	TVM+I	NA
Hypothetical protein LOC564097	<i>ptr</i>	761	<i>2n</i>	TrN	TrN	TVM+G	NA
T-box brain 1	<i>tbr1</i>	705	<i>2n</i>	HKY	F81	HKY+I	NA
Similar to SH3 and PX domain containing 3 gene	<i>sh3px3</i>	760	<i>2n</i>	GTR	K81uf+I	TIM+I	NA
Pleiomorphic adenoma gene-like 2	<i>plagl2</i>	603	<i>2n</i>	GTR	TVM	TVM	NA

NA = the gene does not contain the specified partition.

Collection information for all sampled lineages are provided in Table S1.

MOLECULAR METHODS

Genomic DNA was extracted using the Qiagen DNEasy Kit (Qiagen, Inc., Valencia, CA). Polymerase chain reaction (PCR) was used to amplify nine genes (one mitochondrial and eight nuclear; Table 1) on a set of percopsiform samples that included two individuals per species. PCRs were conducted using the primers and protocols outlined in previous studies (Kocher et al. 1995; Holcroft 2004; Li et al. 2007). We also amplified a 798 bp section of *rhodopsin* corresponding to amino acids 52 to 317 of bovine *rhodopsin* for 361 individuals (Table S1) using primers rho-1 (5'-GTCCATATGAATACCCTCAGTACTACC-3') and rho-2 (5'-TCTTTCCGCAGCACAACGTGG-3'). *Rhodopsin* is comprised of a single exon in teleost fishes (Fitzgibbon et al. 1995; but see Morrow et al. 2011). Clean PCR products were sequenced at the Molecular Systematics and Conservation Genetics Laboratory, Department of Ecology and Evolutionary, Yale University, New Haven, Connecticut, or the Molecular Biology Resource Facility, Division of Biology, University of Tennessee, Knoxville, Tennessee.

GENETIC ANALYSES

Forward and reverse sequences for each template were aligned and edited using SEQUENCHER v4.5 (Gene Codes, Ann Arbor, MI) with ambiguous base calls verified manually by examining the electropherogram for each sequence. Resulting contigs were aligned using SEQUENCHER and MACCLADE v4.08 (Maddison and Maddison 2005). A few individuals contained heterozygous genotypes for the sampled nuclear loci. Haplotype phase of nuclear sequences was inferred using PHASE v2.1 (Stephens et al. 2001; Stephens and Scheet 2005). Unique DNA sequences gen-

erated for this study were accessioned into GenBank (JX459100–JX459566; Table S1).

SPECIES TREE AND DIVERGENCE TIME ESTIMATION

We estimated the species tree topology and divergence times simultaneously under a Bayesian framework using a nine-gene dataset (Table 1; all genes but *rhodopsin*) on a subset of percopsiform samples (two individuals per species). Relative divergence times and the species tree topology were estimated using an uncorrelated lognormal distribution of branch lengths implemented in the *BEAST module (Heled and Drummond 2010) of BEAST v1.6.1 (Drummond et al. 2006; Drummond and Rambaut 2007). *BEAST infers species trees from multilocus data by jointly estimating multiple gene trees embedded in a shared species tree under the multispecies coalescent. We conducted divergence time analyses using the species tree to calibrate nodes following McCormack et al. (2011). Because no amblyopsid fossils exist, we used two fossil-calibrated age prior distributions from non-amblyopsid fossil taxa in all BEAST analyses. †*Tricophanes foliarum* (Cope 1872) is known from the oligocene-aged deposits and shares common ancestry with *Aphredoderus* (Rosen 1962; Rosen and Patterson 1969). The age of the node subtending *Aphredoderus* and Amblyopsidae was calibrated using the age of this fossil. We chose a lognormal prior distribution (mean: 1.0, SD: 1.0) such that the minimum possible sampled age corresponded to 33.9 Mya. †*Lateopisciculus turrifimosus* (Murray and Wilson 1996) is known from the Paleocene and shares common ancestry with *Percopsis* (Murray and Wilson 1996). We calibrated the most recent common ancestor (MRCA) of *Percopsis* and the clade containing *Aphredoderus* and Amblyopsidae using the age of this fossil, choosing a lognormal prior distribution (mean: 1.0, SD: 1.0) such that the minimum possible sampled age corresponded to 58.7 Mya. We hand-edited the XML file to incorporate fossil

priors on the species tree and used a Yule tree prior. We specified the appropriate model of molecular evolution for each data partition (Table 1) after selecting the best-fit model of nucleotide substitution for each gene (including by codon position for protein-coding loci) using MrModeltest v2.3 (Nylander 2004). The clock models and gene trees were unlinked and the uncorrelated lognormal relaxed molecular clock model was used for each locus. Three independent runs of 100 million generations were conducted and the resulting tree and log files for each run were combined using LogCombiner v1.6.1 (<http://beast.bio.ed.ac.uk/LogCombiner>). Convergence of model parameter values was assessed by the effective sample size (ESS) and by examination of convergence and likelihood stationarity in TRACER v1.5 from combined posterior samples to ensure adequate mixing of the MCMC (ESS > 200). A conservative cutoff of 20% was used for the burn-in. The posterior probability density of the combined tree and log files was summarized as a maximum clade credibility tree using TreeAnnotator v1.6.1 (<http://beast.bio.ed.ac.uk/TreeAnnotator>). The mean and 95% highest posterior density estimates of divergence times and the posterior probabilities of inferred clades were visualized on the maximum clade credibility tree using FigTree v1.3.1 (<http://beast.bio.ed.ac.uk/FigTree>).

RECONSTRUCTION OF EYE FUNCTIONALITY

We examined patterns of eye evolution in amblyopsid cavefishes using ML ancestral character state reconstructions implemented in MESQUITE v2.75 (Maddison and Maddison 2011). The functionality of eyes was scored as a binary character (functional vs. nonfunctional) and reconstructed using the time-calibrated species tree phylogeny. Following the recommendations of Goldberg and Iqic (2008), we first compared the relative fit of a model with different rates for gains and losses of eye function (Mk2; Lewis 2001) to a model where eye function is only lost and never regained. The state of the root of the tree was set as an equal probability of either state. We also set root states to be consistent with model rates, but the results were similar to those with model runs with equal root states (results not shown). Because character-associated changes in diversification rate can result in the mistaken rejection of irreversible models (Goldberg and Iqic 2008), we also tested for character-associated diversification using the binary state speciation and extinction approach (BiSSE; Maddison et al. 2007) implemented in MESQUITE. We compared a model in which estimated rates of losses and gains of eye functionality were allowed to vary to a model in which reversals to functional eyes were not allowed, but accounting for the potential effects of the different states on diversification rates for both models. We also compared a model in which rates of diversification associated with each character state were allowed to differ to a model in which these rates were set to be equal,

to evaluate whether there is any evidence that eye functionality influences diversification. All BiSSE analyses used 20 likelihood optimization iterations. Models were compared using differences in Akaike information criterion (AIC), with $AIC = 2k - 2$ in likelihood, where k is the number of parameters in the model.

ESTIMATION OF THE RHODOPSIN GENE TREE

We estimated the *rhodopsin* gene tree using a codon-partitioned Bayesian analysis of unique *rhodopsin* haplotypes. Bayesian posterior probabilities were estimated in MRBAYES 3.1 (Ronquist and Huelsenbeck 2003) using four independent runs using six Markov chains and temperature profiles at the default settings for 10 million generations, sampling every 1000th generation. The first 2 million generations (20%) were discarded as burn-in to ensure stationarity after examination of log-likelihood values for each Bayesian run in TRACER. Samples from the stationary distribution of posterior trees were used to generate a 50% majority-rule consensus tree.

PHYSICOCHEMICAL PROTEIN PROPERTIES OF RHODOPSIN

We estimated several protein physicochemical properties for each surface and cave *rhodopsin* sequence using the CLC Main Workbench software (CLC Bio) and the ProtParam tool on the ExpASY server (Gasteiger et al. 2003). Because the entire *rhodopsin* coding region was not sequenced, the first 51 and last 37 amino acids were added to each *rhodopsin* sequence based on the *rhodopsin* of *Danio rerio* (GenBank accession no. NM_131084; Uniprot P35359). Sequences that contained nonsense mutations or frameshift deletions that resulted in nonsense mutations were excluded from these analyses because they focus specifically on the properties of functional proteins. We tested whether variances of each protein property were higher in cave versus surface lineages with Levene's tests.

We also employed the MutPred analysis score (Li et al. 2009a) to estimate the pathogenicity of nonsynonymous mutations and LOF mutations, where higher scores correspond to a greater likelihood that a mutation is deleterious. MutPred models the changes in protein structural features and functional sites and outputs probabilities of gain or loss of structure and function to distinguish between disease-associated mutations and putatively neutral polymorphisms from Swiss-Prot (Boeckmann et al. 2003). The MutPred analysis was trained on a set of 65,657 reported mutations on 10,150 proteins, including *rhodopsin*, and performs especially well for well-studied proteins, such as *rhodopsin*, where solved structure is available. We first reconstructed the ancestral states of *rhodopsin* in percopsiforms using the parsimony approach in MESQUITE, which allows for missing data. The *D. rerio rhodopsin* was used to fill in flanking regions for which sequence data were missing. MutPred scores were then calculated

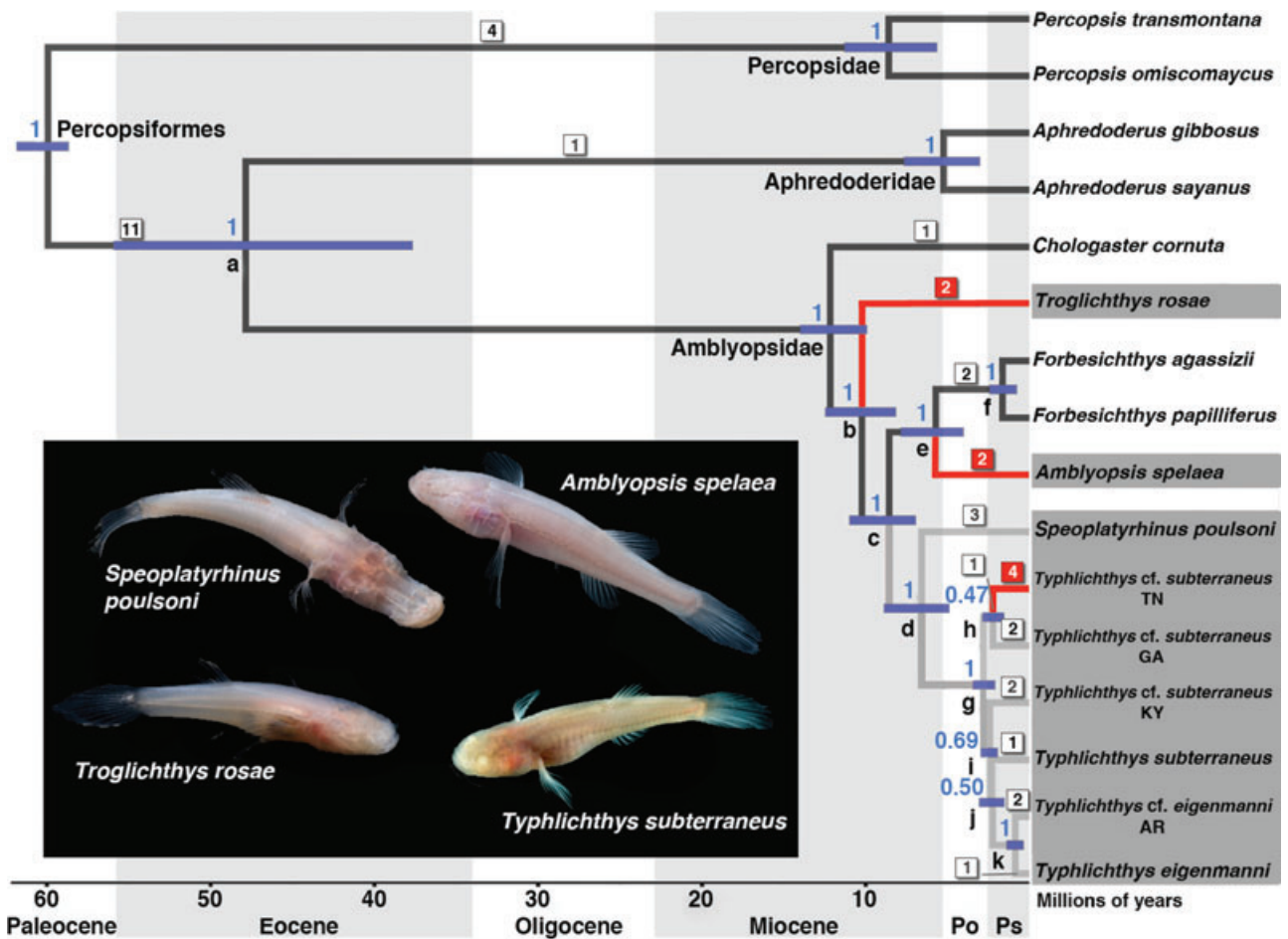


Figure 1. Percopsiform chronogram inferred from a multilocus divergence time analysis. Blue bars at nodes represent 95% highest posterior density intervals of age estimates. Clade posterior probabilities are indicated by blue numbers next to nodes. Cave lineages are indicated by dark gray tip labels. Labeled nodes are the same as those listed in Table 2. Branches in black are reconstructed as surface and gray branches are reconstructed as cave based on a parallel evolution model (M1 model in Table 5). Nonsynonymous substitutions in *rhodopsin* (white or red square) are mapped above branches. Lineages with loss-of-function mutations in *rhodopsin* are indicated by red branches and red squares.

for each mutation away from the reference ancestral sequence. Nonsense mutations and indels were assigned the maximum MutPred pathogenicity score (1.0), because the software only evaluates missense mutations.

The likelihood that a mutation is deleterious was compared between cave and surface lineages using a *t*-test treating each unique mutation as an independent observation. In addition, we used a randomization test to account for phylogenetic nonindependence. Following the logic of Felsenstein (1985), we assumed each branch (internode) in the *rhodopsin* gene tree represents a statistically independent observation of molecular evolution. To compare MutPred scores between cave and surface branches, we recorded the mean MutPred score for each branch and calculated the mean of those values, weighted by the number of nonsynonymous mutations along the branch. The difference in weighted means was the test statistic. Cave and surface branches

were defined based on reconstructions using the irreversible Mk2 model. To generate a null distribution, we randomized branch means across branches with nonzero weight (i.e., branches with no mutations were not included), leaving the tree structure constant (weights and cave vs. surface category). This way, any lineage-specific idiosyncracies are preserved in the null distribution and cannot drive spurious inference. We recorded the fraction of 10,000 randomizations giving a greater than observed weighted mean difference between cave and surface lineages as a one-tailed test.

TESTS FOR SELECTION

To determine if *rhodopsin* is free of functional constraints in subterranean lineages, we obtained maximum-likelihood estimates of the rate of nonsynonymous substitutions (d_N) and the rate of synonymous substitutions (d_S) comparing alternative branch

Table 2. Divergence times (Mya) and proportional likelihoods for eye character states of two-parameter ancestral character state reconstruction models (re-evolution vs. independent evolution) for nodes annotated in Figure 2. Character states: 0, functional eyes; 1, degenerate eyes.

Node	Time	95% CI	Re-evolution (Mk2)		Independent evolution (Mk2—no reversal)	
			0	1	0	1
Percopsiformes	60.2	58.9–62.1	0.731	0.269	1.0	0.0
a	48.1	37.8–56.1	0.696	0.304	1.0	0.0
Percopsidae	8.6	5.7–11.3	0.973	0.027	1.0	0.0
Aphredoderidae	5.3	3.0–7.7	0.988	0.012	1.0	0.0
Amblyopsidae	12.2	10.0–14.0	0.197	0.803	1.0	0.0
b	10.3	8.2–12.5	0.038	0.962	1.0	0.0
c	8.6	7.0–11.0	0.019	0.981	1.0	0.0
d	6.6	4.9–8.9	0.003	0.997	0.149	0.851
e	5.7	4.0–7.9	0.071	0.929	1.0	0.0
f	1.6	0.8–2.4	0.980	0.020	1.0	0.0
g	2.8	2.1–3.5	0.0	1.0	0.0	1.0
h	2.2	1.6–2.9	0.0	1.0	0.0	1.0
i	2.4	1.9–3.0	0.0	1.0	0.0	1.0
j	2.2	1.5–3.1	0.0	1.0	0.0	1.0
k	0.9	0.4–1.4	0.0	1.0	0.0	1.0

selection models implemented in the CODEML module of PAML (Yang 2007) on the *rhodopsin* gene tree. The ratio of d_N/d_S (ω) is <1 under purifying selection, approaches 1 under neutral rates of evolution, and is >1 under positive selection. To maximize the amount of information in the *rhodopsin* dataset, we followed Leys et al. (2005) and substituted nucleotide deletions resulting in frameshifts with the ancestral character states for the sites missing. First, we tested a model (M0) where a single ω was estimated for all branches on the *rhodopsin* tree. This model was compared to a model (M1) with two ratios, a background ω for surface lineages and a separate foreground ω for cave lineages. To determine if estimates of ω differed from rates of neutral evolution, we compared the M1 model to a two-ratio model where ω was fixed at 1.0 in cave lineages (M1fixed). We also compared the M0 model to a model similar to the M1 model, but where each cave lineage (*Amblyopsis spelaea*, *Troglichthys rosae*, and the clade containing *Typhlichthys* and *Speoplatyrhinus*) was allowed to have a separate ω (M1a). We also analyzed a saturated model (M2) where each branch had its own ω . AIC was used to assess significant model improvement.

Results

SPECIES PHYLOGENY

The phylogenetic relationships estimated from the nine-gene dataset are presented in Figure 1 and represents the first molecular phylogeny to include all known species of percopsiforms. We

also conducted analyses using mtDNA alone, nuclear data alone (concatenated and species tree), and mtDNA+nuclear (concatenated), and the topology and support for major nodes in early identical to those from the current species tree analyses (results not shown; Niemiller 2011). Differences are limited to the relationships among *Typhlichthys* lineages, which is also reflected in our species tree analysis with posterior probabilities <0.70 (Fig. 1). As with previous studies, monophyly of Amblyopsidae is strongly supported (Niemiller and Fitzpatrick 2008; Dillman et al. 2011). We also found strong support (posterior probabilities of 1.0) for all the deepest nodes in the phylogeny, as well as monophyly of *Typhlichthys* and *Forbesichthys* (Fig. 1). However, the relationships among species differed considerably from previous phylogenetic hypotheses (Woods and Inger 1957; Swofford 1982; Niemiller and Poulson 2010). Most notable is the resolution of the surface dwelling species of *Forbesichthys* being nested within a clade containing all obligate cave-dwelling lineages (Fig. 1). In addition, *T. rosae* and *A. spelaea* were distantly related, despite both species having previously been classified as *Amblyopsis* (Woods and Inger 1957; Nelson 2006). We found strong support for a clade containing *Forbesichthys* and *A. spelaea*, whereas *T. rosae* was the sister lineage of a clade containing all amblyopsids except *Chologaster*, which was resolved as the earliest diverging amblyopsid lineage. This resolution is different from the phylogeny based on a limited mtDNA dataset presented in Dillman et al. (2011), where *T. rosae* was the sister lineage of a clade containing all other amblyopsids, including *Chologaster*. The

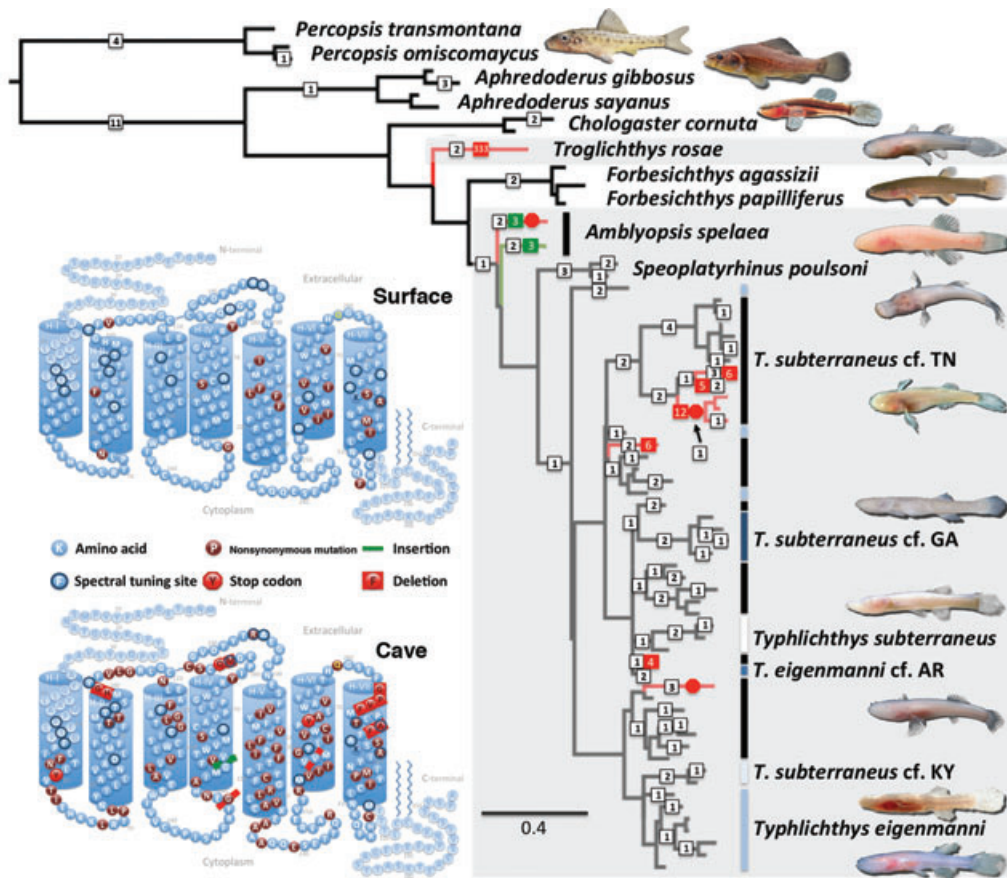


Figure 2. (a) *Rhodopsin* gene tree with nonsynonymous mutations (white square), deletions (red square), insertions (green square), and premature stop codons (red octagon) mapped onto branches. Cave lineages are in gray. The size of indels (in base pairs) is indicated within the red or green square. The inset shows two-dimensional models of *rhodopsin* with cumulative nonsynonymous mutations, deletions, insertions, and premature stop codons indicated for surface and cave lineages. No indels or stop codons were found in surface lineages. The start and end of the 111 amino acid deletion in *Troglitchthys rosae* are indicated by red bars. Amino acid positions demonstrated or suspected to be important in spectral tuning are indicated with a bold outline.

phylogenetic placement of *Speoplatyrhinus* has been uncertain since its description in the 1970s. Dillman et al. (2011) resolved a clade containing *Speoplatyrhinus* and *Forbesichthys*, whereas Proudlove (2006) speculated that *Speoplatyrhinus* was likely phylogenetically distant to other amblyopsids based on the degree of troglomorphy. We resolved a strongly supported clade containing *Speoplatyrhinus* and *Typhlichthys*, as hypothesized by Boschung and Mayden (2004).

DIVERGENCE TIMES

Divergence time estimates (Table 2; Fig. 1) were largely discordant with previous reported ages, which were considerably older (Niemiller and Fitzpatrick 2008; Dillman et al. 2011). Divergence time estimates differ between this and previous studies because of differences in taxa and loci sampled, fossil calibrations, and methodologies employed. In particular, divergence time estimates were based on mtDNA only in both Niemiller and Fitzpatrick (2008) and Dillman et al. (2011). Older dates in previous studies

may be related to issues with saturation. The estimated age of the MRCA of Amblyopsidae was 12.2 Mya (95% highest posterior density [HPD] = 10.0–14.0 Mya) in the Miocene, whereas the age of the MRCA of cave-dwelling amblyopsid species was 10.3 Mya (95% HPD: 8.2–12.5 Mya). The age of the MRCA of the clade containing *Forbesichthys* and *Amblyopsis* was 5.7 Mya (95% HPD: 4.0–7.9 Mya). Diversification within *Typhlichthys* and *Forbesichthys* occurred primarily in the Pleistocene (Fig. 2).

ANCESTRAL RECONSTRUCTIONS OF EYE FUNCTIONALITY

Maximum-likelihood ancestral state reconstruction supports the hypothesis that eye functionality has re-evolved in *Forbesichthys* from a cave-dwelling ancestor (Fig. 3; Tables 2 and 3). An independent evolution (irreversible Mk2) model that estimates a forward rate (functional to degenerate), but constrains the reverse rate (degenerate to functional) to zero was significantly

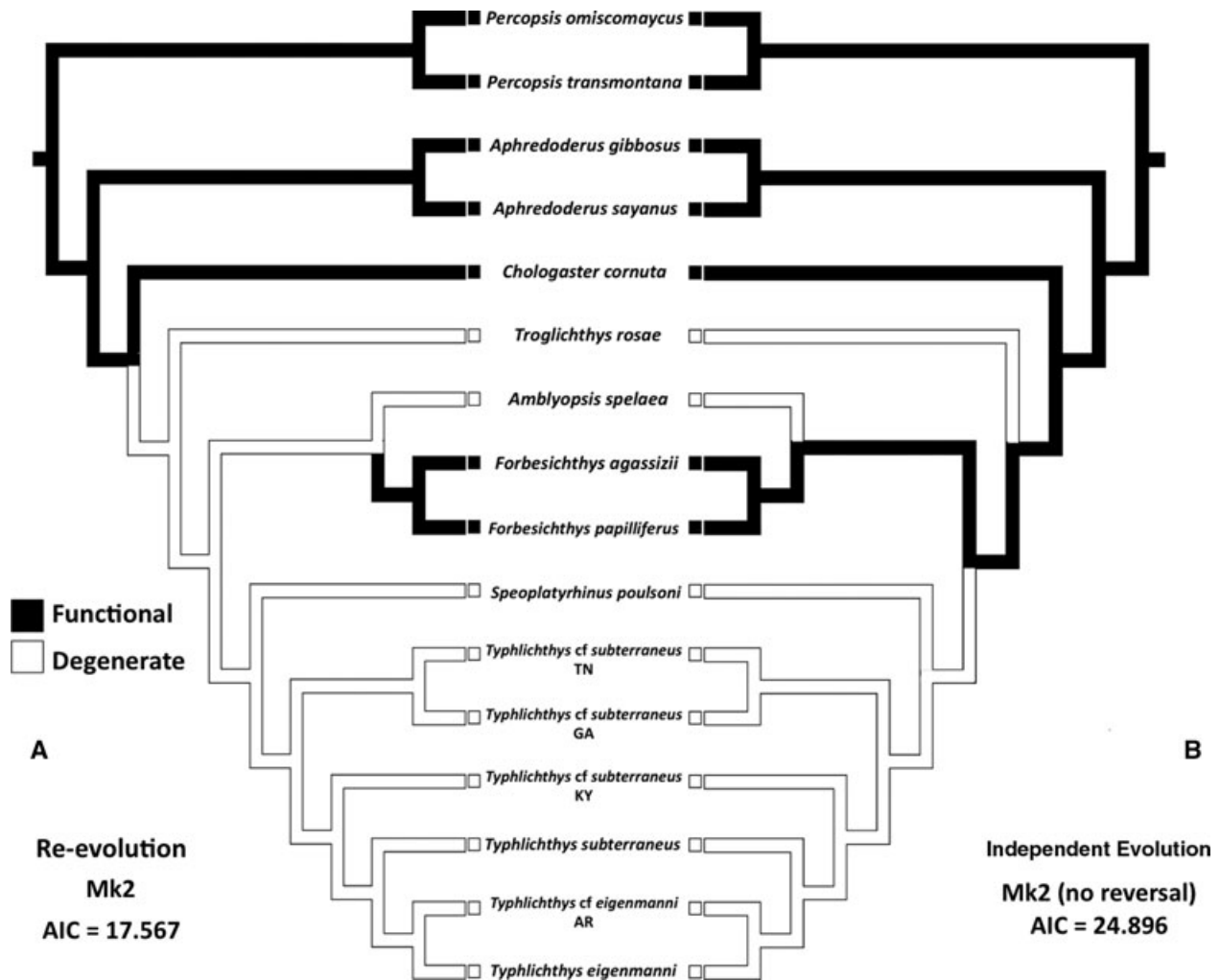


Figure 3. The two contrasting models to explain eye evolution in amblyopsids: a re-evolution model (A) where eyes degenerate once in the ancestor to all subterranean amblyopsids then eye functionality is regained in *Forbesichthys*, and an independent evolution model (B) where eyes independently degenerate in separate subterranean lineages but functionality is retained in *Forbesichthys* throughout its evolutionary history. Likelihood-based character reconstructions of amblyopsid eye evolution under reversible Markov (Mk2) and irreversible Markov (Mk2—no reversal) models using the time-calibrated Bayesian multilocus species phylogeny support the re-evolution model; however, the pattern of degeneration of individual eye structures (Table S3) supports independent degeneration of eyes, as cave lineages differ in individual eye structures that have degenerated or been lost. Character states: black, functional eyes; white, degenerate eyes.

worse than a re-evolution (reversible Mk2) model ($\Delta\text{AIC} = 7.33$; Table 3). The BiSSE model, which accounts for the effects of character states on diversification rate, also supported re-evolution of eye functionality in *Forbesichthys* ($\Delta\text{AIC} = 10.13$; Fig. 3). There was not support for state-dependent diversification, as a constrained model where $a_0 = a_1$ was not significantly better than an unconstrained model ($\Delta\text{AIC} = 2.00$). Likelihood scores for character state reconstruction at the MRCA of Amblyopsidae (Fig. 1) show there is high probability that the ancestor of this clade was subterranean (Table 3). In addition, the MRCA of *Forbesichthys* and *A. spelaea* (node e in Fig. 1) also is reconstructed as having degenerate eyes (Fig. 3).

RHODOPSIN MOLECULAR EVOLUTION

Sixty-six haplotypes were observed, including 12 haplotypes from seven surface lineages and 54 haplotypes from nine cave lineages. The *rhodopsin* gene tree is generally concordant with the multilocus species tree except with regard to the relationships of *Forbesichthys* and *Amblyopsis* (Fig. 2). We found no evidence of relaxation of selective constraint or LOF in surface lineages, whereas evidence of LOF was found in three of the cave lineages (*T. rosae*, *A. spelaea*, and *Typhlichthys* cf. *subterraneus* TN). Seven novel indels (six deletions and one insertion) and three mutations resulting in premature stop codons were discovered, highlighted by an 111 amino acid deletion (31% of the coding region)

Table 3. Results of maximum-likelihood ancestral character state reconstruction analyses of eye evolution comparing models allowing reversals to those in which reversals are not allowed or constrained to have a very low probability using the multilocus species tree derived from Bayesian divergence time estimation (Fig. 3). For two-parameter models (Mk2), parameter estimates include the rate of changes from 0 to 1 (q_{01}) and from 1 to 0 (q_{10}). For the BiSSE model, parameter estimates include q_{01} and q_{10} , as well as speciation/extinction rate with state 0 (a_0), speciation/extinction rate with state 1 (a_1), net diversification rate with state 0 (r_0), and net diversification rate with state 1 (r_1). Character states: 0, functional eyes; 1, degenerate eyes.

Reversals allowed (re-evolution)	Reversals prohibited (independent evolution)
Mk2	Mk2 (no reversal)
$-\ln L = 6.783$	$-\ln L = 11.448$
AIC = 17.567	AIC = 24.896
$q_{01} = 0.013$	$q_{01} = 0.015$
$q_{10} = 0.037$	$q_{10} = 0$
BiSSE (unconstrained)	BiSSE (no reversal)
$-\ln L = 55.973$	$-\ln L = 61.040$
AIC = 123.946	AIC = 134.081
$q_{01} = 0.008$	$q_{01} = 0.031$
$q_{10} = 0.056$	$q_{10} = 1.0 \times 10^{-14}$
$a_0 = 7.1 \times 10^{-6}$	$a_0 = 2.0 \times 10^{-4}$
$a_1 = 9.5 \times 10^{-4}$	$a_1 = 0.482$
$r_0 = 0.024$	$r_0 = 0.058$
$r_1 = 0.149$	$r_1 = 3.3 \times 10^{-4}$

in all *T. rosae* sampled (Figs. 2 and 4A). The insertion in all *A. spelaea* as well as four deletions in *Troglichthys* and *Typhlichthys* were in frame, whereas two deletions in *Typhlichthys* cf. *subterraneus* TN resulted in frameshifts causing premature stop codons. Of the 86 nonsynonymous nucleotide substitutions observed (Figs. 2 and 4A), 63 occurred exclusively in cave lineages, compared to just 10 in surface lineages. Thirteen nonsynonymous substitutions were shared between cave and surface lineages. Nonsynonymous substitutions in cave lineages occurred in every structural domain of *rhodopsin* sequenced, with the greatest concentration in transmembrane domains V–VII (Figs. 2 and 4A). Several *rhodopsin* sequences in cave lineages lacked LOF mutations, including several cryptic lineages in *Typhlichthys* and *Speoplatyrhinus* (Fig. 2). To explore the evolution of relaxation of selection, we mapped LOF mutations and nonsynonymous substitutions onto both the species tree (Fig. 1) and *rhodopsin* gene tree (Fig. 2). No LOF mutations were shared among cave lineages. Likewise, no nonsynonymous substitutions were shared among cave lineages that were not also shared with surface lineages. Specifically, no LOF mutations or nonsynonymous substitutions occurred during the 4.6 Mya period from the MRCA of all cave amblyopsids and

the MRCA of *Forbesichthys* and *Amblyopsis* (internal branches between nodes b and e in Fig. 1).

MUTATIONAL EFFECTS ON STRUCTURE AND FUNCTION

Although several *rhodopsin* sequences in cave lineages are presumably functional, we predicted that the *rhodopsin* of cave lineages would exhibit greater variation in physicochemical properties compared to surface lineages. For several physicochemical protein properties, the *rhodopsin* of cave lineages did show greater variation compared to surface lineages (Fig. 4B; Table 4). Ten of the 63 nonsynonymous substitutions in cave lineages affect amino acids that are conserved or group-conserved in Class A GPCRs or are conserved in >90% of genes in the visual receptor subfamily (Smith 2010). As an additional method of estimating the effects of mutations on *rhodopsin* structure and function, we estimated the probability that a given mutation is deleterious based on sets of features reflecting protein structure and function using the MutPred analysis score. Mutations in cave lineages had a greater likelihood of being deleterious compared to surface lineages ($t = 2.35$, $df = 81$, $P = 0.022$; Fig. 4C). Moreover, 49% of mutations in cave lineages had a high probability ($P > 0.7$) of being deleterious compared to just a single mutation in surface lineages (Table S2). The phylogenetic randomization test agreed that mutations in cave lineages had higher average MutPred scores than mutations in surface lineages (one-tailed $P = 0.0276$; 10,000 randomizations).

TESTS OF SELECTIVE CONSTRAINT

To test whether *rhodopsin* in cave lineages evolves at different relative rates compared to surface lineages, we compared a series of ML branch-based models of selection (Table 5). A two- ω ratio model (M1) was favored where cave lineages had a separate ω from surface lineages over a model (M0) where all branches on the tree had the same ω ($\Delta AIC = 36.24$) and a saturated model (M2) where each branch had its own ω ($\Delta AIC = 141.52$). In addition, a model where each major cave lineage (M1a) had its own ω also was favored over model M0 ($\Delta AIC = 29.29$) but this model was not favored over the model M1 where all cave lineages had the same ω ($\Delta AIC = 6.95$). In the M1 model, ω in cave lineages was over five times greater than ω for surface lineages, indicating a relaxation of selection in cave lineages. However, ω in cave lineages have not yet approached the expected equilibrium of 1.0 under neutral evolution (model M1fixed; $\Delta AIC = 28.96$), but ω varies among cave lineages ranging from 0.115 in *T. rosae* to 0.697 in *A. spelaea* based on estimates under the M1a model. We also compared the M1 model to a model that conforms to the re-evolution scenario suggested by ancestral character state reconstructions (M1rev) where a third ω was included for the branch leading to surface *Forbesichthys*. The M1 model was again favored ($\Delta AIC = 7.92$;

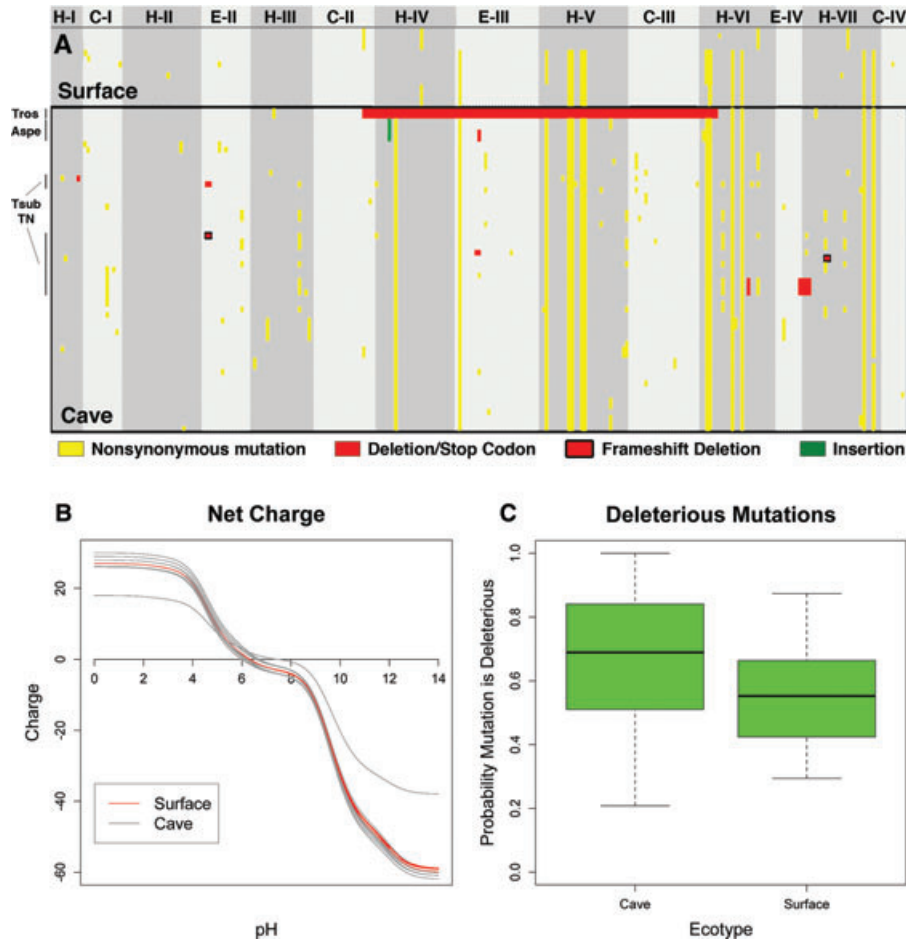


Figure 4. Structural and functional effects of loss-of-function mutations and nonsynonymous substitutions in *rhodopsin*. (A) Sequence alignment of *rhodopsin* haplotypes showing the location and size of loss-of-function and nonsynonymous substitutions with respect to structural domains of the coding region in cave and surface lineages. Mutations cause greater variation in several physicochemical properties of *rhodopsin*, such as net charge (B). The plot in B shows the variation in net charge of *rhodopsin* given the pH between surface haplotypes ($n = 12$) in red and cave haplotypes ($n = 54$) in gray. In C, mutations in cave lineages are more likely to be deleterious compared to mutations in surface lineages.

Table 5) and there was no evidence for loss and then regain of a functional *rhodopsin* protein in *Forbesichthys*.

Discussion

LOSS OF EYE FUNCTIONALITY

Understanding the genetic basis and evolutionary processes that lead to the degeneration of eyes has been a central focus in studies of regressive evolution in subterranean organisms. We found compelling evidence for the loss of functional constraint in the eye photoreceptor gene *rhodopsin* associated with eye degeneration in amblyopsid cavefishes, as three cave lineages possessed novel LOF mutations, while cave lineages overall had increased rates of nonsynonymous mutations indicative of relaxation of selective constraint compared to surface lineages exhibiting a pattern consistent with purifying selection. Despite increased study of the developmental and molecular basis of eye regression, few studies

have found evidence of loss of functionality in genes associated with visual perception. In particular, no studies of subterranean organisms to date have found evidence of LOF or relaxed selective constraint in *rhodopsin*. Therefore, important questions involve the evolutionary mechanism behind eye degeneration in amblyopsid cavefishes, and the underlying reason for the lack of evidence for loss of *rhodopsin* gene function in other studies of subterranean organisms.

The potential mechanisms behind regressive phenotypes in subterranean organisms are highly debated (Culver and Wilkens 2000; Jeffery 2009; Wilkens 2010). Contemporary studies of regressive evolution, and of the loss of visual function in particular, have focused on two main competing hypotheses. The first hypothesis is that degeneration of eyes is caused by the accumulation of selectively neutral mutations and indels in genes responsible for the development, structure, and function of the eye (Kosswig 1940; Wilkens 1988). These mutations are free to accumulate

Table 4. Summary, distributional, and protein physicochemical statistics of missense mutations and indels for surface and cave perciform *rhodopsin* sequences.

Statistic	Surface	Cave	Significance
Sample size	104	257	
No. of haplotypes	12	54	
No. of nonsynonymous mutations ¹	22	76	
No. of premature stop codons	0	3	
No. of deletions	0	6	
No. of insertions	0	1	
ω ratio	0.070	0.361	
MutPred pathogenicity score	0.546 ± 0.179	0.684 ± 0.212	<i>P</i> < 0.05
Distribution of nonsynonymous mutations			
H-I ¹	0	3	
C-I ¹	1	5	
H-II ¹	1	2	
E-II ¹	1	4	
H-III ¹	0	7	
C-II ¹	1	2	
H-IV ¹	1	2	
E-III ¹	1	5	
H-V ¹	5	18	
C-III ¹	0	6	
H-VI ¹	6	9	
E-IV ¹	0	1	
H-VII ¹	4	11	
C-IV ¹	1	1	
Protein physicochemical properties			
Net charge at pH 7.0	-2.213 ± 0.002	-2.390 ± 0.868	<i>P</i> < 0.01
Isoelectric point	6.32 ± 0.00	6.31 ± 0.25	<i>P</i> < 0.01
Hydrophobicity	0.463 ± 0.006	0.467 ± 0.018	<i>P</i> < 0.05
Grand average of hydropathicity (GRAVY)	0.447 ± 0.006	0.450 ± 0.016	<i>P</i> < 0.05
Extinction coefficient (all)	66,742 ± 608.3	66,842 ± 3222	<i>P</i> > 0.05
Extinction coefficient (Cys residues reduced)	65,857 ± 630.2	64,655 ± 9224	<i>P</i> > 0.05
Absorption at 280 nm (all)	1.686 ± 0.012	1.701 ± 0.051	<i>P</i> < 0.05
Absorption at 280 nm (Cys residues reduced)	1.663 ± 0.012	1.677 ± 0.051	<i>P</i> < 0.05
Instability index	44.12 ± 0.80	44.08 ± 0.98	<i>P</i> > 0.05
Aliphatic index	85.56 ± 0.36	84.80 ± 1.23	<i>P</i> > 0.05

Abbreviations for *rhodopsin* domains include H = transmembrane helix; C = cytoplasm; and E = extracellular. Reported values are mean ± 1 standard deviation. Significant differences in mean (MutPred pathogenicity score) or variance are indicated in bold.

¹Mutations shared between surface and cave lineages.

because of relaxation of selective constraints and are ultimately fixed in a population through genetic drift. Over enough time, a character is destined to disappear if not maintained by purifying selection. According to the second hypothesis, eye regression may be driven by direct or indirect selection in aphotic environments if eye degeneration is associated with increased fitness (Barr 1968; Poulson and White 1969; Jeffery 2005, 2009). Natural selection may act directly to reduce or eliminate eyes in cave habitats because having eyes in such environments is deleterious (Barr 1968) or their development and maintenance are energetically costly in energy-limited subterranean habitats (Culver 1982; Culver and

Wilkens 2000). Eye regression might also involve indirect selection whereby degeneration of eyes arises as a correlated response on another trait through pleiotropic developmental trade-offs (Jeffery 2005, 2009).

Several studies have found no evidence for loss of functionality in vision-related genes, including *rhodopsin*. For example, gene expression and direct sequencing of vision-related loci, including those functioning at the base of regulatory cascades, have shown that LOF mutations likely have not occurred in most eye genes in *Astyanax* cavefish (Behrens et al. 1997; Jeffery 2005, 2009; Wilkens 2010). The absence of LOF mutations or

Table 5. AIC scores and ω estimates for various branch-based models testing for heterogeneous selection pressures for *rhodopsin*. The best-fit model is indicated in bold.

Model	Description	AIC	ω estimates
M0	One ω for all branches	5793.53	0.171
M1	Two-ratio model with background (surface) ω and single ω for cave branches [independent evolution model]	5757.29	Surface: 0.070, cave: 0.361
M1fixed	Two-ratio model with background (surface) ω and single ω for cave branches fixed at neutral evolution ($\omega = 1$)	5786.25	Surface: 0.071, cave: 1.0
M1a	Four-ratio model with background (surface) ω and a single ω for each cave lineage (<i>Amblyopsis spelaea</i> , <i>A. rosae</i> , and <i>Typhlichthys</i> + <i>Speoplatyrhinus</i>) [independent evolution model]	5764.24	Surface: 0.077, <i>A. spelaea</i> : 0.697, <i>A. rosae</i> : 0.115, <i>Typh</i> + <i>Speo</i> : 0.360
M1rev	Three-ratio model with background (surface) ω , an ω for cave lineages, and a third ω for branch leading to <i>Forbesichthys</i> [re-evolution model]	5765.22	Surface: 0.068, cave: 0.325, <i>Forbes</i> : 0.162
M2	ω for each branch	5898.81	Surface: 0.001–0.309, cave: 0.001–0.935

signatures of relaxed selection has led some authors to speculate that these genes have retained functionality, perhaps associated with nonvisual traits (Crandall and Hillis 1997; Janssen et al. 2000; Smulders et al. 2002; Li and He 2009). However, another plausible explanation is that most vision-related loci near the ends of developmental and regulatory cascades are freed of selective constraint but insufficient time has occurred for the accumulation of LOF mutations or to detect a strong signature of relaxed selection (Leebens-Mack and dePamphilis 2002; Leys et al. 2005). Consequently, the molecular integrity of a gene may remain intact for considerable periods of time due to chance alone before LOF mutations develop and become fixed in a population (Marshall et al. 1994). Our interpretation in amblyopsid cavefishes is that loss of *rhodopsin* functionality is driven by the accumulation of neutral mutations. The ω of cave lineages was over five times greater than ω for surface lineages in the best model (M1) of selective constraint (Table 5). Some lineages, such as *Troglichthys* and *Amblyopsis*, have accumulated LOF mutations whereas many other lineages, such as *Speoplatyrhinus* and most *Typhlichthys* lineages, have not. Insufficient time for the accumulation of neutral mutations may also explain functionality of *rhodopsin* in other cave organisms. For example, cave *Astyanax* populations possess a functional *rhodopsin* but have only colonized caves from surface habitats sometime in the last 2.2 million years (Porter et al. 2007). The accurate estimation of timing of subterranean colonization is difficult for many groups, however, as related lineages have often gone extinct.

Under the neutral mutation hypothesis, traits that are biologically functionless may not only degenerate in size, but also are predicted to exhibit increased genetic and phenotypic variability,

particularly just after the ecological shift into subterranean habitats before mutations become fixed in a population (Koswig 1940; Wilkens 1988, 2010). Although studies of intra- and interpopulation variation in eye morphology are lacking for amblyopsids, genetic variation in *rhodopsin* is increased in cave lineages (Figs. 2 and 4A), and that translates into increased variability in the structure and function of the protein (Fig. 4B; Table 4), especially within *Typhlichthys*. Rather than signifying maintenance of pleiotropic functionality, these lineages have more recently colonized subterranean habitats and there has been insufficient time for not only LOF mutations to accumulate, but also to become fixed in cave lineages.

It has been hypothesized that presumed functionality of *rhodopsin* in subterranean organisms is due to potential involvement in the mediation of circadian rhythms (Crandall and Hillis 1997; Janssen et al. 2000; Li and He 2009). Other opsin genes, such as *exo-rhodopsin* and *melanopsin*, have been implicated in the regulation of circadian rhythms in vertebrates (Ruby et al. 2002; Moutsaki et al. 2003; Bertolucci and Foa 2004; Shin et al. 2012), including subterranean organisms (Janssen et al. 2000; Cavallari et al. 2011; Mejia 2011). In a recent study, Shin et al. (2012) suggest *rhodopsin* might also mediate effects of the environmental photocycle on circadian rhythms. We cannot rule out the possibility that *rhodopsin* might be involved in the mediation of circadian rhythms in some amblyopsid cavefishes. However, our results suggest that this potential function also has been lost in cave lineages and some behavioral evidence also supports this hypothesis, as cave amblyopsids studied as well as *Forbesichthys* cannot be entrained to light–dark cycles (Poulson and Jegla 1969; Poulson and White 1969). Therefore, *rhodopsin*

functionality may be retained in cave lineages until circadian rhythms degrade, even after functional constraint for vision is lost.

INDEPENDENT EVOLUTION OF THE CAVE PHENOTYPE OR RE-EVOLUTION OF EYE FUNCTIONALITY?

Our ancestral reconstructions of eye functionality in amblyopsid cavefishes raise the intriguing possibility that vision and utilization of surface aquatic habitats have re-evolved from a subterranean ancestor. A fundamental question in evolutionary biology is whether evolution is reversible (Dollo 1893, 1922; Gould 1970; Collin & Miglietta 2008). Dollo's law (Dollo 1893, 1922) posits that a complex character is unlikely to be reacquired once lost. However, the recent literature is replete with putative examples of re-evolution of complex traits, including shell coiling in snails (Collin and Cipriani 2003; Pagel 2004), digits in lizards (Kohlsdorf and Wagner 2006; Brandley et al. 2008), developmental stages in amphibians (Chippindale et al. 2004; Mueller et al. 2004; Wiens et al. 2007), mandibular teeth in frogs (Wiens 2011), and oviparity in snakes (Lynch and Wagner 2010). Taken at face value, these examples reject Dollo's law and shift the debate from the possibility of re-evolution to the evolutionary and developmental mechanisms potentially responsible for resurrecting complex character structure and function (Marshall et al. 1994; Porter and Crandall 2003; Collin and Miglietta 2008).

Cave-dwelling organisms are often viewed as "evolutionary dead-ends" unable to recolonize or adapt to surface habitats. However, the re-evolution of a surface phenotype is not a novel proposition. Culver et al. (1995) hypothesized that eyed amphipods (*Gammarus minus*) living in karst windows re-evolved from related cave-adapted populations with reduced eyes. Likewise, re-evolution also has been proposed in cave scorpions (Prendini et al. 2010), cavefishes (Dillman et al. 2011), and cave salamanders (Trajano and Cobolli 2012). Eye function could be regained even if all individuals in a population were functionally blind if functional alleles still occurred at low frequencies at degenerate vision loci. Under neutrality, the random fixation of mutations causing degeneration likely is a slow process and, consequently, the probability that a trait can be reacquired is higher (Marshall et al. 1994; Collin and Miglietta 2008). Such standing genetic variation could provide the molecular basis necessary for re-evolution of eye function in the ecological shift from cave to surface habitats (Colosimo et al. 2005). In addition, the maintenance of genetic components through pleiotropy or other mechanisms could also facilitate the reacquisition of functional eyes (Syme and Oakley 2012).

Although our ancestral reconstructions reject irreversible evolution of eye functionality in amblyopsid cavefishes in favor of a re-evolution scenario (Fig. 3), several lines of evidence support at least three independent colonization events into subterranean

habitats and subsequent losses of eye functionality. First, cave lineages do not share the same set LOF mutations in *rhodopsin* (Figs. 1 and 2), and not even a single nonsynonymous substitution is observed in the 4.6 Mya interval from the MRCA of all cave amblyopsids to the MRCA of *Forbesichthys* and *Amblyopsis* (node b–e in Fig. 1), as would be expected under a single cave colonization and re-evolution of eyes and surface-dwelling scenario. In addition, a two-ratio branch-based ML model corresponding to independent evolution of the cave phenotype (M1 model) was favored over a re-evolution model (M1rev model) that included a third ω for the branch in the *rhodopsin* gene tree leading to surface-dwelling *Forbesichthys* (Table 5). Second, eye histological data of the cave-dwelling species are inconsistent with re-evolution. The pattern of degeneration of individual eye structures (Table S3) supports independent degeneration of eyes, as cave lineages differ in individual eye structures that have degenerated or been lost (Eigenmann 1897, 1899a,b, 1909; Niemiller and Poulson 2010); however, the degenerate eyes of several cave lineages have yet to be examined. Third, a single cave colonization and re-evolution scenario requires significant subterranean dispersal to explain the broad geographical distribution of cave amblyopsids. Most subterranean organisms are viewed as having a limited opportunity to disperse (Caccone and Sbordoni 2001; Porter 2007; Culver and Pipan 2009), resulting in high genetic differentiation with little gene flow among populations, even at local scales. Niemiller et al. (2012) found that genetic variation in *Typhlichthys* was strongly associated with hydrological boundaries implicating a significant role for geographic isolation and limited dispersal. Therefore, groundwater dispersal for hundreds of kilometers through subterranean corridors across major hydrological boundaries and barriers, such as the Mississippi River and Ohio River, is highly implausible to explain the geographic extent of cave amblyopsids. Finally, phylogenetic studies of most subterranean organisms support the independent derivation of cave populations and lineages, particularly in young subterranean species where surface ancestral populations have not yet gone extinct (Culver et al. 1995; Niemiller et al. 2008; Carlini et al. 2009; Jeffery 2009; Bradic et al. 2012). Thus, an inference of re-evolution might be an artifact of extinction of surface lineages, as posited by the climate-relict model of speciation in temperate subterranean faunas (Holsinger 1988, 2000; Ashmole 1993).

Conclusions

Our study provides compelling evidence for repeated loss of functional constraint of *rhodopsin* in amblyopsid cavefishes, as at least three cave lineages have independently accumulated LOF mutations. Although adaptive hypotheses cannot be ruled out for other aspects of eye degeneration, our results are consistent with the neutral accumulation of mutations responsible for degeneration

of *rhodopsin*. In lineages that still possess a functional *rhodopsin*, we hypothesize that retained functionality is due to recent subterranean colonization and the stochastic nature of mutation accumulation, rather than unknown pleiotropic functions.

Although naïve ancestral reconstructions support re-evolution of eye functionality in amblyopsid cavefishes, several lines of evidence support multiple, independent subterranean colonization events and losses of eye functionality, including eye histological data, lack of shared LOF mutations in *rhodopsin* in cave lineages, and results of previous studies of cave organisms. Ancestral reconstructions can occasionally produce strongly supported yet misleading results. Thorough analysis of molecular evolution of genes associated with a phenotype of interest can provide important insights in studies of trait evolution.

ACKNOWLEDGMENTS

We thank B. Miller, J. Cooley, R. McCandless, W. Pearson, V. Walenta, N. Mann, G. Moni, A. Moni, J. Page, B. Gee, B. Walden, L. Simpson, B. Kuhajda, J. Jensen, D. Fenolio, M. Slay, G. Graening, R. Long, J. Eberly, S. House, J. Buhay, B. Biddix, B. Walter, B. Fluker, B. Glorioso, J. Todd, T. Niemiller, P. Hollingsworth, A. Romero, G. Graening, J. A. Miller, J. H. Miller, and R. G. Reynolds for assistance with field sampling or providing samples. G. Wagner, M. Donaghue, N. Sanders, R. G. Reynolds, and R. Eytan provided comments on previous versions of this manuscript as well two anonymous reviewers and associate editor. We thank the Alabama Department of Conservation and Natural Resources, Arkansas Fish and Game Commission, Georgia Department of Natural Resources, Illinois Department of Natural Resources, Indiana Department of Natural Resources, Kentucky Department of Fish and Wildlife Resources, Missouri Department of Conservation, North Carolina Wildlife Resources Commission, Tennessee Department of Environment and Conservation, Tennessee Wildlife Resources Agency, National Park Service, U.S. Fish and Wildlife Service, U.S. Forest Service, Southeastern Cave Conservancy, Inc., Carroll Cave Conservancy, National Speleological Society, and private landowners for permitting access to caves on their properties and the collection of samples. We thank the Tennessee Cave Survey, the Tennessee Department of Environment and Conservation, and the Missouri Department of Conservation for providing locality data. This work was funded by the Tennessee Wildlife Resources Agency (contract nos. ED-06-02149-00 and ED-08023417-00 to MLN and BMF), Kentucky Department of Fish and Wildlife Resources (contract no. PON2 660 1000003354 to MLN and BMF), National Science Foundation (DEB-1011216 to MLN; DEB-0716155, DEB-1061806, and ANT-0839007 to TJN), American Museum of Natural History (to MLN), Cave Research Foundation (MLN), National Speleological Society (to MLN), and American Society of Ichthyologists and Herpetologists (to MLN). PS acknowledges support from a Burroughs Wellcome Fund Career Award and David & Lucille Packard Foundation Fellowship awarded to J. B. Plotkin. We also thank Dante Fenolio for use of photographs.

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Associate Editor: L. Harmon

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Locality information for *rhodopsin* samples included in the study and associated GenBank accession numbers.

Table S2. MutPred scores and significant structural or functional hypotheses representing the probability that a mutation is deleterious for each unique *rhodopsin* mutation in cave and surface percopsiform lineages.

Table S3. Eye structures in amblyopsid cavefishes. The eyes of *Speoplatyrhinus poulsoni* and newly recognized *Typhlichthys* lineages have not been histologically examined.