# Promotion and constraint of adaptive evolution in cave-dwelling lineages

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**Dissertation Abstract**

Experiencing a novel environment is a common driver of evolutionary change. This change can be promoted by a variety of mechanisms and outcomes, such as novel selection, decreases in genetic variation due to founder effects, drift effects, and genetic admixture. Equally important are forces that impede or prevent change, including lack of suitable mutations, genetic correlations, developmental constraints, or antagonistic selection. A valuable system for studying questions related to evolutionary change following colonization of a novel habitat is the cave habitat, which has induced dramatic adaptive changes in many species that inhabit caves as a result of both adaptive and neutral forces. I studied the evolution of cave-dwelling salamanders, and in particular in the cave salamander, *Eurycea lucifuga*. *Eurycea lucifuga* is an evolutionary puzzle, inhabiting caves while having maintained brilliant coloration more typical of ancestral, surface dwelling ancestors.

I first compared body shape among salamanders within *Eurycea* to examine the prediction from many previous studies that cave-dwellers are elongated relative to non-cave-dwellers. I found that terrestrial species were larger than aquatic species, and also that cave species had shorter tails relative to their body size than non-cave species. These results contrast with trends generally discussed in the cave literature, and reflect a need for explicit testing of how habitat impacts morphology in salamanders. I next investigated the phylogeographic history of *Eurycea lucifuga* and found that divergence among major lineages happened millions of years ago, with more recent expansion within each clade. This is similar to other cave-dwellers that show extreme morphological specialization to the subterranean climate. Our results do not support the idea that a lack

of time underground acts as a constraint on adaptive evolution in this species. Lastly, I compared phenotypic differentiation in color traits with neutral genetic differentiation. The findings indicated that at small scales there is evidence of local differentiation in color relative to the neutral expectation. However, the spatial structuring of differentiation differs between the color phenotype and genotype. Population genetic analyses within a cave system indicated that migration likely occurs by surface corridors, suggesting that coloration may be maintained by a more substantial reliance on non-cave habitats than has been documented for this species.

I concluded from these results that the phenotype of the cave salamander, *Eurycea lucifuga*, is greatly impacted by a relatively minor aspect of its ecology, and hypothesize that its non-cave morphology is selectively maintained by use of surface habitats. In general, this work emphasizes the need to examine trait change in a broad context, considering phylogenetics, ecology, and neutral processes.

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**Introduction**

Understanding the parameters that impact a species' ability to adapt and survive in a novel environment has been crucial to evolutionary biology, conservation biology, and ecology (Bradshaw 1991; Charlesworth & Hughes 2000; Reed *et al.* 2003). Exposure to novel environments can come about primarily two ways: either a change occurs in a population's current range, or there is dispersal and colonization of a new space. Perhaps the most ubiquitous example of these processes is the disruption of environment that accompanied glaciation in the Pleistocene, which not only altered the physiology and behavior of many species, but also dramatically impacted many species' ranges (Avise 2000). There are many features of both populations and habitats that will make colonization more or less likely. Colonization success varies across species with differing biotic traits such as taxon, species, body size, and functional group (Eggleston *et al.* 1999), and is more likely in species that display omnivory, gregariousness, and asexuality (Ehrlich 1989; Lodge 1993). Features such as the type and size of a species' abiotic habitat also impact colonization success (Eggleston *et al.* 1999). It has been suggested that some colonizers gain a benefit from preadaptation, for example an ability to use the widest range or commonest type of habitat (Thomas *et al.* 2001), or from phenotypic plasticity (Baldwin 1896; Ehrlich 1989; Holway & Suarez 1999).

Once a novel environment has been experienced, populations can change in a variety of ways. The most obvious mechanism through which environment causes phenotypic change is that populations are exposed to new selection pressures, with simultaneous relaxation of selection from the previous environment (Mitchell & Power 2003; Torchin *et al.* 2003). A well-known example of adaptive morphological change is

the industrial melanism observed in the Peppered Moth (Cook *et al.* 1986; Kettlewell 1961), where the introduction of a new selection pressure (darkening of the trees due to pollution) caused a change in pigment to be beneficial for certain populations. Colonization can also impact the amount of genetic variation available for selection to work with. Dispersal to a new habitat is often accompanied by genetic bottleneck events due to the relatively small number of individuals in the new population (Chakraborty & Nei 1977; Nei *et al.* 1975). This affects adaptive evolution not only because of the composition of the founder population, but also because of its vulnerability to stochastic changes (Santos *et al.* 2012). Neutral forces such as drift and admixture may also cause phenotypic and genetic changes in founder populations (Clegg *et al.* 2002). Drift was the primary force behind geographic genetic and morphometric variation in the Common Chaffinch, *Fringilla coelebs*, which has led to both intra- and interspecific divergence (Baker *et al.* 1990). Variation in lamella number and body size in introduced populations of the Brown Anole, *Anolis sagrei* was found to be a result of admixture between different source populations and not of natural selection (Kolbe *et al.* 2004; Kolbe *et al.* 2007). Other factors that may affect genetic variation of colonizing populations include the mutation rate and patterns of dominance, epistasis, and pleiotropy of mutations (Reed *et al.* 2003).

Often we think about ways that evolutionary change can be promoted in colonizing populations, but equally important to the evolutionary trajectory can be constraining factors on adaptive change. Futuyma (2010) outlines several limits on response to selection: First, there may be a lack of suitable mutations on which selection may act, or the mutation rate may be slow enough to limit genetic variation. Next, a

character may be genetically correlated with another character under antagonistic selection. There may also be a lack of genetic correlation among traits, which could make selection in a particular direction ineffective if the benefits of a phenotype require change in multiple traits (Futuyma 2010). Gene flow among populations can also act as a constraint when gene swamping overwhelms rare alleles, particularly in small marginal populations, although the effects of gene flow will vary depending on whether the migrating alleles are beneficial, neutral, or deleterious (Antonovics 1976). Limits to adaptation also come from development, since the structure, character composition, or dynamics of the developmental system may not allow for certain changes (Smith *et al.* 1985). For example, a constraint local to Palms involves a lack of secondary thickening in trunk tissue, which prevents branching structures from being successful in that taxon. A more universal constraint involves a skeletal trade-off, where change to favor speed necessarily causes a decrease in potential for applied force (Smith *et al.* 1985). Developmental constraints can generally be identified using measurements of selection and heritability, or comparisons among taxa. A further constraint involves the idea that adaptive evolution requiring multiple changes can only happen if the intermediate steps are beneficial, or at least non-deleterious (Smith *et al.* 1985).

There may be benefits to populations that avoid adapting completely to one environment, although evidence for this has been mixed. The jack-of-all-trades, master of none hypothesis (Ehinger *et al.* 2014; Huey & Hertz 1984) predicts that specialization to a resource will increase a population's fitness in that environment, but will cause a trade-off in the population's fitness in other environments. This hypothesis has been supported by findings that inhabiting spatially or temporally heterogeneous environment

results in more genetic and phenotypic diversity (Buckling & Rainey 2002; Porter & Rice 2013; Reed *et al.* 2003; Travisano *et al.* 1995) (but see (Hawthorne 1997)) and that specialists obtained a greater benefit from their symbionts than did generalists (Ehinger *et al.* 2014). This view of specialists and generalists also supports the niche width-variation hypothesis, which predicts that species with more or broader niches should be more polymorphic (Soulé 1971). There is also evidence that populations inhabiting variable environments are more successful when faced with a novel stressful environment (Reed *et al.* 2003). The limitations imposed by adaptive specialization seem to vary among taxa. In a comparison of generalist and specialist lady beetles in the genus *Harmonia* (Coccinelidae), similar fitness measurements of both species raised on the resource of the specialist indicated that host range in these species is not motivated by intrinsic suitability (Noriyuki & Osawa 2012). Lack of evidence for performance trade-offs on different hosts was also found in other systems (Agosta & Klemens 2009; Futuyma 2008), which suggests that specialization may evolve through multiple mechanisms. The consequences of specialization seem to vary also in different systems: while specialization within some species in the angiosperm genus *Ruellia* (Acanthacaea) (for example, pollination by hawkmoths or bats) have lead to evolutionary 'dead-ends', limiting the amount of further diversification, others (such as hummingbird pollination) have not (Tripp & Manos 2008).

*Study System*

Cave communities have been the focus of a large body of literature investigating interactions between environment and phenotype because of the repeated evolution of a consistent collection of traits known as troglomorphy across many taxa. Troglomorphy

includes such phenotypic change as reduced eyes and blindness, limb and body elongation, depigmentation, reduced metabolism, slower life histories, and paedomorphosis (Porter 2007). Caves represent a relatively simple habitat in terms of environmental consistency, selection pressure, and community structure (Barr & Holsinger 1985; Howarth 1993), and the mechanism behind phenotypic change in cave-dwellers is well characterized (Jeffery 2005; Jeffery 2009; Protas *et al.* 2007; Protas *et al.* 2011; Yamamoto & Jeffery 2000; Yamamoto *et al.* 2004). There is debate surrounding the process of colonization, which tends to center around two alternative hypotheses: exploratory use of caves by some populations, which are then isolated when climatic change causes extinction events in surface dwelling populations (Barr & Holsinger 1985), or sympatric isolation between surface and cave populations caused by adaptive divergence in the cave-dwellers (Howarth 1973). Regardless of the causal order of phenotypic and genetic divergence between surface and cave populations, troglomorphy is generally viewed as a relatively simple one-way path to adaptive specialization resulting in the restriction of taxa to a subterranean habitat.

Cave-restricted individuals, or troglobites, often share the cave habitat with other classes of cave-dwellers with different ecological and phenotypic characteristics. Troglophiles are a class of cave species that establish permanent populations in caves, but are not restricted to that habitat (Sket 2008). These species lack many or most of the traits characterized by troglomorphy, and for many years were thought to represent younger lineages acting as a stepping stone between surface-dweller and cave-relict (Sket 2008). However, genetic studies have shown no clear correlation between age of a cave lineage and its degree of troglomorphy (Wessel *et al.* 2007). Although troglophiles tend

to exhibit more gene flow between populations than troglobites (Porter 2007), the degree to which they are able to traverse non-cave environments varies greatly depending on characteristics of both the landscape and the species (Caccone 1985).

Most of this dissertation focuses on a troglophilic salamander, *Eurycea lucifuga*, which is a common inhabitant of karstic caves across the central and Eastern United States. Cave-dwelling has evolved independently at least five times within *Eurycea* (Bonett *et al.* 2014), and the genus includes the greatest number of troglobites of any vertebrate taxon (Culver *et al.* 2000). While *E. lucifuga* is restricted to caves during the egg and larval stages of its life cycle, adults are occasionally found outside of caves, (Hutchison 1958; Petranka 1998). Despite its ubiquity in the cave habitat and the contrast between its habitat and phenotype, little is known about the evolutionary history or ecology of *Eurycea lucifuga*.

*Purpose*

My goal in this body of work was to examine how the evolutionary history and ecology of *Eurycea lucifuga* have led to its phenotype, given its divergent evolutionary trajectory from that of closely related cave-dwellers. More specifically, I aimed to examine potential reasons why many species, after colonizing the cave environment, gain troglomorphic characteristics and become restricted to caves, and yet *Eurycea lucifuga* and other troglophilic species retained the ancestral phenotype following colonization of caves. I formed two hypotheses, which are not mutually exclusive: that this species represents a very recent colonization of the cave habitat, or that maintenance of the ancestral phenotype is a result of antagonistic selection due to its use of the surface habitat. This dissertation is composed of three chapters and an Appendix.

*Chapter 1*: I performed morphometric comparisons among terrestrial and aquatic, and among cave and non-cave species within a broad sampling of salamander species. These comparisons were performed using standard generalized linear mixed models, as well as phylogenetic ANOVA. I was specifically interested in testing a long-held hypothesis that elongation increases following cave colonization. Results indicate that the majority of variation among habitat groups is found between terrestrial and aquatic species and do not reflect previously reported differences in limb length among cave and non-cave species.

*Chapter 2*: I reconstructed the phylogeographic history of *Eurycea lucifuga* across its range using tree-building methods as well as genetic clustering algorithms. Additionally, I used molecular dating methods to infer lineage ages within the species. Results suggest that there are three major lineages within *E. lucifuga*, each of which show signs of either recent population expansion and/or persistent among-population gene flow. Additionally, dating methods place the split among the major lineages on the scale of millions of years. *Eurycea lucifuga* has inhabited caves for as much time, or in some case much longer, than other taxa that show extreme forms of morphological adaptation to caves.

*Chapter 3*: I compared phenotypic differentiation with neutral genetic variation at a range-wide scale and within a single cave system in order to detect patterns of migration and signatures of selection on color in *E. lucifuga*. These analyses revealed that the spatial structure of differentiation varies among phenotypic traits and neutral genetic loci, suggesting that variation in color is influenced by selection. Higher relatedness between populations connected by above-ground routes rather than between those connected

within the cave system, in addition to the presentation of a river as an effective barrier to dispersal, suggest that a possible source of this selection may come from use of the surface habitat for dispersal.

Though many studies focus on the adaptive response to selection pressure, these are often focused on character change in response to one or a few variables in its primary environment. The most important message from this body of work is that it is crucial to keep a species' evolutionary history and its complete ecology in mind when studying trait evolution. By incorporating phylogenetic relatedness into comparative models studying a broad group of taxa I showed that body shape evolution is more influenced by the transition between terrestrial to aquatic habitats than by colonization of caves, as previously thought. Similarly, I demonstrated that even limited use of an alternative habitat, in this case possibly as a conduit for dispersal, is enough to influence patterns of population structure in *Eurycea lucifuga*. Thus, it was only by uncovering the complicated ecology and evolutionary history of this species and other cave-dwelling salamanders that I was able to explain the disjunction between its habitat and morphology.

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# CHAPTER 1

## THE PHYLOGENETIC HISTORY OF BODY SHAPE VARIATION IN SALAMANDERS OF THE GENUS *EURYCEA* (PLETHODONTIDAE)

**Abstract**

Body shape is directly related to many aspects of a species' evolution and ecology. Body and limb elongation are associated with a general phenotype exhibited by species inhabiting cave environments. However, studies explicitly testing for differences in body shape between cave-dwelling and non-cave-dwelling lineages are rare. Here we examine the variation in body shape among 20 species in the salamander genus *Eurycea* (Plethodontidae) in species inhabiting aquatic or terrestrial, and cave or non-cave habitats. After analyzing morphometric data in a phylogenetic context, we found that there is no evidence of differences in limb elongation between cave and non-cave species. Instead, we find significant differences in general body size between aquatic and terrestrial species, and significant differences in tail length between cave and non-cave species. These results suggest that habitat does impact body shape in this group of salamanders, but perhaps differently than previously thought.

**Background**

Body shape is a key part of morphological variation among vertebrates, with impacts on function and ecology (Carroll 1997; Collar *et al.* 2013). Variation in shape may be a result of environmental effects, structural or functional constraints, adaptive differentiation, or shared phylogenetic history (Blomberg *et al.* 2003; Gould 2002; Losos 2011). There are many examples of body shape divergence that have been attributed completely to adaptation to ecological circumstance (Schluter *et al.* 2004; Shine 1986; Walker & Bell 2000), differences of function (e.g. the use of limbs for running across open ground versus clinging to rocky outcrops) (Cunha *et al.* 2009; Kamiya 2011; Losos 1990; Walker 1997), or a combination of the two (Klingenberg *et al.* 2003; Wikramanayake 1990). Often, patterns of morphological variation are shaped by shared phylogenetic history (Álvarez *et al.* 2013; Stayton 2005), which may influence variation in function or behavior (Bergmann & Irschick 2010). Understanding what causes variation in body shape is important for understanding how it may impact a species' evolutionary trajectory: for example, increased fitness from the evolution of a certain body shape may prevent divergence from that shape, whereas similarity due to shared evolutionary history may not limit future changes in morphology.

Elongation of body or limb is a specific axis of morphological variation that has long been included in a suite of traits associated with cave-dwelling species (Brandon 1971; Christiansen 1961; Mitchell & Reddell 1965; Sket 2008; Weber 2004; White & Culver 2012; Wilkens *et al.* 2000). Cave-dwelling taxa have been of particular interest for many years because of their dramatic morphological and physiological changes, the simplicity of the selection regime within the cave habitat and the resulting parallel

evolution of cave-associated traits (Barr & Holsinger 1985; Culver 1982). These traits, known collectively as troglomorphy, include other features such as regression of eyes, depigmentation, enhanced extra-optic sensory structures, and reduced metabolism. Troglomorphic traits result from both a relaxation of selection pressures formerly experienced in the ancestral surface habitats, and as a result of directional selection experienced within the cave environment (Pipan & Culver 2012). Though most cases of troglomorphic elongation have been studied in invertebrates (Barr & Holsinger 1985; Christiansen 1961), studies of cave vertebrates, and salamanders in particular, also associate elongation with cave-dwelling (Bendik *et al.* 2013; Mitchell & Reddell 1965; Wiens *et al.* 2003).

In this study we compared body shape variation among different habitat groups by comparing morphological measurements among (1) aquatic and terrestrial and (2) cave and surface-dwelling species in the genus *Eurycea* and several outgroups. This work addresses a number of issues with our current knowledge of the evolution of body elongation as it relates to habitat occupancy: First, we analyzed the relationship between habitat and trait evolution using phylogenetically corrected models. Though it is important to consider trait evolution in the context of patterns of relatedness in order to avoid bias (Brooks *et al.* 1995; Felsenstein 1985), to our knowledge troglomorphic elongation has not been assessed using phylogenetically based statistical methods. Additionally, the majority of the previous studies comparing cave-dwelling and non-cave-dwelling salamanders have focused on aquatic cave-restricted species exhibiting all troglomorphic traits. By studying a broader ecological and morphological sampling of the genus, with appropriate outgroups, we were able to compare species in a variety of

habitats, providing a greater insight into the relationship between ecology, phylogeny, and morphology.

**Methods**

*Morphometric data collection*

In December, 2013 and February, 2015 we took photographs of 480 preserved specimens representing 20 species of *Eurycea* and 8 representative species of outgroup genera in the family Plethodontidae in the herpetology collections of the American Museum of Natural History (New York City, New York) and the Smithsonian Institution National Museum of Natural History (Washington, D.C.) (Supplementary Table S1). Photographs included three angles (dorsal, ventral, and lateral views), and included a penny as a size standard. Because sexual size dimorphism is minor relative to individual size variance in salamanders (Petranka 1998) we did not attempt to collect data on sex from these specimens. We measured nine morphometric traits from these photographs using the image processing software ImageJ (NIH). These traits include: head width, front leg length, front leg width, body width at the widest part between the front and back legs, back leg length, back leg width, the length of the fourth back digit, tail length, and snout-vent length. One person (J. Allen) performed all of the digital processing to avoid among-researcher error in measurement. Using information from the IUCN Redlist website (IUCN Global Species Programme Red List Unit), AmphibiaWeb, and Petranka (1998), we assigned each species to a 'habitat' category, including whether it is aquatic or non-aquatic, and cave-dwelling or non-cave-dwelling (Supplementary Table S1). These designations reflect the habitat in which each species is most likely to spend the majority of its adult life stage.

*Phylogenetic reconstruction*

In order to analyze these data in a phylogenetic context we aggregated sequence data from the gene fragments 16s, cytb, POMC, and rag1 for each species from GenBank (Benson *et al.* 2011). Sequence information and accession numbers are available in Supplementary Table 1. We aligned, trimmed, and edited each locus using MEGAv.5.2.2 (Tamura *et al.* 2011). Species with no data available for a locus were represented with a string of missing data characters so as to make all four loci identical in number and order of species. We used jModelTest v2.1.4 (Posada 2008) using the Akaike Information Criterion, Bayesian Information Criterion, and Decision Theory methods to find the most appropriate models of substitution for each locus, then reconstructed the phylogenetic history using each locus as a separate partition with raxmlGUI v1.31 (Silvestro & Michalak 2012). While it was not included in the comparative analyses, we included data from *Proteus anguinus* in the phylogenetic reconstruction as an outgroup in order to root the tree. We ran the analysis with 1000 bootstrap replicates, and visualized the resulting Maximum Likelihood tree using FigTree v1.4.0 (Ronquist *et al.* 2012).

*Uncorrected statistical analyses*

All statistical analyses were performed using R v.3.1.2 (R Core Team, 2014) interfaced through RStudio v.0.98.1091 (RStudio, Inc.). We first collapsed the data set to include trait means for each species using the function summaryBy() in the package *doBy* (Hojsgaard & Halekoh 2015) in order to mitigate any effects of unequal sampling within the species. We performed Principal Components Analysis (PCA) on the nine log-transformed body shape measurements in order to obtain axes of variation using the function prcomp() in the R package *stats* (R Core Team, 2014). We tested these data for

normality and heteroscedasticity using the shapiro.test() function in the R package *stats* and leveneTest() function in the R package *car* (Fox & Weisberg 2011), respectively. We then employed two separate linear models to assess the relationship between habitat and each principal component, including PC1 and PC2 as the dependent variable in each model and Aquatic/Terrestrial, Cave/Non-cave, and the interaction term as the independent variables using the function lm() in the R package *stats*. We performed Type III tests to assess the significance of the linear model using the function Anova() in the package *car*, and visualized plots of these data with ggplot() in the package *ggplot2* (Wickham 2009).

*Phylogenetically-corrected statistical analysis*

Since relatedness among species may impinge on the independence of these data, we also analyzed them in a phylogenetic context. We performed phylogenetic comparative analyses using the Maximum Likelihood tree obtained previously with four separate analyses. In each model we included either PC1 PC2 as each dependent variable. Since phylogenetic ANOVA cannot include multiple independent factors, we ran two models for each dependent variable, one comparing Cave/Non-cave groups, and one comparing Aquatic/Terrestrial groups. These analyses used simulations to obtain a distribution of empirical F statistics with which to test each hypothesis. We chose to run each analysis using the default assumption of evolution by Brownian motion. We used the function aov.phylo() in the R package *geiger (Garland et al. 1993)*, specifying the Wilks test, and performed 1000 simulations.

## Results

### *Morphology*

Principal Component Analysis (PCA) was performed on all 25 species, including 20 species of *Eurycea* and 5 outgroup species. The first two principal components accounted for almost 100% of the cumulative variance (Table 1). PC1, which accounted for 83% of the variance, was representative largely of increased snout-vent length and tail length, and had positive loadings of all other traits, so we consider this to be representative of general size. PC2, which accounted for 16% of the variance, had a strong positive loading of tail length, and a strong negative loading of snout-vent length. Summary plots of each principal component can be seen in Figure 1a,b. Tests of normality indicated that PC2 violates this assumption ($W=0.906$, $p=0.025$), but visual inspection of the data indicated that these violations are mild. Additionally, Levene tests indicated that our data do not violate assumptions of homoscedasticity.

### *Phylogenetic models*

Sequence data were collected from Genbank for all 25 species for Rag1 and cytb, and for the majority of species for POMC and 16s. jModelTest predicted the most appropriate model of evolution for each locus to be GTR+I+G (POMC, Rag1) and GTR+G (cytb, 16s). The Maximum Likelihood tree generated from the four loci recapitulated what most phylogenetic reconstructions of this group have shown, with bootstrap values of over 50 for almost all branches, and most near 100 (Figure 2). We estimate by visual inspection that there are four to six transitions to cave-dwelling within the species we sampled, and possibly three to five transitions from terrestrial to aquatic.

*Comparative statistical analyses*

ANOVA comparing both PC1 and PC2 among the different habitat groups indicated that terrestrial species had significantly higher values of PC1 (general body size) than aquatic species. However, there were no significant differences among the habitat groups in PC2 (tail length relative to body size; Table 2, Figure 3). Analyzing these data using phylogenetic comparative analyses, we found that again PC1 (general body size) was significantly larger in terrestrial species than aquatic species. We also found that when analyzed in the phylogenetic ANOVA cave species had significantly higher values of PC2 (tail length relative to body length) than non-cave species, meaning that they have relatively shorter tails than non-cave species (Table 3).

**Discussion**

We compared body shape measurements among 20 species of *Eurycea* salamanders, together with 8 outgroup species from distinct genera. Our results indicate that the first principal component, which represents body size generally, accounts for the vast majority of the morphological variation that we measured among species. Relative tail length is represented by the second principal component, which accounts for 16% of the variation. One of our aims was to examine the differences between cave and non-cave species; however, morphological variation classically associated with cave-dwelling (relative length of limbs) was only included in a principal component accounting for one percent of the total variance. The primary differences we found in morphology among habitat groups were that terrestrial species were generally larger than aquatic species, and that cave species had shorter tails than non-cave species. Visual inspection suggests that this difference in tail length is comprised largely of differences between terrestrial cave

and non-cave species. While the difference in general size between aquatic and terrestrial species was revealed by both the phylogenetically uncorrected ANOVA and the phylogenetic comparative analyses, only in the phylogenetic ANOVA did we find a difference in tail length between cave and non-cave species. The discordance between the ANOVA and phylogenetic ANOVA results suggests that phylogenetic signal was masking the effect of tail length within this group.

Variation in morphology arises through many different mechanisms including environmental influences, structural or functional constraints, or shared evolutionary history (Blomberg *et al.* 2003; Gould 2002; Losos 2011). Some taxa show distinct differences among populations due primarily to ecological differences (Álvarez *et al.* 2013; Clabaut *et al.* 2007; Kamiya 2011) which may be driven primarily by functional differences in how traits benefit organisms in those habitats (e.g., climbing requires different attributes than swimming or burrowing) (Blankers *et al.* 2012). Other taxa exhibit a combination of ecology-driven and phylogeny-driven variation among lineages (Blankers *et al.* 2012; Jockusch 1997). The similarity between the results of the phylogenetically uncorrected ANOVA and the phylogenetic comparative models we performed suggests that any significant differences in morphology cannot be explained only by shared evolutionary history. A role of ecological difference in variation of tail length particularly may be due to functional differences in tails between aquatic and terrestrial salamanders. While in aquatic species the tail is mainly used for locomotion, in terrestrial species it also has functional significance in fat storage and predator defense strategies (Arnold 1982; Brodie 1977; Maiorana 1977; Vaglia *et al.* 1997). Such

differences in function may necessitate differences in form, such as the differences in length we see among non-cave terrestrial and aquatic species.

A surprising part of our results is the small amount of body shape variation represented by limb length relative to body size, which has been described as one of the traits distinguishing cave-dwelling species (Brandon 1971; Christiansen 1961; Mitchell & Reddell 1965; Sket 2008; Weber 2004; White & Culver 2012; Wilkens *et al.* 2000). Past comparisons that find differences in shape among cave-dwelling and non-cave-dwelling populations (Bendik *et al.* 2013; Mitchell & Reddell 1965; Wiens *et al.* 2003), have focused mainly on the Texas clade of cave dwelling and non-cave dwelling *Eurycea* which are entirely aquatic, and none have included phylogenetically corrected statistical models. Our study, in contrast, suggests that variation between cave and non-cave species exists in the length of the tail, rather than limb elongation.

Historical transitions between habitats, characterized by Bonett *et al.* (2014), are complicated in the *Eurycea*. The ancestral group to the Spelerpines, which includes *Eurycea* and *Gyrinophilus*, was most likely a non-cave terrestrial species. Within the Spelerpines, the change from a terrestrial to an aquatic habitat occurred multiple times followed by at least one reversal back to terrestriality. The transition to cave-dwelling also occurred multiple times, mainly within those groups that transitioned to paedomorphosis, and possibly followed by at least one reversal to non-cave occupancy. Though there was no statistical support for either the transition from terrestriality to aquatic or from non-cave to cave-dwelling being earlier, and the two were likely temporally close, the authors predicted that the transition to cave-dwelling was the more recent change, and that paedomorphosis had allowed populations to make use of limited

cave resources (Bonett *et al.* 2014). An initial transition to the aquatic habitat followed by cave colonization is also thought to have occurred in the non-Spelerpine cave salamander *Proteus anguinus* (Proteidae), as all proteids are paedomorphic, and most *Proteus* are cave-dwellers (Sket & Arntzen 1994).

The complex transition history within these salamanders has interesting implications for the results presented here because of the patterns we see among habitat groups in body shape. Because there are similar body shapes among cave and non-cave species within the aquatic and terrestrial groups, it is likely that convergent evolution of shape traits followed each habitat transition. If, as Bonett *et al.* (2014) hypothesize, the first transition was of terrestrial species into aquatic habitats followed by independent cave colonizations, then aquatic species first became smaller generally than terrestrial species. Subsequently, the colonization of caves by aquatic species led to shortened tails, and cave colonization by terrestrial species resulted in both shortened tails and larger general size. Alternatively, if the transition to the cave habitat was followed by independent transitions to aquatic habitats, then cave-dwelling species initially experienced a reduction in tail length. This would have been followed by a reduction in general size among aquatic species both in and out of caves. Either scenario makes it likely that in each transition there were multiple independent changes in body form, which suggests that body shape in these species is strongly influenced by ecological factors. Similar independent evolution due to ecology has been found in other systems, such as the convergent reductions in bone size in freshwater threespine sticklebacks (Walker & Bell 2000) and repeated elongation within families of reef fish (Claverie & Wainwright 2014).

## Conclusions

This study examined variation in body shape among 25 species of salamander inhabiting two habitat categories: cave vs. non-cave and aquatic vs. terrestrial in order to characterize whether ecological habitat was predictive of form across a broad sampling of taxa. Although limb elongation in vertebrates has previously been considered a trait included in a suite of cave-associated traits, an examination of a broader sampling of *Eurycea* including both aquatic and terrestrial species as well as cave and non-cave species indicates that limb length does not contribute substantially to variation among habitat groups. There were significant differences among habitat groups in both general body size and tail length, wherein terrestrial species were larger than aquatic species, and non-cave species had larger tails than cave species. These results suggest that explicit testing is important for our understanding of the relationships between body shape and habitat in salamander species.

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## Figures

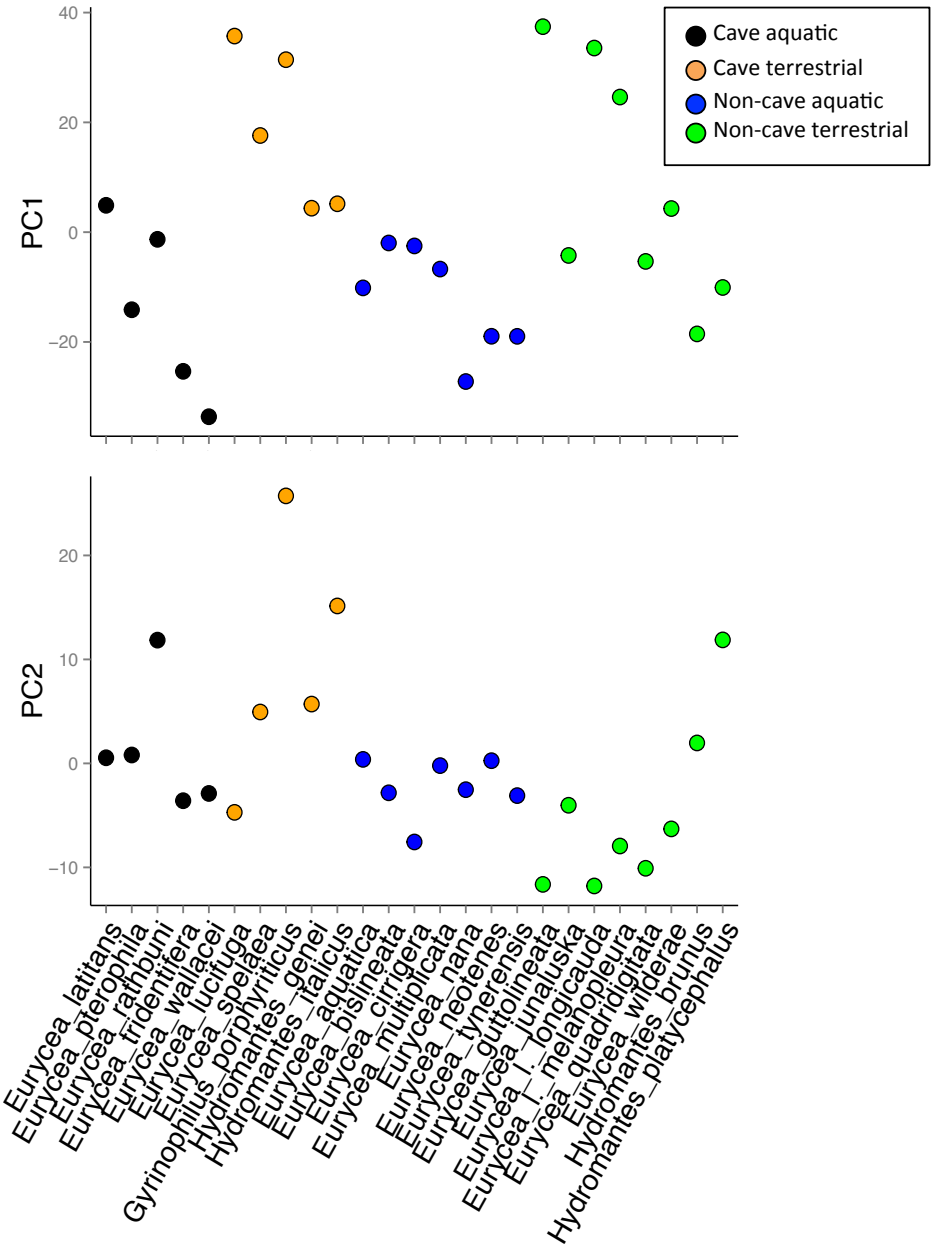


Figure 1. Species means of PC1 (general body size) and PC2 (relative tail length), grouped according to habitat preference. Habitat designation is indicated by color, as seen in the key.

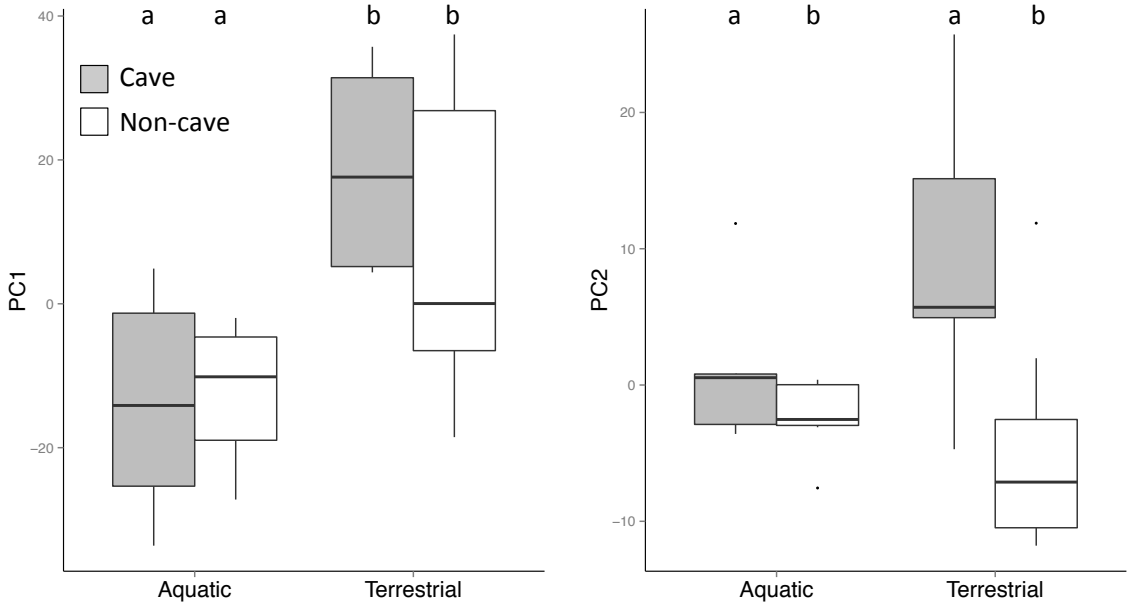


Figure 2. The relationships between PC1 (general body size) and PC2 (relative tail length) and habitat among species means were explored using a linear model and then in a phylogenetically corrected ANOVA. Box and whisker plots indicate the median, first and third quartiles, and whiskers extend to 1.5 for the interquartile range. We found significant differences among aquatic and terrestrial species in PC1 ($p=0.015$), as well as significant differences among cave and non-cave species in PC2 ($p=0.011$).

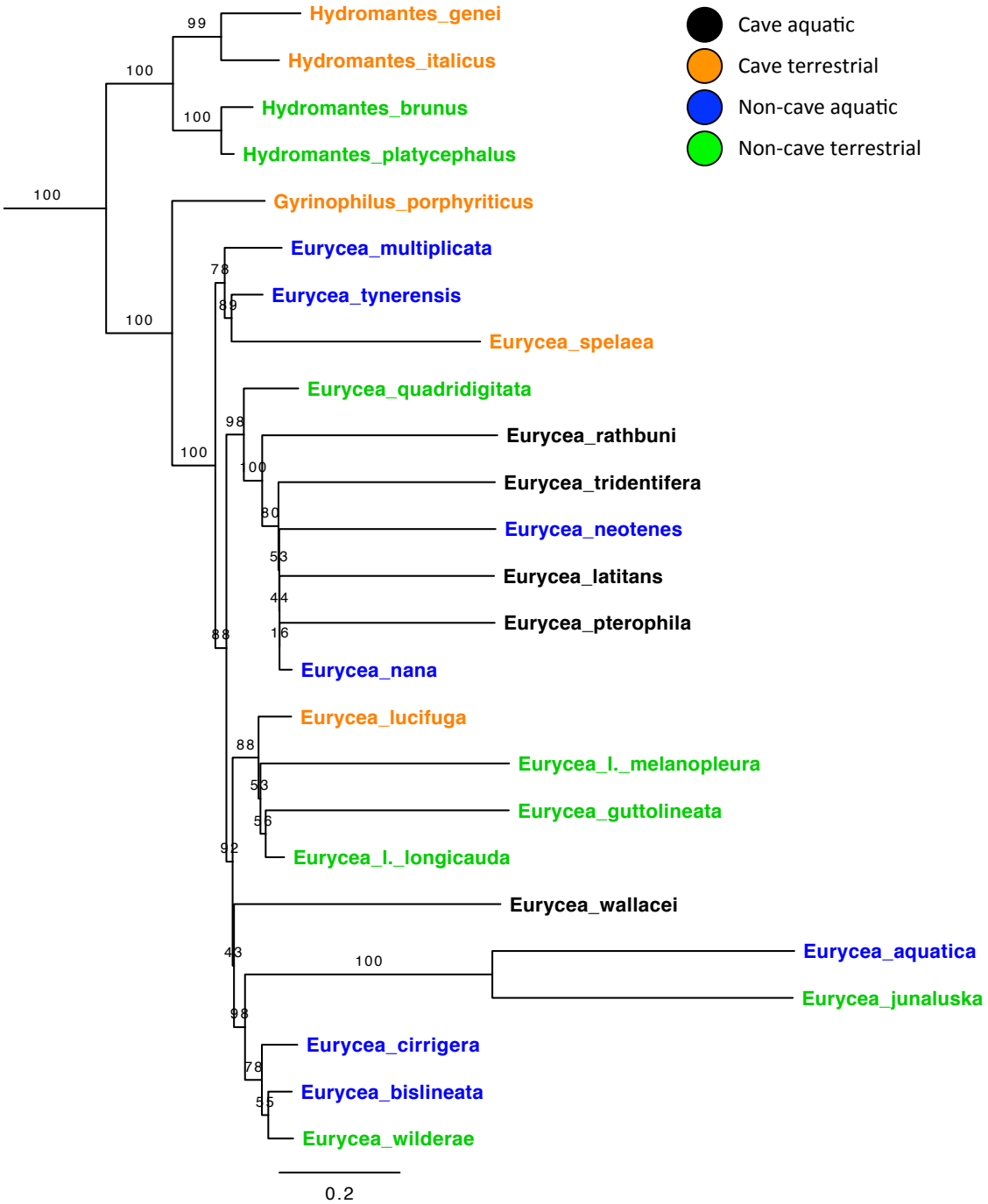


Figure 3. Phylogenetic relationships among these species were compared using a maximum likelihood tree-building approach, with *Proteus anguinus* included as an outgroup (not shown). Branches are labeled with bootstrap scores, and color represents habitat assignment (black=cave aquatic, orange=cave terrestrial, blue=non-cave aquatic, green=non-cave terrestrial).

## Tables

Table 1. Components of variance of the Principal Components Analysis, and loadings of each trait on each PC. PC1 and PC2 were used in further analyses.

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 | PC9 |
|---|---|---|---|---|---|---|---|---|---|
| **Standard** | 20.288 | 8.790 | 2.793 | 0.666 | 0.486 | 0.292 | 0.192 | 0.103 | 0.056 |
| **Proportion** | 0.827 | 0.155 | 0.016 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **Cumulative** | 0.827 | 0.983 | 0.998 | 0.999 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 | PC9 |
|---|---|---|---|---|---|---|---|---|---|
| **Head Width** | 0.079 | 0.179 | -0.200 | 0.704 | -0.443 | -0.305 | 0.238 | -0.272 | -0.076 |
| **Front Limb Length** | 0.115 | 0.152 | -0.669 | -0.109 | -0.393 | 0.460 | -0.329 | 0.170 | 0.026 |
| **Front Limb Width** | 0.026 | 0.032 | 0.001 | 0.094 | 0.140 | 0.376 | 0.193 | -0.424 | 0.782 |
| **Body Width** | 0.088 | 0.137 | 0.008 | 0.603 | 0.517 | 0.063 | -0.483 | 0.312 | 0.084 |
| **Back Limb Length** | 0.141 | 0.158 | -0.635 | -0.214 | 0.511 | -0.430 | 0.222 | -0.101 | 0.021 |
| **Back Limb Width** | 0.033 | 0.030 | -0.025 | 0.088 | 0.296 | 0.489 | 0.050 | -0.541 | -0.607 |
| **Digit Length** | 0.018 | 0.018 | -0.047 | 0.163 | 0.101 | 0.354 | 0.716 | 0.562 | -0.081 |
| **Tail Length** | 0.808 | -0.587 | 0.007 | 0.038 | -0.026 | -0.011 | -0.002 | -0.003 | 0.002 |
| **Snout-Vent Length** | 0.545 | 0.745 | 0.325 | -0.194 | -0.058 | 0.006 | 0.022 | 0.018 | -0.013 |

Table 2. ANOVA results from models including each principal component as the dependent variable, and both aquatic/terrestrial and cave/non-cave comparisons as independent variables, as well as an interaction term. Significance is denoted by bold.

| | SS | DF | F | p |
|---|---|---|---|---|
| **PC1** | | | | |
| **Cave/Non-cave** | 7 | 1,21 | 0.026 | 0.873 |
| **Aquatic/Terrestrial** | 2683.4 | 1,21 | 10.083 | **0.005** |
| **Interaction** | 241.1 | 1,21 | 0.906 | 0.352 |
| **PC2** | | | | |
| **Cave/Non-cave** | 37.17 | 1,21 | 0.655 | 0.428 |
| **Aquatic/Terrestrial** | 160.65 | 1,21 | 2.829 | 0.107 |
| **Interaction** | 166.13 | 1,21 | 2.926 | 0.102 |

Table 3. Results from separate Phylogenetic ANOVAs including each PC as the dependent variable, and habitat as the independent variable.

| | DF | SS | F | p |
|---|---|---|---|---|
| **PC1** | | | | |
| **Aquatic/Terrestrial** | 1,23 | 3900.8 | 15.009 | **0.015** |
| **Cave/Non-cave** | 1,23 | 102.3 | 0.241 | 0.671 |
| **PC2** | | | | |
| **Aquatic/Terrestrial** | 1,23 | 12.58 | 0.157 | 0.850 |
| **Cave/Non-cave** | 1,23 | 477.43 | 7.976 | **0.011** |

**Supplementary Materials**

Supplementary Table 1. Accession numbers of taxa included in phylogenetically corrected comparisons.

| **Species** | **16s** | **cytb** | **POMC** | **Rag1** |
|---|---|---|---|---|
| Eurycea aquatica | | KF562543.1 | | FJ750235 |
| Eurycea bislineata | JQ920581.1 | AY528402 | EU275815 | EU275784 |
| Eurycea cirrigera | JQ920583.1 | KF562548.1 | JQ920728.1 | FJ750245 |
| Eurycea guttolineata | JQ920586.1 | FJ866207.1 | | JQ920770.1 |
| Eurycea junaluska | | KF562550.1 | | FJ750246 |
| Eurycea latitans | | KF562551.1 | | KF562652.1 |
| Eurycea longicauda longicauda | JQ920586.1 | AY528403 | JQ920730.1 | AY650121 |
| Eurycea longicauda melanopleura | FJ866478.1 | KF562552.1 | | KF562653.1 |
| Eurycea lucifuga | JQ920584.1 | EF044248 | JQ920729.1 | FJ917632 |
| Eurycea multiplicata | JQ920580.1 | AY528341 | JQ920725.1 | AY691707 |
| Eurycea nana | JQ920590.1 | AY014846 | JQ920735.1 | EF443113 |
| Eurycea neotenes | | AY528400 | | AY650122 |
| Eurycea pterophila | | AY014851 | | KF562658.1 |
| Eurycea quadridigitata | AY523777 | AY528401 | JQ920759.1 | AY691708 |
| Eurycea rathbuni | | AY014845 | | KF562663.1 |
| Eurycea spelaea | | FJ866336.1 | | KF562667.1 |
| Eurycea tridentifera | | KF562565.1 | | KF562669.1 |
| Eurycea tynerensis | | AY528373 | JF768990.1 | KF562676.1 |
| Eurycea wallacei | | AF252380 | | KF562693.1 |
| Eurycea wilderae | JQ920582.1 | AF252379 | JQ920727.1 | KF562680.1 |
| Gyrinophilus porphyriticus | JQ920577.1 | JQ920616.1 | EU275853 | AY583349 |
| Hydromantes brunus | HM989444.1 | U89614.1 | EU275825.1 | HM797660.1 |
| Hydromantes genei | FJ602156.1 | U89617.1 | EU275840.1 | FJ602343.1 |
| Hydromantes italicus | EF107189.1 | FJ602303.1 | EU275827.1 | EF107312.1 |
| Hydromantes platycephalus | EF107215.1 | U89612 | EU275828 | EU275793 |
| Proteus anguinus | EF107180 | GQ368659 | KC295576.1 | AY650138 |

Supplementary Table 2. Raw measurement data and habitat assignments for each species included in the comparative analyses.

| | | | | Forelimb | | | Hindlimb | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Institution** | **Catalog #** | **Species** | **Head Width** | **Length** | **Width** | **Body Width** | **Length** | **Width** | **Digit Length** | **Tail Length** | **Snout-Vent Length** | **Eye Width** | **Habitat** |
| AMNH | 69032 | Eurycea aquatica | 6.596 | 7.664 | 1.343 | 6.368 | 9.659 | 2 | 0.857 | 42.676 | 36.807 | 2.221 | Non-cave_Aquatic |
| AMNH | 69033 | Eurycea aquatica | 6.525 | 8.656 | 1.302 | 6.521 | 9.682 | 1.802 | 0.46 | 44.696 | 41.854 | 2.086 | Non-cave_Aquatic |
| AMNH | 90687 | Eurycea aquatica | 5.454 | 6.337 | 1.37 | 4.639 | 9.036 | 1.435 | 0.648 | 36.587 | 36.99 | | Non-cave_Aquatic |
| AMNH | 108781 | Eurycea aquatica | 6.659 | 7.704 | 1.21 | 6.024 | 8.715 | 1.837 | 1.205 | 41.699 | 42.159 | 1.683 | Non-cave_Aquatic |
| AMNH | 108782 | Eurycea aquatica | 5.803 | 7.809 | 1.201 | 5.589 | 9.619 | 1.761 | 0.999 | 30.493 | 40.378 | 1.748 | Non-cave_Aquatic |
| AMNH | 108783 | Eurycea aquatica | 6.017 | 8.232 | 1.186 | 6.385 | 9.325 | 1.865 | 0.99 | 36.24 | 40.081 | 1.574 | Non-cave_Aquatic |
| AMNH | 108784 | Eurycea aquatica | 5.556 | 4.624 | 0.937 | 5.587 | 5.293 | 0.917 | 0.724 | 25.907 | 32.118 | 1.074 | Non-cave_Aquatic |
| AMNH | 108785 | Eurycea aquatica | 4.846 | 6.908 | 1.013 | 4.809 | 8.244 | 1.463 | 0.663 | 22.73 | 33.252 | 1.197 | Non-cave_Aquatic |
| AMNH | 108786 | Eurycea aquatica | 6.361 | 6.786 | 1.069 | 5.828 | 6.739 | 1.503 | 0.846 | 25.766 | 32.603 | 1.28 | Non-cave_Aquatic |
| AMNH | 108787 | Eurycea aquatica | 4.841 | 4.999 | 0.629 | 3.909 | 4.234 | 0.907 | 0.656 | 19.91 | 24.657 | 1.296 | Non-cave_Aquatic |
| AMNH | 108788 | Eurycea aquatica | 5.048 | 6.829 | 1.01 | 4.421 | 9.008 | 1.145 | 1.102 | 21.35 | 30.818 | 1.55 | Non-cave_Aquatic |
| AMNH | 6632 | Eurycea bislineata | 5.862 | 5.518 | 0.873 | 5.103 | 8.95 | 1.626 | 1.483 | 23.406 | 42.618 | 1.868 | Non-cave_Aquatic |
| AMNH | 7540 | Eurycea bislineata | 5.8 | 5.918 | 1.156 | 5.096 | 8.055 | 1.283 | 1.074 | 22.317 | 38.189 | 1.844 | Non-cave_Aquatic |
| AMNH | 7543 | Eurycea bislineata | 5.269 | 5.212 | 1.281 | 4.961 | 6.981 | 1.376 | 0.813 | 35.74 | 32.817 | 1.503 | Non-cave_Aquatic |
| AMNH | 13099 | Eurycea bislineata | 4.8 | 5.516 | 1.121 | 5.363 | 7.972 | 1.855 | 1.187 | 45.4 | 41.929 | 1.227 | Non-cave_Aquatic |
| AMNH | 13100 | Eurycea bislineata | 6.404 | 4.529 | 1.407 | 6.271 | 5.194 | 1.331 | 1.058 | 44.798 | 47.714 | 1.637 | Non-cave_Aquatic |
| AMNH | 15748 | Eurycea bislineata | 4.864 | 6.013 | 0.966 | 4.055 | 7.556 | 1.425 | 0.962 | 28.567 | 32.072 | 1.3 | Non-cave_Aquatic |
| AMNH | 32864 | Eurycea bislineata | 4.647 | 6.225 | 0.926 | 4.881 | 7.393 | 1.226 | 0.801 | 37.494 | 33.456 | 1.464 | Non-cave_Aquatic |
| AMNH | 51541 | Eurycea bislineata | 4.809 | 5.091 | 1.369 | 5.515 | 7.849 | 1.667 | 0.943 | 34.418 | 36.436 | 1.189 | Non-cave_Aquatic |
| AMNH | 51697 | Eurycea bislineata | 4.669 | 4.384 | 1.371 | 4.013 | 6.545 | 1.395 | 1.028 | 40.424 | 25.77 | 1.169 | Non-cave_Aquatic |
| AMNH | 60780 | Eurycea bislineata | 5.091 | 6.272 | 1.333 | 6.8 | 8.236 | 1.674 | 0.773 | 33.587 | 38.623 | 1.266 | Non-cave_Aquatic |
| AMNH | 60781 | Eurycea bislineata | 5.023 | 5.48 | 1.32 | 6.444 | 8.652 | 1.618 | 1.136 | 32.311 | 38.273 | 1.492 | Non-cave_Aquatic |
| AMNH | 116809 | Eurycea bislineata | 5.729 | 6.862 | 1.643 | 6.763 | 7.903 | 2.129 | 0.898 | 54.252 | 46.117 | 2.483 | Non-cave_Aquatic |
| AMNH | 116810 | Eurycea bislineata | 5.17 | 7.504 | 1.164 | 4.812 | 7.909 | 1.641 | 1.073 | 49.009 | 39.356 | 2.043 | Non-cave_Aquatic |
| AMNH | 116811 | Eurycea bislineata | 5.758 | 6.83 | 1.672 | 5.973 | 9.356 | 1.846 | 1.36 | 61.378 | 44.261 | 1.882 | Non-cave_Aquatic |
| AMNH | 116812 | Eurycea bislineata | 4.856 | 7.55 | 1.14 | 5.405 | 8.238 | 1.75 | 0.841 | 44.381 | 36.811 | 1.797 | Non-cave_Aquatic |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 116813 | Eurycea bislineata | 4.5 | 5.956 | 1.02 | 4.195 | 7.736 | 1.353 | 1.106 | 41.202 | 30.659 | 1.813 | Non-cave_Aquatic |
| AMNH | 164483 | Eurycea bislineata | 5.974 | 7.377 | 1.338 | 6.129 | 8.612 | 1.873 | 1.319 | 51.906 | 41.782 | 1.725 | Non-cave_Aquatic |
| AMNH | 164484 | Eurycea bislineata | 5.896 | 7.734 | 1.39 | 5.859 | 10.23 | 1.804 | 1.442 | 41.826 | 42.704 | 1.34 | Non-cave_Aquatic |
| AMNH | 44200 | Eurycea cirrigera | 6.152 | 7.166 | 1.918 | 7.337 | 11.241 | 2.389 | 1.498 | 60.864 | 41.973 | 4.241 | Non-cave_Aquatic |
| AMNH | 50866 | Eurycea cirrigera | 4.98 | 5.362 | 1.363 | 5.139 | 6.78 | 1.749 | 1.042 | 53.248 | 34.472 | 3.122 | Non-cave_Aquatic |
| AMNH | 50867 | Eurycea cirrigera | 5.502 | 7.267 | 1.21 | 6.571 | 8.297 | 1.667 | 0.737 | 38.319 | 38.065 | 2.032 | Non-cave_Aquatic |
| AMNH | 50869 | Eurycea cirrigera | 5.766 | 7.072 | 1.42 | 5.899 | 9.201 | 2.149 | 1.059 | 47.309 | 40.323 | 2.079 | Non-cave_Aquatic |
| AMNH | 60784 | Eurycea cirrigera | 5.09 | 5.826 | 1.048 | 4.899 | 6.684 | 1.771 | 0.526 | 42.854 | 32.216 | 1.453 | Non-cave_Aquatic |
| AMNH | 62827 | Eurycea cirrigera | 5.907 | 7.028 | 1.509 | 6.377 | 9.068 | 2.075 | 1.341 | 45.157 | 37.28 | 1.812 | Non-cave_Aquatic |
| AMNH | 100411 | Eurycea cirrigera | 6.146 | 7.207 | 1.412 | 5.751 | 8.256 | 2.148 | 1.102 | 32.231 | 36.042 | 2.786 | Non-cave_Aquatic |
| AMNH | 100412 | Eurycea cirrigera | 7.018 | 8.088 | 1.51 | 6.48 | 9.04 | 1.943 | 1.503 | 51.787 | 40.952 | 1.641 | Non-cave_Aquatic |
| AMNH | 100415 | Eurycea cirrigera | 5.614 | 7.959 | 1.034 | 5.067 | 9.726 | 1.306 | 0.981 | 42.946 | 36.996 | 2.18 | Non-cave_Aquatic |
| AMNH | 135499 | Eurycea cirrigera | 3.458 | 4.606 | 0.631 | 3.389 | 5.775 | 0.841 | 0.619 | 39.811 | 27.57 | 1.027 | Non-cave_Aquatic |
| AMNH | 135500 | Eurycea cirrigera | 3.477 | 4.579 | 0.756 | 3.243 | 5.953 | 0.994 | 0.631 | 26.641 | 25.499 | 1.367 | Non-cave_Aquatic |
| AMNH | 135501 | Eurycea cirrigera | 3.304 | 3.871 | 0.657 | 3.013 | 6.302 | 1.143 | 0.624 | 25.623 | 24.329 | 1.172 | Non-cave_Aquatic |
| AMNH | 143130 | Eurycea cirrigera | 5.224 | 5.798 | 1.147 | 4.924 | 7.457 | 1.728 | 1.158 | 50.784 | 33.427 | 1.803 | Non-cave_Aquatic |
| AMNH | 143131 | Eurycea cirrigera | 5.108 | 6.705 | 1.116 | 4.81 | 9.243 | 1.656 | 1.43 | 45.408 | 30.927 | 1.705 | Non-cave_Aquatic |
| AMNH | 143133 | Eurycea cirrigera | 4.02 | 4.122 | 0.656 | 3.649 | 4.57 | 1.029 | 0.582 | 42.314 | 25.825 | 1.278 | Non-cave_Aquatic |
| AMNH | 143134 | Eurycea cirrigera | 3.648 | 4.666 | 0.978 | 3.833 | 5.866 | 1.02 | 0.876 | 35.24 | 26.397 | 1.626 | Non-cave_Aquatic |
| AMNH | 143135 | Eurycea cirrigera | 5.302 | 6.008 | 1.277 | 4.293 | 7.114 | 1.377 | 1.028 | 24.618 | 30.77 | 1.692 | Non-cave_Aquatic |
| AMNH | 143136 | Eurycea cirrigera | 6.006 | 7.098 | 1.504 | 5.547 | 9.602 | 2.025 | 0.927 | 48.744 | 36.11 | 1.81 | Non-cave_Aquatic |
| AMNH | 182150 | Eurycea cirrigera | 5.292 | 5.457 | 1.306 | 6.614 | 8.003 | 2.029 | 1.179 | 51.56 | 38.85 | 1.766 | Non-cave_Aquatic |
| AMNH | 182151 | Eurycea cirrigera | 5.329 | 7.499 | 1.197 | 5.656 | 8.309 | 1.684 | 1.255 | 44.949 | 39.814 | 2.495 | Non-cave_Aquatic |
| AMNH | 182154 | Eurycea cirrigera | 4.941 | 6.313 | 1.15 | 4.707 | 8.222 | 1.531 | 0.943 | 38.861 | 33.243 | 2.11 | Non-cave_Aquatic |
| AMNH | 182155 | Eurycea cirrigera | 5.364 | 5.753 | 1.075 | 4.623 | 6.151 | 1.496 | 0.953 | 37.393 | 32.596 | 1.69 | Non-cave_Aquatic |
| AMNH | 182156 | Eurycea cirrigera | 5.967 | 8.115 | 1.145 | 6.175 | 8.897 | 1.822 | 1.358 | 50.585 | 36.248 | 2.49 | Non-cave_Aquatic |
| AMNH | 3090 | Eurycea guttolineata | 8.439 | 8.283 | 1.747 | 10.291 | 12.06 | 2.838 | 1.386 | 81.382 | 58.541 | 2.308 | Non-cave_Terrestrial |
| AMNH | 3997 | Eurycea guttolineata | 8.136 | 12.874 | 2.239 | 8.484 | 13.201 | 2.905 | 2.312 | 85.061 | 50.057 | 3.011 | Non-cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 3998 | Eurycea guttolineata | 6.696 | 11.197 | 2.221 | 9.107 | 12.703 | 3.143 | 1.656 | 88.048 | 54.398 | 3.028 | Non-cave_Terrestrial |
| AMNH | 3999 | Eurycea guttolineata | 8.637 | 12.55 | 2.255 | 9.023 | 15.072 | 3.129 | 2.645 | 86.514 | 59.065 | 2.421 | Non-cave_Terrestrial |
| AMNH | 21195 | Eurycea guttolineata | 7.297 | 11.377 | 1.778 | 7.161 | 13.229 | 2.597 | 1.683 | 81.635 | 52.149 | 1.646 | Non-cave_Terrestrial |
| AMNH | 127073 | Eurycea guttolineata | 8.329 | 11.91 | 2.613 | 9.513 | 13.81 | 3.583 | 1.607 | 93.552 | 52.956 | 2.686 | Non-cave_Terrestrial |
| AMNH | 127075 | Eurycea guttolineata | 9.094 | 11.847 | 2.147 | 10.526 | 14.692 | 4.21 | 1.285 | 92.233 | 59.267 | 2.652 | Non-cave_Terrestrial |
| AMNH | 127076 | Eurycea guttolineata | 9.283 | 11.814 | 2.316 | 9.965 | 14.93 | 2.991 | 2.102 | 67.39 | 58.518 | 2.602 | Non-cave_Terrestrial |
| AMNH | 127087 | Eurycea guttolineata | 7.21 | 10.188 | 1.648 | 6.442 | 11.553 | 2.363 | 1.252 | 52.617 | 45.636 | 2.057 | Non-cave_Terrestrial |
| AMNH | 127088 | Eurycea guttolineata | 8.519 | 11.398 | 2.199 | 9.631 | 7.951 | 2.83 | 1.465 | 103.749 | 54.979 | 2.823 | Non-cave_Terrestrial |
| AMNH | 127089 | Eurycea guttolineata | 7.837 | 11.338 | 2.012 | 9.484 | 12.812 | 2.943 | 1.556 | 102.123 | 55.773 | 2.557 | Non-cave_Terrestrial |
| AMNH | 127092 | Eurycea guttolineata | 8.259 | 11.553 | 1.957 | 9.261 | 12.478 | 3.06 | 2.084 | 99.577 | 54.318 | 2.368 | Non-cave_Terrestrial |
| AMNH | 127093 | Eurycea guttolineata | 8.874 | 11.895 | 2.292 | 9.4 | 11.902 | 3.154 | 1.356 | 87.293 | 53.873 | 2.777 | Non-cave_Terrestrial |
| AMNH | 127102 | Eurycea guttolineata | 10.264 | 11.264 | 2.452 | 9.774 | 15.086 | 3.625 | 2.021 | 71.686 | 58.569 | 2.912 | Non-cave_Terrestrial |
| AMNH | 182160 | Eurycea guttolineata | 6.978 | 9.025 | 1.738 | 5.926 | 12.176 | 2.002 | 1.55 | 55.687 | 41.405 | 2.579 | Non-cave_Terrestrial |
| AMNH | 182161 | Eurycea guttolineata | 6.363 | 7.386 | 1.39 | 5.892 | 9.847 | 1.709 | 1.453 | 55.099 | 38.238 | 2.828 | Non-cave_Terrestrial |
| AMNH | 187780 | Eurycea guttolineata | 7.818 | 9.88 | 2.006 | 9.165 | 15.731 | 2.845 | 2.616 | 80.138 | 54.632 | 2.821 | Non-cave_Terrestrial |
| AMNH | 187781 | Eurycea guttolineata | 6.873 | 8.28 | 1.754 | 7.448 | 10.079 | 2.207 | 1.466 | 34.516 | 39.863 | 1.975 | Non-cave_Terrestrial |
| AMNH | 187782 | Eurycea guttolineata | 7.32 | 12.39 | 1.66 | 7.126 | 13.978 | 1.979 | 1.741 | 56.304 | 46.076 | 2.528 | Non-cave_Terrestrial |
| AMNH | 187783 | Eurycea guttolineata | 8.767 | 13.635 | 2.275 | 9.529 | 15.032 | 3.151 | 2.192 | 94.428 | 55.381 | 2.653 | Non-cave_Terrestrial |
| AMNH | 187784 | Eurycea guttolineata | 8 | 12.136 | 1.836 | 8.485 | 13.779 | 3.113 | 1.626 | 51.937 | 53.589 | 2.192 | Non-cave_Terrestrial |
| AMNH | 171578 | Eurycea junaluska | 4.141 | 5.944 | 0.778 | 3.748 | 7.576 | 1.357 | 0.646 | 43.76 | 30.914 | 1.443 | Non-cave_Terrestrial |
| AMNH | 172184 | Eurycea junaluska | 5.801 | 9.147 | 0.997 | 6.429 | 9.713 | 1.605 | 1.168 | 34.19 | 40.11 | 1.798 | Non-cave_Terrestrial |
| AMNH | 38057 | Eurycea l. longicauda | 6.859 | 11.758 | 1.84 | 5.537 | 14.402 | 1.981 | 1.203 | 87.513 | 54.983 | 2.149 | Non-cave_Terrestrial |
| AMNH | 43662 | Eurycea l. longicauda | 6.109 | 7.662 | 1.428 | 5.414 | 11.561 | 1.885 | 1.747 | 73.492 | 41.951 | 2.78 | Non-cave_Terrestrial |
| AMNH | 51698 | Eurycea l. longicauda | 7.633 | 13.903 | 2.212 | 10.087 | 15.2 | 3.356 | 1.607 | 93.134 | 51.571 | 2.542 | Non-cave_Terrestrial |
| AMNH | 58232 | Eurycea l. longicauda | 9.138 | 13.048 | 2.145 | 8.461 | 15.117 | 2.581 | 1.796 | 87.131 | 54.254 | 2.342 | Non-cave_Terrestrial |
| AMNH | 59810 | Eurycea l. longicauda | 7.391 | 10.025 | 1.433 | 5.951 | 14.01 | 2.387 | 0.794 | 61.405 | 51.295 | 2.188 | Non-cave_Terrestrial |
| AMNH | 79728 | Eurycea l. longicauda | 6.285 | 10.997 | 1.278 | 5.652 | 11.723 | 1.991 | 0.741 | 24.404 | 37.336 | 2.396 | Non-cave_Terrestrial |
| AMNH | 89783 | Eurycea l. longicauda | 8.072 | 13.612 | 1.769 | 8.154 | 16.433 | 2.409 | 1.919 | 101.849 | 57.656 | 2.919 | Non-cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 99284 | Eurycea l. longicauda | 7.746 | 11.152 | 2.268 | 8.14 | 15.684 | 2.784 | 1.715 | 97.844 | 57.65 | 2.38 | Non-cave_Terrestrial |
| AMNH | 99286 | Eurycea l. longicauda | 8.415 | 14.088 | 1.76 | 6.858 | 18.345 | 2.386 | 1.458 | 59.503 | 62.019 | 2.513 | Non-cave_Terrestrial |
| AMNH | 112043 | Eurycea l. longicauda | 5.893 | 9.206 | 1.553 | 4.935 | 12.257 | 1.741 | 1.015 | 61.414 | 41.73 | 2.033 | Non-cave_Terrestrial |
| AMNH | 112044 | Eurycea l. longicauda | 5.75 | 9.066 | 1.25 | 5.344 | 9.953 | 2.057 | 1.041 | 61.321 | 40.555 | 1.82 | Non-cave_Terrestrial |
| AMNH | 112045 | Eurycea l. longicauda | 5.809 | 10.127 | 1.173 | 5.005 | 11.166 | 1.921 | 1.38 | 69.868 | 49.388 | 2.41 | Non-cave_Terrestrial |
| AMNH | 114012 | Eurycea l. longicauda | 6.994 | 9.902 | 1.986 | 8.222 | 12.442 | 2.682 | 1.496 | 88.872 | 54.015 | 1.983 | Non-cave_Terrestrial |
| AMNH | 114385 | Eurycea l. longicauda | 7.26 | 15.338 | 1.992 | 9.043 | 13.504 | 2.411 | 1.207 | 77.347 | 53.23 | 2.135 | Non-cave_Terrestrial |
| AMNH | 114386 | Eurycea l. longicauda | 5.82 | 10.936 | 1.397 | 5.748 | 12.109 | 2.419 | 1.041 | 61.822 | 39.017 | 1.959 | Non-cave_Terrestrial |
| AMNH | 115838 | Eurycea l. longicauda | 7.181 | 12.544 | 1.692 | 6.415 | 13.788 | 1.916 | 1.817 | 80.307 | 49.503 | 2.214 | Non-cave_Terrestrial |
| AMNH | 135240 | Eurycea l. longicauda | 7.924 | 14.599 | 1.816 | 8.067 | 15.588 | 2.837 | 1.598 | 87.949 | 58.252 | 3.157 | Non-cave_Terrestrial |
| AMNH | 135241 | Eurycea l. longicauda | 7.423 | 12.282 | 1.923 | 8.129 | 14.971 | 2.493 | 1.614 | 79.745 | 51.717 | 2.605 | Non-cave_Terrestrial |
| AMNH | 135782 | Eurycea l. longicauda | 6.495 | 12.608 | 1.861 | 6.806 | 15.264 | 2.426 | 2.079 | 93.709 | 48.254 | 2.535 | Non-cave_Terrestrial |
| AMNH | 136646 | Eurycea l. longicauda | 7.23 | 11.555 | 1.708 | 6.453 | 13.847 | 2.37 | 2.007 | 60.713 | 50.936 | 2.155 | Non-cave_Terrestrial |
| AMNH | 148944 | Eurycea l. longicauda | 6.659 | 9.453 | 1.481 | 5.831 | 12.119 | 1.719 | 1.447 | 55.706 | 42.587 | 2.453 | Non-cave_Terrestrial |
| AMNH | 148950 | Eurycea l. longicauda | 6.399 | 10.474 | 1.485 | 6.365 | 12.847 | 1.838 | 1.089 | 64.379 | 45.55 | 2.128 | Non-cave_Terrestrial |
| AMNH | 151305 | Eurycea l. longicauda | 7.889 | 12.968 | 1.826 | 7.231 | 13.499 | 2.235 | 1.737 | 74.055 | 54.626 | 2.333 | Non-cave_Terrestrial |
| AMNH | 40345 | Eurycea l. melanopleura | 7.62 | 10.747 | 1.718 | 6.213 | 13.113 | 2.111 | 1.937 | 74.188 | 49.066 | 2.499 | Non-cave_Terrestrial |
| AMNH | 40346 | Eurycea l. melanopleura | 8.626 | 11.891 | 1.638 | 6.858 | 11.772 | 2.796 | 1.577 | 80.155 | 44.461 | 2.467 | Non-cave_Terrestrial |
| AMNH | 40347 | Eurycea l. melanopleura | 7.692 | 9.978 | 1.522 | 6.396 | 12.388 | 2.021 | 1.102 | 64.487 | 47.485 | 2.343 | Non-cave_Terrestrial |
| AMNH | 40349 | Eurycea l. melanopleura | 6.261 | 10.283 | 1.236 | 6.171 | 10.886 | 1.757 | 1.708 | 36.223 | 40.633 | 2.125 | Non-cave_Terrestrial |
| AMNH | 40351 | Eurycea l. melanopleura | 7.402 | 10.454 | 1.919 | 7.08 | 12.816 | 2.195 | 1.388 | 82.262 | 49.768 | 2.672 | Non-cave_Terrestrial |
| AMNH | 40354 | Eurycea l. melanopleura | 6.273 | 8.259 | 1.519 | 5.715 | 11.478 | 1.664 | 1.1 | 62.701 | 55.342 | 2.259 | Non-cave_Terrestrial |
| AMNH | 40356 | Eurycea l. melanopleura | 8.712 | 9.951 | 1.524 | 7.28 | 12.056 | 2.576 | 1.685 | 83.172 | 45.669 | 2.478 | Non-cave_Terrestrial |
| AMNH | 52071 | Eurycea l. melanopleura | 9.054 | 8.712 | 2.087 | 8.768 | 12.798 | 2.697 | 1.959 | 83.032 | 54.316 | 2.458 | Non-cave_Terrestrial |
| AMNH | 52072 | Eurycea l. melanopleura | 8.617 | 12.106 | 2.318 | 9.463 | 13.303 | 3.309 | 1.55 | 53.551 | 56.266 | 2.787 | Non-cave_Terrestrial |
| AMNH | 52073 | Eurycea l. melanopleura | 8.293 | 11.984 | 2.082 | 10.27 | 15.429 | 2.973 | 1.802 | 86.971 | 57.069 | 2.657 | Non-cave_Terrestrial |
| AMNH | 52075 | Eurycea l. melanopleura | 7.898 | 9.522 | 1.524 | 7.377 | 12.393 | 2.441 | 1.175 | 60.643 | 43.999 | 2.048 | Non-cave_Terrestrial |
| AMNH | 52077 | Eurycea l. melanopleura | 9.649 | 12.335 | 2.349 | 10.192 | 13.62 | 3.174 | 1.954 | 74.178 | 58.875 | 2.789 | Non-cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 52078 | Eurycea l. melanopleura | 8.505 | 12.655 | 1.903 | 9.481 | 14.589 | 2.927 | 1.554 | 67.296 | 60.688 | 2.6 | Non-cave_Terrestrial |
| AMNH | 59797 | Eurycea l. melanopleura | 6.142 | 9.774 | 1.086 | 5.621 | 11.682 | 1.595 | 1.523 | 54.255 | 41.083 | 2.363 | Non-cave_Terrestrial |
| AMNH | 59798 | Eurycea l. melanopleura | 5.64 | 7.349 | 1.182 | 4.93 | 10.199 | 1.595 | 0.8 | 40.804 | 36.57 | 1.671 | Non-cave_Terrestrial |
| AMNH | 59799 | Eurycea l. melanopleura | 6.239 | 8.882 | 1.307 | 4.872 | 11.034 | 1.704 | 1.301 | 32.847 | 40.588 | 2.259 | Non-cave_Terrestrial |
| AMNH | 59800 | Eurycea l. melanopleura | 5.588 | 8.096 | 1.03 | 4.83 | 9.752 | 1.321 | 0.895 | 41.353 | 35.52 | 2.297 | Non-cave_Terrestrial |
| AMNH | 149001 | Eurycea l. melanopleura | 5.213 | 8.581 | 1.055 | 4.727 | 9.625 | 1.698 | 1.069 | 44.608 | 35.411 | 2.033 | Non-cave_Terrestrial |
| AMNH | 149201 | Eurycea l. melanopleura | 7.794 | 11.172 | 1.656 | 7.241 | 12.367 | 2.551 | 1.262 | 61.83 | 45.241 | 2.271 | Non-cave_Terrestrial |
| AMNH | 149212 | Eurycea l. melanopleura | 8.786 | 11.507 | 1.956 | 10.484 | 13.034 | 2.795 | 1.684 | 87.046 | 53.768 | 2.568 | Non-cave_Terrestrial |
| AMNH | 149230 | Eurycea l. melanopleura | 7.97 | 12.033 | 2.284 | 9.056 | 13.588 | 3.044 | 1.566 | 77.246 | 56.029 | 2.873 | Non-cave_Terrestrial |
| AMNH | 149249 | Eurycea l. melanopleura | 7.532 | 11.992 | 2.02 | 7.827 | 12.347 | 2.511 | 1.197 | 73.182 | 48.489 | 2.452 | Non-cave_Terrestrial |
| NMNH | 123594 | Eurycea latitans | 7.991 | 6.943 | 1.546 | 7.115 | 8.871 | 1.895 | 1.477 | 45.758 | 46.945 | 1.15 | Cave_Aquatic |
| NMNH | 545379 | Eurycea latitans | 8.499 | 8.677 | 1.401 | 7.199 | 8.519 | 1.227 | 1.779 | 41.844 | 41.392 | 1.074 | Cave_Aquatic |
| AMNH | 11867 | Eurycea lucifuga | 8.835 | 13.762 | 2.078 | 7.478 | 17.943 | 2.627 | 1.742 | 72.338 | 53.867 | 2.407 | Cave_Terrestrial |
| AMNH | 11868 | Eurycea lucifuga | 9.114 | 14.758 | 2.387 | 8.402 | 20.227 | 2.47 | 2.772 | 87.605 | 63.245 | 1.943 | Cave_Terrestrial |
| AMNH | 16826 | Eurycea lucifuga | 10.045 | 17.462 | 2.189 | 8.95 | 19.346 | 2.529 | 2.191 | 78.219 | 64.906 | 3.057 | Cave_Terrestrial |
| AMNH | 22915 | Eurycea lucifuga | 6.289 | 10.881 | 1.473 | 4.878 | 9.346 | 1.288 | 1.562 | 60.8 | 37.313 | 2.654 | Cave_Terrestrial |
| AMNH | 22916 | Eurycea lucifuga | 8.262 | 16.264 | 2.227 | 9.052 | 16.463 | 2.993 | 1.963 | 80.656 | 55.416 | 3.519 | Cave_Terrestrial |
| AMNH | 22917 | Eurycea lucifuga | 8.599 | 13.923 | 1.873 | 7.828 | 13.071 | 2.066 | 2.324 | 70.119 | 46.463 | 3.164 | Cave_Terrestrial |
| AMNH | 22918 | Eurycea lucifuga | 5.751 | 8.802 | 1.125 | 5.171 | 10.6 | 2.305 | 1.44 | 22.33 | 39.554 | 1.945 | Cave_Terrestrial |
| AMNH | 22920 | Eurycea lucifuga | 8.752 | 13.1 | 2.084 | 8.638 | 14.213 | 2.783 | 2.228 | 82.81 | 55.115 | 3.156 | Cave_Terrestrial |
| AMNH | 22921 | Eurycea lucifuga | 6.841 | 12.186 | 1.75 | 5.777 | 11.454 | 2.104 | 1.884 | 45.598 | 62.327 | 2.337 | Cave_Terrestrial |
| AMNH | 22922 | Eurycea lucifuga | 7.085 | 10.437 | 1.161 | 5.575 | 11.326 | 2.071 | 1.731 | 56.376 | 42.017 | 2.34 | Cave_Terrestrial |
| AMNH | 32175 | Eurycea lucifuga | 6.137 | 9.108 | 1.222 | 4.474 | 10.765 | 1.796 | 1.199 | 44.7 | 35.17 | 2.103 | Cave_Terrestrial |
| AMNH | 32176 | Eurycea lucifuga | 5.73 | 8.213 | 1.201 | 4.819 | 10.41 | 1.682 | 1.023 | 43.592 | 35.055 | 2.049 | Cave_Terrestrial |
| AMNH | 33407 | Eurycea lucifuga | 9.573 | 15.588 | 2.152 | 7.873 | 15.226 | 2.797 | 1.741 | 83.79 | 60.548 | 2.759 | Cave_Terrestrial |
| AMNH | 33408 | Eurycea lucifuga | 8.628 | 14.404 | 2.035 | 6.82 | 14.613 | 2.577 | 1.36 | 83.463 | 57.146 | 2.769 | Cave_Terrestrial |
| AMNH | 33409 | Eurycea lucifuga | 8.642 | 14.66 | 2.128 | 7.829 | 16.127 | 2.889 | 2.182 | 83.135 | 56.52 | 2.973 | Cave_Terrestrial |
| AMNH | 36112 | Eurycea lucifuga | 5.479 | 6.601 | 1.201 | 4.549 | 8.843 | 1.704 | 0.814 | 19.463 | 35.544 | 2.299 | Cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 36113 | Eurycea lucifuga | 5.666 | 7.943 | 1.208 | 4.785 | 9.452 | 1.899 | 1.255 | 42.509 | 34.784 | 2.058 | Cave_Terrestrial |
| AMNH | 36115 | Eurycea lucifuga | 5.854 | 9.905 | 1.269 | 4.624 | 10.76 | 1.331 | 1.012 | 39.087 | 35.959 | 1.893 | Cave_Terrestrial |
| AMNH | 36116 | Eurycea lucifuga | 5.895 | 8.599 | 1.193 | 5.216 | 10.132 | 1.673 | 1.039 | 17.884 | 36.321 | 2.342 | Cave_Terrestrial |
| AMNH | 36120 | Eurycea lucifuga | 6.144 | 10.216 | 1.487 | 4.942 | 9.36 | 1.926 | 1.129 | 36.264 | 36.771 | 2.069 | Cave_Terrestrial |
| AMNH | 38500 | Eurycea lucifuga | 10.297 | 17.352 | 2.507 | 8.234 | 19.074 | 2.435 | 2.231 | 63.567 | 62.796 | 2.562 | Cave_Terrestrial |
| AMNH | 40342 | Eurycea lucifuga | 9.337 | 16.878 | 2.469 | 7.99 | 18.258 | 2.556 | 1.618 | 64.874 | 60.08 | 3.005 | Cave_Terrestrial |
| AMNH | 41467 | Eurycea lucifuga | 6.947 | 12.175 | 1.817 | 6.946 | 12.443 | 2.964 | 1.951 | 70.418 | 47.096 | 1.851 | Cave_Terrestrial |
| AMNH | 41786 | Eurycea lucifuga | 8.038 | 13.998 | 1.702 | 6.476 | 16.564 | 2.31 | 2.816 | 37.623 | 49.144 | 2.382 | Cave_Terrestrial |
| AMNH | 50036 | Eurycea lucifuga | 6.985 | 12.247 | 1.569 | 6.095 | 13.82 | 2.147 | 1.319 | 62.894 | 44.728 | 1.895 | Cave_Terrestrial |
| AMNH | 52068 | Eurycea lucifuga | 10.278 | 16.933 | 3.042 | 10.61 | 15.891 | 3.282 | 1.658 | 87.868 | 64.13 | 3.395 | Cave_Terrestrial |
| AMNH | 52069 | Eurycea lucifuga | 8.639 | 14.282 | 1.736 | 7.631 | 14.167 | 2.619 | 1.814 | 72.62 | 51.597 | 3.566 | Cave_Terrestrial |
| AMNH | 52104 | Eurycea lucifuga | 10.491 | 16.401 | 2.258 | 10.586 | 17.421 | 3.174 | 1.99 | 86.751 | 61.561 | 3.053 | Cave_Terrestrial |
| AMNH | 52105 | Eurycea lucifuga | 9.854 | 15.691 | 2.75 | 10.368 | 15.457 | 3.523 | 1.805 | 80.444 | 57.544 | 3.246 | Cave_Terrestrial |
| AMNH | 52106 | Eurycea lucifuga | 10.251 | 15.926 | 2.153 | 7.403 | 18.124 | 3.525 | 2.005 | 68.362 | 61.454 | 3.404 | Cave_Terrestrial |
| AMNH | 52107 | Eurycea lucifuga | 10.173 | 15.134 | 2.688 | 7.26 | 19.516 | 2.968 | 2.141 | 90.776 | 65.173 | 3.727 | Cave_Terrestrial |
| AMNH | 52108 | Eurycea lucifuga | 9.806 | 15.095 | 1.865 | 8.91 | 15.143 | 3.194 | 1.444 | 89.484 | 63.845 | 3.486 | Cave_Terrestrial |
| AMNH | 52109 | Eurycea lucifuga | 9.674 | 15.36 | 2.13 | 9.039 | 18.062 | 2.765 | 1.543 | 86.221 | 59.878 | 3.459 | Cave_Terrestrial |
| AMNH | 52110 | Eurycea lucifuga | 10.704 | 13.747 | 2.104 | 9.621 | 15.946 | 3.178 | 1.88 | 98.029 | 63.134 | 3.653 | Cave_Terrestrial |
| AMNH | 52111 | Eurycea lucifuga | 10.397 | 12.453 | 1.953 | 7.084 | 14.422 | 3.004 | 1.71 | 63.746 | 54.043 | 3.113 | Cave_Terrestrial |
| AMNH | 52112 | Eurycea lucifuga | 8.406 | 12.906 | 1.959 | 7.27 | 13.592 | 2.628 | 1.836 | 76.394 | 51.894 | 3.257 | Cave_Terrestrial |
| AMNH | 52113 | Eurycea lucifuga | 9.035 | 14.87 | 1.901 | 7.787 | 14.286 | 2.353 | 2.083 | 75.554 | 57.213 | 3.886 | Cave_Terrestrial |
| AMNH | 52114 | Eurycea lucifuga | 7.847 | 12.663 | 1.513 | 6.243 | 15.845 | 2.734 | 2.395 | 69.316 | 51.308 | 3.138 | Cave_Terrestrial |
| AMNH | 52115 | Eurycea lucifuga | 8.653 | 14.162 | 1.919 | 6.493 | 13.455 | 2.762 | 1.574 | 87.37 | 54.992 | 3.824 | Cave_Terrestrial |
| AMNH | 52117 | Eurycea lucifuga | 9.951 | 17.052 | 2.243 | 8.677 | 17.232 | 2.711 | 1.826 | 65.411 | 57.671 | 3.354 | Cave_Terrestrial |
| AMNH | 52118 | Eurycea lucifuga | 9.046 | 12.949 | 1.926 | 8.073 | 13.624 | 1.786 | 2.118 | 75.353 | 55.074 | 2.315 | Cave_Terrestrial |
| AMNH | 52119 | Eurycea lucifuga | 9.602 | 16.117 | 2.283 | 8.855 | 16.591 | 2.938 | 1.678 | 89.114 | 62.928 | 3.454 | Cave_Terrestrial |
| AMNH | 52121 | Eurycea lucifuga | 9.743 | 16.259 | 2.429 | 8.228 | 19.049 | 3.449 | 1.987 | 65.683 | 60.547 | 2.875 | Cave_Terrestrial |
| AMNH | 52443 | Eurycea lucifuga | 10.743 | 17.62 | 2.661 | 8.708 | 18.105 | 3.555 | 1.957 | 73.129 | 72.792 | 3.286 | Cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 52444 | Eurycea lucifuga | 10.357 | 13.532 | 2.728 | 7.985 | 14.888 | 2.745 | 1.838 | 75.317 | 60.682 | 3.608 | Cave_Terrestrial |
| AMNH | 52445 | Eurycea lucifuga | 10.248 | 13.398 | 2.194 | 10.52 | 16.237 | 3.181 | 1.348 | 49.48 | 58.432 | 4.019 | Cave_Terrestrial |
| AMNH | 52446 | Eurycea lucifuga | 11.948 | 16.155 | 3.284 | 11.944 | 15.76 | 4.098 | 2.489 | 95.947 | 68.362 | 3.668 | Cave_Terrestrial |
| AMNH | 52475 | Eurycea lucifuga | 9.555 | 12.817 | 2.156 | 8.004 | 15.557 | 3.29 | 1.684 | 90.28 | 57.608 | 3.556 | Cave_Terrestrial |
| AMNH | 52527 | Eurycea lucifuga | 9.038 | 13.866 | 1.661 | 5.681 | 15.204 | 1.903 | 1.991 | 61.356 | 44.06 | 2.201 | Cave_Terrestrial |
| AMNH | 54554 | Eurycea lucifuga | 11.179 | 14.243 | 2.55 | 7.484 | 17.492 | 3.168 | 1.493 | 58.82 | 68.111 | 3.705 | Cave_Terrestrial |
| AMNH | 54555 | Eurycea lucifuga | 9.748 | 15.288 | 2.157 | 7.053 | 15.356 | 2.411 | 1.561 | 81.683 | 62.531 | 3.829 | Cave_Terrestrial |
| AMNH | 56367 | Eurycea lucifuga | 7.081 | 11.155 | 1.206 | 5.408 | 13.487 | 1.936 | 1.668 | 52.959 | 44.481 | 2.434 | Cave_Terrestrial |
| AMNH | 56368 | Eurycea lucifuga | 9.97 | 18.553 | 2.136 | 8.033 | 19.41 | 2.738 | 1.854 | 65.803 | 64.546 | 2.924 | Cave_Terrestrial |
| AMNH | 58233 | Eurycea lucifuga | 9.843 | 16.616 | 1.92 | 9.065 | 21.288 | 3.119 | 2.327 | 111.287 | 61.433 | 2.798 | Cave_Terrestrial |
| AMNH | 58234 | Eurycea lucifuga | 9.627 | 14.66 | 2.246 | 7.74 | 17.59 | 2.924 | 2.926 | 112.952 | 60.326 | 2.778 | Cave_Terrestrial |
| AMNH | 58235 | Eurycea lucifuga | 9.706 | 16.969 | 1.763 | 7.695 | 17.375 | 2.949 | 2.652 | 97.394 | 57.321 | 2.982 | Cave_Terrestrial |
| AMNH | 58236 | Eurycea lucifuga | 7.199 | 14.036 | 2.215 | 7.212 | 15.273 | 2.873 | 2.362 | 83.224 | 49.416 | 2.616 | Cave_Terrestrial |
| AMNH | 59020 | Eurycea lucifuga | 9.949 | 16.082 | 2.142 | 7.899 | 14.404 | 2.294 | 2.254 | 100.55 | 62.842 | 3.609 | Cave_Terrestrial |
| AMNH | 59021 | Eurycea lucifuga | 9.577 | 11.977 | 2.144 | 8.923 | 13.735 | 2.777 | 2.285 | 95.046 | 58.132 | 3.096 | Cave_Terrestrial |
| AMNH | 59022 | Eurycea lucifuga | 8.323 | 12.185 | 2.028 | 6.822 | 16.135 | 2.6 | 2.096 | 83.843 | 54.214 | 3.174 | Cave_Terrestrial |
| AMNH | 59758 | Eurycea lucifuga | 11.37 | 16.852 | 2.448 | 8.768 | 16.981 | 3.076 | 1.445 | 87.448 | 66.974 | 3.381 | Cave_Terrestrial |
| AMNH | 59759 | Eurycea lucifuga | 9.307 | 15.717 | 1.663 | 7.019 | 18.186 | 2.619 | 2.306 | 62.508 | 52.743 | 3.089 | Cave_Terrestrial |
| AMNH | 59760 | Eurycea lucifuga | 9.685 | 15.949 | 2.489 | 7.204 | 16.144 | 2.464 | 1.948 | 45.587 | 60.544 | 3.139 | Cave_Terrestrial |
| AMNH | 59761 | Eurycea lucifuga | 8.92 | 15.7 | 2.109 | 7.063 | 18.241 | 2.487 | 2.673 | 89.711 | 59.922 | 2.915 | Cave_Terrestrial |
| AMNH | 59762 | Eurycea lucifuga | 7.506 | 11.725 | 1.669 | 5.282 | 14.393 | 1.861 | 1.598 | 61.957 | 45.279 | 2.43 | Cave_Terrestrial |
| AMNH | 59764 | Eurycea lucifuga | 11.543 | 16.953 | 2.635 | 9.708 | 18.043 | 3.287 | 3.113 | 79.978 | 62.149 | 3.125 | Cave_Terrestrial |
| AMNH | 59765 | Eurycea lucifuga | 8.696 | 11.597 | 1.93 | 7.039 | 13.93 | 2.152 | 1.76 | 74.496 | 55.809 | 2.905 | Cave_Terrestrial |
| AMNH | 59767 | Eurycea lucifuga | 11.14 | 17.152 | 2.499 | 9.193 | 16.625 | 3.04 | 1.27 | 76.619 | 61.347 | 3.035 | Cave_Terrestrial |
| AMNH | 59768 | Eurycea lucifuga | 8.309 | 14.032 | 1.721 | 6.803 | 15.873 | 2.292 | 1.933 | 77.071 | 54.986 | 2.666 | Cave_Terrestrial |
| AMNH | 59769 | Eurycea lucifuga | 8.67 | 14.313 | 1.984 | 8.171 | 16.535 | 2.687 | 1.584 | 90.558 | 58.845 | 3.246 | Cave_Terrestrial |
| AMNH | 59770 | Eurycea lucifuga | 10.702 | 15.783 | 2.314 | 7.951 | 16.923 | 2.65 | 2.598 | 100.495 | 71.033 | 3.348 | Cave_Terrestrial |
| AMNH | 59771 | Eurycea lucifuga | 10.17 | 15.257 | 2.206 | 7.253 | 16.894 | 2.845 | 2.543 | 76.438 | 59.669 | 2.763 | Cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 59772 | Eurycea lucifuga | 8.507 | 13.026 | 1.813 | 6.025 | 13.548 | 2.352 | 1.934 | 65.271 | 53.001 | 2.801 | Cave_Terrestrial |
| AMNH | 59780 | Eurycea lucifuga | 11.349 | 13.46 | 2.057 | 9.513 | 18.492 | 2.911 | 2.17 | 90.315 | 58.543 | 3.014 | Cave_Terrestrial |
| AMNH | 59781 | Eurycea lucifuga | 12.442 | 17.857 | 3.02 | 12.245 | 17.289 | 4.107 | 2.615 | 84.226 | 66.225 | 3.765 | Cave_Terrestrial |
| AMNH | 59782 | Eurycea lucifuga | 10.478 | 14.301 | 2.661 | 10.406 | 17.016 | 2.903 | 1.666 | 68.78 | 59.79 | 3.183 | Cave_Terrestrial |
| AMNH | 59803 | Eurycea lucifuga | 8.874 | 13.955 | 8.144 | 6.166 | 9.843 | 2.428 | 1.754 | 78.01 | 55.488 | 3.164 | Cave_Terrestrial |
| AMNH | 59804 | Eurycea lucifuga | 9.575 | 17.61 | 2.076 | 8.774 | 18.407 | 3.063 | 1.84 | 70.975 | 65.827 | 3.298 | Cave_Terrestrial |
| AMNH | 59805 | Eurycea lucifuga | 11.045 | 17.125 | 2.399 | 8.515 | 16.043 | 3.219 | 2.196 | 81.658 | 64.328 | 3.391 | Cave_Terrestrial |
| AMNH | 59806 | Eurycea lucifuga | 10.402 | 14.001 | 2.382 | 8.31 | 17.322 | 3.372 | 1.701 | 81.556 | 61.264 | 3.365 | Cave_Terrestrial |
| AMNH | 59807 | Eurycea lucifuga | 9.513 | 15.933 | 2.39 | 8.618 | 18.279 | 3.248 | 1.904 | 91.709 | 59.594 | 3.388 | Cave_Terrestrial |
| AMNH | 137469 | Eurycea lucifuga | 9.785 | 18.695 | 2.085 | 9.018 | 17.12 | 2.706 | 2.373 | 77.406 | 65.295 | 3.534 | Cave_Terrestrial |
| AMNH | 137470 | Eurycea lucifuga | 10.119 | 15.641 | 2.003 | 8.227 | 20.534 | 2.357 | 2.336 | 98.909 | 66.436 | 3.528 | Cave_Terrestrial |
| AMNH | 137471 | Eurycea lucifuga | 9.758 | 18.251 | 1.487 | 7.804 | 19.245 | 2.498 | 2.446 | 82.319 | 63.379 | 2.932 | Cave_Terrestrial |
| AMNH | 137472 | Eurycea lucifuga | 10.462 | 17.448 | 2.443 | 8.883 | 20.597 | 2.574 | 2.546 | 89.321 | 65.703 | 2.457 | Cave_Terrestrial |
| AMNH | 137473 | Eurycea lucifuga | 9.583 | 16.614 | 1.423 | 6.607 | 16.783 | 2.25 | 2.695 | 46.575 | 60.052 | 3.043 | Cave_Terrestrial |
| AMNH | 143530 | Eurycea lucifuga | 8.386 | 12.359 | 2.001 | 7.814 | 14.892 | 2.94 | 2.093 | 74.072 | 53.145 | 2.682 | Cave_Terrestrial |
| AMNH | 143532 | Eurycea lucifuga | 10.463 | 15.148 | 2.152 | 9.828 | 16.696 | 2.84 | 2.453 | 44.834 | 64.299 | 3.116 | Cave_Terrestrial |
| AMNH | 143533 | Eurycea lucifuga | 8.025 | 12.146 | 1.495 | 6.425 | 15.303 | 2.094 | 1.56 | 82.282 | 54.298 | 2.001 | Cave_Terrestrial |
| AMNH | 143534 | Eurycea lucifuga | 11.342 | 15.318 | 2.426 | 8.268 | 18.668 | 4.103 | 2.118 | 71.429 | 62.393 | 3.532 | Cave_Terrestrial |
| AMNH | 143535 | Eurycea lucifuga | 7.701 | 12.23 | 1.552 | 6.355 | 15.195 | 2.798 | 1.643 | 53.655 | 48.355 | 2.409 | Cave_Terrestrial |
| AMNH | 143536 | Eurycea lucifuga | 8.836 | 13.615 | 1.588 | 6.5 | 15.323 | 2.572 | 1.925 | 75.323 | 58.023 | 2.38 | Cave_Terrestrial |
| AMNH | 143537 | Eurycea lucifuga | 9.026 | 14.207 | 1.953 | 7.592 | 15.52 | 2.657 | 2.054 | 58.387 | 50.401 | 3.259 | Cave_Terrestrial |
| AMNH | 143539 | Eurycea lucifuga | 9.979 | 16.261 | 2.514 | 7.97 | 16.788 | 3.136 | 2.021 | 58.636 | 62.492 | 3.543 | Cave_Terrestrial |
| AMNH | 143540 | Eurycea lucifuga | 10.03 | 15.801 | 2.238 | 7.711 | 17.335 | 2.858 | 2.418 | 83.866 | 58.229 | 3.204 | Cave_Terrestrial |
| AMNH | 143541 | Eurycea lucifuga | 10.909 | 17.206 | 2.814 | 8.283 | 18.966 | 3.75 | 1.32 | 36.301 | 58.08 | 3.677 | Cave_Terrestrial |
| AMNH | 143542 | Eurycea lucifuga | 10.483 | 13.325 | 2.025 | 7.933 | 14.178 | 3.251 | 2.056 | 74.078 | 57.939 | 3.202 | Cave_Terrestrial |
| AMNH | 143543 | Eurycea lucifuga | 9.08 | 14.55 | 2.138 | 8.493 | 17.079 | 2.902 | 1.924 | 47.94 | 56.601 | 2.912 | Cave_Terrestrial |
| AMNH | 143545 | Eurycea lucifuga | 10.735 | 12.88 | 2.309 | 8.474 | 17.499 | 3.09 | 2.045 | 65.707 | 63.167 | 2.539 | Cave_Terrestrial |
| AMNH | 143547 | Eurycea lucifuga | 9.182 | 11.639 | 1.805 | 8.366 | 13.759 | 2.414 | 2.338 | 82.181 | 59.8 | 2.745 | Cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 143548 | Eurycea lucifuga | 10.002 | 16.141 | 2.095 | 8.379 | 17.307 | 3.759 | 1.434 | 93.631 | 59.452 | 1.926 | Cave_Terrestrial |
| AMNH | 143549 | Eurycea lucifuga | 9.882 | 14.817 | 2.166 | 7.482 | 14.943 | 3.223 | 2.063 | 44.798 | 62.504 | 2.75 | Cave_Terrestrial |
| AMNH | 143552 | Eurycea lucifuga | 8.214 | 12.744 | 1.876 | 8.514 | 13.741 | 2.63 | 2.515 | 81.115 | 57.078 | 2.737 | Cave_Terrestrial |
| AMNH | 143553 | Eurycea lucifuga | 6.574 | 14.214 | 1.95 | 7.743 | 13.677 | 1.891 | 1.902 | 81.083 | 57.566 | 4.087 | Cave_Terrestrial |
| AMNH | 143554 | Eurycea lucifuga | 8.041 | 12.743 | 1.787 | 6.697 | 17.589 | 2.362 | 2.464 | 73.88 | 50.818 | 2.927 | Cave_Terrestrial |
| AMNH | 143555 | Eurycea lucifuga | 7.85 | 12.4 | 1.957 | 6.718 | 14.624 | 2.399 | 1.626 | 59.124 | 50.298 | 2.468 | Cave_Terrestrial |
| AMNH | 143556 | Eurycea lucifuga | 6.589 | 9.574 | 0.978 | 5.36 | 13.938 | 2.043 | 1.609 | 71.062 | 49.159 | | Cave_Terrestrial |
| AMNH | 143557 | Eurycea lucifuga | 8.955 | 13.147 | 2.549 | 7.755 | 18.593 | 2.973 | 1.561 | 50.697 | 49.244 | 3.013 | Cave_Terrestrial |
| AMNH | 143558 | Eurycea lucifuga | 10.425 | 12.598 | 1.904 | 8.034 | 11.013 | 3.448 | 1.896 | 89.405 | 61.082 | 3.802 | Cave_Terrestrial |
| AMNH | 143559 | Eurycea lucifuga | 7.832 | 14.936 | 1.333 | 6.034 | 17.038 | 1.885 | 2.117 | 88.793 | 56.807 | 3.632 | Cave_Terrestrial |
| AMNH | 143560 | Eurycea lucifuga | 9.462 | 16.111 | 1.825 | 7.624 | 16.25 | 3.368 | 2.241 | 86.722 | 58.721 | 3.515 | Cave_Terrestrial |
| AMNH | 143561 | Eurycea lucifuga | 10.039 | 15.521 | 2.329 | 8.375 | 15.871 | 3.504 | 2.373 | 78.51 | 57.3 | 3.176 | Cave_Terrestrial |
| AMNH | 143562 | Eurycea lucifuga | 8.167 | 11.716 | 1.83 | 6.747 | 14.169 | 2.735 | 1.191 | 73.148 | 51.369 | 2.514 | Cave_Terrestrial |
| AMNH | 143563 | Eurycea lucifuga | 10.271 | 13.971 | 1.986 | 8.794 | 15.583 | 3.216 | 2.04 | 88.6 | 59.777 | 2.585 | Cave_Terrestrial |
| AMNH | 143564 | Eurycea lucifuga | 7.97 | 15.332 | 1.735 | 5.886 | 15.421 | 3.107 | 2.039 | 72.532 | 51.914 | 2.585 | Cave_Terrestrial |
| AMNH | 143565 | Eurycea lucifuga | 9.258 | 12.581 | 1.694 | 7.139 | 13.251 | 2.812 | 1.457 | 73.747 | 56.157 | 2.879 | Cave_Terrestrial |
| AMNH | 143566 | Eurycea lucifuga | 7.393 | 13.879 | 2.062 | 6.73 | 15.838 | 3.111 | 2.187 | 70.714 | 48.198 | 3.176 | Cave_Terrestrial |
| AMNH | 143567 | Eurycea lucifuga | 8.658 | 13.403 | 1.834 | 4.439 | 9.598 | 2.866 | 1.57 | 65.834 | 48.489 | 2.586 | Cave_Terrestrial |
| AMNH | 143568 | Eurycea lucifuga | 8.85 | 12.962 | 2.313 | 7.242 | 15.296 | 2.711 | 1.591 | 74.059 | 54.036 | 3.303 | Cave_Terrestrial |
| AMNH | 143569 | Eurycea lucifuga | 7.312 | 10.6 | 1.839 | 6.537 | 15.152 | 2.725 | 1.675 | 67.174 | 45.214 | 2.475 | Cave_Terrestrial |
| AMNH | 143570 | Eurycea lucifuga | 9.894 | 16.72 | 2.323 | 8.365 | 18.454 | 2.87 | 1.508 | 69.779 | 56.122 | 3.265 | Cave_Terrestrial |
| AMNH | 143573 | Eurycea lucifuga | 9.644 | 12.236 | 2.447 | 7.002 | 15.183 | 2.576 | 1.619 | 89.082 | 53.277 | 3.242 | Cave_Terrestrial |
| AMNH | 143574 | Eurycea lucifuga | 9.434 | 13.015 | 1.808 | 7.142 | 16.121 | 2.452 | 1.622 | 79.805 | 53.557 | 3.017 | Cave_Terrestrial |
| AMNH | 143575 | Eurycea lucifuga | 8.096 | 11.406 | 1.877 | 5.959 | 15.389 | 2.308 | 1.598 | 58.546 | 48.468 | 2.74 | Cave_Terrestrial |
| AMNH | 143576 | Eurycea lucifuga | 8.779 | 13.452 | 1.841 | 6.018 | 14.972 | 2.553 | 1.597 | 72.889 | 50.533 | 2.494 | Cave_Terrestrial |
| AMNH | 143577 | Eurycea lucifuga | 9.033 | 13.316 | 2.07 | 7.64 | 17.159 | 2.796 | 1.298 | 60.537 | 53.441 | 2.796 | Cave_Terrestrial |
| AMNH | 143578 | Eurycea lucifuga | 10.283 | 15.83 | 2.37 | 8.68 | 16.283 | 3.241 | 1.82 | 77.822 | 61.63 | 2.45 | Cave_Terrestrial |
| AMNH | 143579 | Eurycea lucifuga | 10.546 | 16.943 | 2.637 | 9.462 | 18.707 | 3.033 | 1.82 | 99.526 | 59.269 | 2.626 | Cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 143580 | Eurycea lucifuga | 9.653 | 14.189 | 2.398 | 7.195 | 16.168 | 3.049 | 1.974 | 87.148 | 61.679 | 3.115 | Cave_Terrestrial |
| AMNH | 143582 | Eurycea lucifuga | 7.807 | 12.654 | 1.768 | 6.244 | 13.902 | 2.221 | 1.583 | 51.365 | 46.775 | 2.315 | Cave_Terrestrial |
| AMNH | 143583 | Eurycea lucifuga | 9.582 | 16.395 | 2.178 | 7.391 | 15.697 | 2.884 | 2.249 | 21.468 | 55.945 | 3.617 | Cave_Terrestrial |
| AMNH | 143584 | Eurycea lucifuga | 8.378 | 15.014 | 1.727 | 7.195 | 14.738 | 2.334 | 1.837 | 72.467 | 50.98 | 3.622 | Cave_Terrestrial |
| AMNH | 143585 | Eurycea lucifuga | 7.745 | 12.745 | 1.65 | 5.28 | 14.466 | 1.788 | 1.626 | 32.056 | 49.879 | 2.783 | Cave_Terrestrial |
| AMNH | 143588 | Eurycea lucifuga | 6.928 | 11.422 | 1.187 | 5.359 | 11.242 | 1.835 | 1.355 | 26.582 | 43.049 | 2.476 | Cave_Terrestrial |
| AMNH | 143589 | Eurycea lucifuga | 10.32 | 16.165 | 2.447 | 7.715 | 18.073 | 3.563 | 1.948 | 47.928 | 60.009 | 3.936 | Cave_Terrestrial |
| AMNH | 155760 | Eurycea lucifuga | 10.837 | 14.761 | 2.78 | 8.583 | 18.214 | 3.438 | 1.775 | 100.342 | 65.176 | 2.822 | Cave_Terrestrial |
| AMNH | 155761 | Eurycea lucifuga | 9.833 | 16.186 | 2.138 | 9.522 | 17.33 | 2.72 | 1.864 | 88.877 | 60.004 | 2.982 | Cave_Terrestrial |
| AMNH | 182172 | Eurycea lucifuga | 8.97 | 15.588 | 2.196 | 8.599 | 16.512 | 2.84 | 2.283 | 75.127 | 55.547 | 3.08 | Cave_Terrestrial |
| AMNH | 182173 | Eurycea lucifuga | 7.836 | 9.328 | 1.842 | 8.731 | 15.839 | 2.919 | 2.743 | 80.571 | 55.312 | 2.543 | Cave_Terrestrial |
| AMNH | 187787 | Eurycea lucifuga | 8.655 | 12.518 | 1.617 | 7.666 | 13.602 | 2.062 | 2.5222 | 71.617 | 54.139 | 1.489 | Cave_Terrestrial |
| AMNH | 187788 | Eurycea lucifuga | 10.953 | 14.363 | 2.188 | 9.298 | 15.391 | 3.256 | 2.166 | 71.541 | 66.2 | 2.668 | Cave_Terrestrial |
| AMNH | 187789 | Eurycea lucifuga | 8.032 | 13.29 | 1.665 | 6.528 | 15.495 | 2.684 | 1.854 | 52.427 | 51.987 | 3.321 | Cave_Terrestrial |
| AMNH | 11865 | Eurycea multiplicata | 4.354 | 6.491 | 1.02 | 5.009 | 6.727 | 1.092 | 0.516 | 29.727 | 45.457 | 1.232 | Non-cave_Aquatic |
| AMNH | 32100 | Eurycea multiplicata | 4.78 | 6.747 | 1.143 | 4.495 | 7.688 | 0.936 | 0.679 | 22.446 | 33.738 | 1.456 | Non-cave_Aquatic |
| AMNH | 40358 | Eurycea multiplicata | 8.954 | 11.331 | 2.207 | 10.124 | 14.946 | 3.071 | 1.713 | 87.019 | 55.592 | 2.959 | Non-cave_Aquatic |
| AMNH | 40360 | Eurycea multiplicata | 5.039 | 5.629 | 0.902 | 3.741 | 7.338 | 1.49 | 0.567 | 37.834 | 39.348 | 1.161 | Non-cave_Aquatic |
| AMNH | 40361 | Eurycea multiplicata | 5.32 | 6.945 | 1.294 | 5.286 | 9.274 | 1.653 | 0.867 | 32.81 | 48.767 | 1.241 | Non-cave_Aquatic |
| AMNH | 40362 | Eurycea multiplicata | 5.476 | 6.584 | 0.949 | 4.613 | 8.236 | 1.358 | 0.701 | 23.828 | 42.503 | 1.455 | Non-cave_Aquatic |
| AMNH | 40363 | Eurycea multiplicata | 5.174 | 5.024 | 1.277 | 5.595 | 7.978 | 1.582 | 0.787 | 38.617 | 39.142 | 1.071 | Non-cave_Aquatic |
| AMNH | 52461 | Eurycea multiplicata | 4.014 | 4.943 | 0.778 | 4.127 | 6.104 | 1.146 | 0.919 | 38.646 | 34.283 | 0.836 | Non-cave_Aquatic |
| AMNH | 52462 | Eurycea multiplicata | 4.251 | 5.217 | 0.928 | 4.836 | 5.651 | 1.083 | 0.845 | 25.241 | 29.515 | 1.35 | Non-cave_Aquatic |
| AMNH | 59813 | Eurycea multiplicata | 5.724 | 7.197 | 1.209 | 6.445 | 8.046 | 1.595 | 1.533 | 43.672 | 42.993 | 1.539 | Non-cave_Aquatic |
| AMNH | 143651 | Eurycea multiplicata | 6.275 | 7.502 | 1.252 | 5.899 | 9.353 | 1.604 | 1.014 | 36.441 | 43.965 | 1.583 | Non-cave_Aquatic |
| AMNH | 151182 | Eurycea multiplicata | 6.087 | 4.948 | 1.119 | 5.605 | 6.609 | 1.538 | 0.724 | 31.773 | 35.056 | 1.399 | Non-cave_Aquatic |
| AMNH | 151191 | Eurycea multiplicata | 4.402 | 4.435 | 0.657 | 4.537 | 5.161 | 0.875 | 0.692 | 20.003 | 28.072 | 1.106 | Non-cave_Aquatic |
| AMNH | 151197 | Eurycea multiplicata | 4.612 | 3.678 | 0.78 | 3.555 | 4.343 | 0.714 | 0.512 | 18.615 | 24.05 | 0.859 | Non-cave_Aquatic |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 151199 | Eurycea multiplicata | 4.553 | 6.243 | 1.169 | 4.155 | 6.226 | 1.143 | 0.563 | 29.565 | 37.328 | 1.441 | Non-cave_Aquatic |
| AMNH | 151200 | Eurycea multiplicata | 5.549 | 7.118 | 1.153 | 5.155 | 7.411 | 1.408 | 0.709 | 43.643 | 35.579 | 1.514 | Non-cave_Aquatic |
| AMNH | 151201 | Eurycea multiplicata | 4.659 | 5.967 | 1.149 | 3.974 | 7.376 | 1.286 | 0.677 | 32.258 | 33.998 | 1.324 | Non-cave_Aquatic |
| AMNH | 151202 | Eurycea multiplicata | 4.561 | 5.988 | 0.73 | 3.641 | 6.972 | 1.056 | 0.856 | 19.928 | 25.097 | 1.28 | Non-cave_Aquatic |
| AMNH | 182174 | Eurycea multiplicata | 5.815 | 6.828 | 1.34 | 6.09 | 7.238 | 1.456 | 0.657 | 42.875 | 41.026 | 1.826 | Non-cave_Aquatic |
| AMNH | 182175 | Eurycea multiplicata | 5.159 | 5.288 | 1.118 | 5.799 | 5.862 | 1.594 | 0.868 | 40.177 | 39.775 | 1.577 | Non-cave_Aquatic |
| AMNH | 60789 | Eurycea nana | 2.986 | 3.264 | 0.48 | 2.347 | 5.164 | 0.821 | 0.596 | 19.642 | 27.167 | 1.001 | Non-cave_Aquatic |
| AMNH | 108768 | Eurycea nana | 3.882 | 5.843 | 0.601 | 4.271 | 5.877 | 0.902 | 0.664 | 19.53 | 29.33 | 1.143 | Non-cave_Aquatic |
| AMNH | 108769 | Eurycea nana | 3.368 | 3.89 | 0.594 | 3.537 | 3.359 | 0.629 | 0.52 | 20.229 | 24.692 | 0.991 | Non-cave_Aquatic |
| AMNH | 108771 | Eurycea nana | 3.638 | 4.533 | 0.602 | 2.824 | 4.949 | 0.613 | 0.424 | 20.188 | 22.547 | 0.707 | Non-cave_Aquatic |
| AMNH | 108772 | Eurycea nana | 3.417 | 5.002 | 0.516 | 2.73 | 4.618 | 0.626 | 0.34 | 20.988 | 27.376 | 1.199 | Non-cave_Aquatic |
| AMNH | 108773 | Eurycea nana | 3.723 | 5.191 | 0.381 | 2.287 | 6.105 | 0.558 | 0.774 | 24.302 | 28.077 | 1.419 | Non-cave_Aquatic |
| AMNH | 108774 | Eurycea nana | 3.613 | 4.718 | 0.586 | 3.562 | 4.78 | 0.72 | 0.809 | 20.604 | 25.641 | 0.999 | Non-cave_Aquatic |
| AMNH | 108776 | Eurycea nana | 3.617 | 4.129 | 0.503 | 2.773 | 4.515 | 0.803 | 0.566 | 17.902 | 22.201 | 0.951 | Non-cave_Aquatic |
| AMNH | 108778 | Eurycea nana | 3.122 | 3.718 | 0.376 | 2.476 | 4.01 | 0.493 | 0.438 | 18.686 | 25.354 | 1.14 | Non-cave_Aquatic |
| AMNH | 108779 | Eurycea nana | 3.235 | 3.815 | 0.467 | 2.206 | 4.426 | 0.59 | 0.571 | 15.101 | 19.991 | 0.908 | Non-cave_Aquatic |
| AMNH | 182176 | Eurycea nana | 3.32 | 3.12 | 0.489 | 3.161 | 3.91 | 0.64 | 0.35 | 23.853 | 31.601 | 1.003 | Non-cave_Aquatic |
| AMNH | 182177 | Eurycea nana | 2.971 | 2.73 | 0.336 | 1.935 | 4.549 | 0.397 | 0.401 | 14.66 | 24.445 | 0.813 | Non-cave_Aquatic |
| AMNH | 182178 | Eurycea nana | 2.976 | 2.653 | 0.35 | 2.36 | 3.242 | 0.357 | 0.306 | 16.872 | 23.01 | 0.814 | Non-cave_Aquatic |
| AMNH | 182179 | Eurycea nana | 3.015 | 3.206 | 0.327 | 2.273 | 3.566 | 0.495 | 0.347 | 18.317 | 23.455 | 0.727 | Non-cave_Aquatic |
| NMNH | 545578 | Eurycea nana | 3.942 | 4.365 | 0.671 | 4.111 | 4.805 | 0.663 | 1.18 | 24.67 | 25.87 | 0.901 | Non-cave_Aquatic |
| NMNH | 545579 | Eurycea nana | 3.85 | 4.328 | 0.557 | 3.774 | 4.751 | 0.593 | 0.658 | 23.052 | 29.037 | 0.739 | Non-cave_Aquatic |
| NMNH | 545581 | Eurycea nana | 3.459 | 3.624 | 0.428 | 2.733 | 3.445 | 0.613 | 0.883 | 16.847 | 21.704 | 0.902 | Non-cave_Aquatic |
| NMNH | 545584 | Eurycea nana | 3.849 | 4.198 | 0.726 | 3.413 | 4.944 | 0.763 | 0.859 | 24.114 | 24.876 | 0.966 | Non-cave_Aquatic |
| NMNH | 545585 | Eurycea nana | 3.717 | 4.445 | 0.561 | 2.654 | 4.42 | 0.616 | 1.063 | 16.24 | 25.344 | 1.033 | Non-cave_Aquatic |
| NMNH | 545587 | Eurycea nana | 3.526 | 3.725 | 0.566 | 2.78 | 3.862 | 0.693 | 0.754 | 17.544 | 21.285 | 0.64 | Non-cave_Aquatic |
| NMNH | 545588 | Eurycea nana | 3.455 | 3.387 | 0.408 | 3.009 | 3.648 | 0.458 | 0.55 | 17.417 | 19.928 | 0.834 | Non-cave_Aquatic |
| NMNH | 545589 | Eurycea nana | 3.343 | 3.643 | 0.408 | 3.31 | 4.756 | 0.479 | 0.822 | 19.483 | 19.707 | 0.701 | Non-cave_Aquatic |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 62054 | Eurycea neotenes | 4.413 | 6.355 | 0.791 | 3.876 | 7.014 | 0.89 | 0.805 | 27.319 | 31.503 | 0.901 | Non-cave_Aquatic |
| AMNH | 62057 | Eurycea neotenes | 4.267 | 5.273 | 0.855 | 3.45 | 5.815 | 0.9 | 0.478 | 23.643 | 32.456 | 1.126 | Non-cave_Aquatic |
| AMNH | 62058 | Eurycea neotenes | 3.797 | 4.597 | 0.87 | 3.011 | 5.412 | 0.895 | 0.698 | 16.88 | 23.318 | 1.232 | Non-cave_Aquatic |
| AMNH | 188124 | Eurycea neotenes | 4.441 | 5.759 | 0.959 | 5.434 | 6.721 | 0.994 | 0.618 | 31.609 | 35.816 | 1.138 | Non-cave_Aquatic |
| AMNH | 188126 | Eurycea neotenes | 4.474 | 4.929 | 0.729 | 3.432 | 5.57 | 0.808 | 0.642 | 22.39 | 27.117 | 0.849 | Non-cave_Aquatic |
| AMNH | 188127 | Eurycea neotenes | 5.318 | 6.163 | 0.934 | 4.463 | 6.899 | 1.032 | 0.675 | 26.234 | 36.746 | 1.173 | Non-cave_Aquatic |
| AMNH | 188128 | Eurycea neotenes | 4.612 | 5.763 | 0.656 | 3.941 | 5.792 | 1.034 | 0.536 | 26.781 | 31.19 | 1.026 | Non-cave_Aquatic |
| AMNH | 188129 | Eurycea neotenes | 4.174 | 5.522 | 0.627 | 4.139 | 6.166 | 0.696 | 0.581 | 29.217 | 32.044 | 1.028 | Non-cave_Aquatic |
| AMNH | 188130 | Eurycea neotenes | 4.792 | 5.857 | 1.021 | 4.846 | 4.551 | 1.163 | 0.809 | 24.549 | 31.537 | 1.226 | Non-cave_Aquatic |
| AMNH | 188133 | Eurycea neotenes | 4.751 | 5.524 | 0.699 | 4.385 | 6.061 | 1.081 | 0.508 | 28.723 | 34.204 | 0.727 | Non-cave_Aquatic |
| AMNH | 188134 | Eurycea neotenes | 3.666 | 3.985 | 0.45 | 3.959 | 5.51 | 0.956 | 0.628 | 18.375 | 26.718 | 0.65 | Non-cave_Aquatic |
| AMNH | 188137 | Eurycea neotenes | 4.316 | 5.227 | 0.861 | 4.166 | 5.205 | 0.65 | 0.775 | 24.528 | 33.44 | 0.795 | Non-cave_Aquatic |
| AMNH | 188138 | Eurycea neotenes | 4.296 | 4.019 | 0.598 | 3.799 | 3.672 | 0.637 | 0.549 | 20.881 | 26.094 | 0.846 | Non-cave_Aquatic |
| AMNH | 188139 | Eurycea neotenes | 5.276 | 5.353 | 0.834 | 4.311 | 6.129 | 0.933 | 0.462 | 25.186 | 32.159 | 1.065 | Non-cave_Aquatic |
| AMNH | 188145 | Eurycea neotenes | 5.43 | 5.677 | 0.674 | 4.332 | 5.998 | 0.774 | 0.504 | 25.324 | 35.402 | 0.785 | Non-cave_Aquatic |
| AMNH | 188150 | Eurycea neotenes | 4.429 | 3.347 | 0.664 | 4.815 | 4.675 | 0.9 | 0.47 | 24.182 | 29.331 | 0.827 | Non-cave_Aquatic |
| AMNH | 188151 | Eurycea neotenes | 4.714 | 4.733 | 0.752 | 4.325 | 5.967 | 0.773 | 0.49 | 23.555 | 29.318 | 0.496 | Non-cave_Aquatic |
| AMNH | 188172 | Eurycea neotenes | 3.738 | 3.956 | 0.569 | 3.424 | 4.509 | 0.842 | 0.345 | 20.232 | 25.652 | 0.713 | Non-cave_Aquatic |
| AMNH | 188198 | Eurycea neotenes | 4.991 | 5.099 | 0.961 | 6.135 | 7.017 | 0.866 | 0.954 | 25.301 | 36.295 | 1.09 | Non-cave_Aquatic |
| AMNH | 188199 | Eurycea neotenes | 5.853 | 6.264 | 0.993 | 5.131 | 6.411 | 1.2 | 0.996 | 26.958 | 35.955 | 1.067 | Non-cave_Aquatic |
| AMNH | 188200 | Eurycea pterophila | 5.911 | 5.176 | 1.194 | 5.35 | 4.985 | 1.358 | 0.782 | 28.249 | 34.534 | 0.967 | Cave_Aquatic |
| AMNH | 44336 | Eurycea quadridigitata | 4.828 | 5.844 | 0.803 | 5.204 | 7.532 | 1.128 | 0.926 | 51.089 | 35.927 | 1.381 | Non-cave_Terrestrial |
| AMNH | 44337 | Eurycea quadridigitata | 3.627 | 4.051 | 0.963 | 4.226 | 6.384 | 1.167 | 0.5 | 29.046 | 29.681 | 0.775 | Non-cave_Terrestrial |
| AMNH | 53905 | Eurycea quadridigitata | 3.848 | 5.036 | 0.55 | 3.78 | 6.018 | 1.036 | 0.603 | 46.393 | 30.356 | 1.053 | Non-cave_Terrestrial |
| AMNH | 72593 | Eurycea quadridigitata | 3.817 | 5.975 | 0.745 | 4.101 | 7.549 | 1.105 | 0.826 | 36.709 | 28.646 | 1.465 | Non-cave_Terrestrial |
| AMNH | 89818 | Eurycea quadridigitata | 3.421 | 4.879 | 0.533 | 2.91 | 6.565 | 0.807 | 0.881 | 31.827 | 26.088 | 1.075 | Non-cave_Terrestrial |
| AMNH | 89821 | Eurycea quadridigitata | 4.334 | 5.826 | 0.968 | 5.19 | 6.495 | 1.246 | 0.772 | 48.685 | 33.625 | 1.557 | Non-cave_Terrestrial |
| AMNH | 89822 | Eurycea quadridigitata | 4.217 | 6.459 | 0.866 | 4.352 | 7.419 | 1.149 | 0.925 | 53.357 | 34.102 | 1.47 | Non-cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 89828 | Eurycea quadridigitata | 4.04 | 5.009 | 0.882 | 4.944 | 6.397 | 1.151 | 0.806 | 41.638 | 29.982 | 1.161 | Non-cave_Terrestrial |
| AMNH | 89834 | Eurycea quadridigitata | 3.985 | 5.4 | 0.698 | 4.061 | 7.704 | 1.092 | 0.848 | 47.968 | 32.708 | 1.444 | Non-cave_Terrestrial |
| AMNH | 89835 | Eurycea quadridigitata | 3.24 | 4.664 | 0.735 | 3.839 | 5.792 | 1.086 | 0.833 | 36.601 | 28.894 | 1.172 | Non-cave_Terrestrial |
| AMNH | 93055 | Eurycea quadridigitata | 4.078 | 3.639 | 0.98 | 3.969 | 6.284 | 1.13 | 0.555 | 37.025 | 27.249 | 1.118 | Non-cave_Terrestrial |
| AMNH | 93058 | Eurycea quadridigitata | 3.697 | 3.735 | 0.859 | 3.466 | 5.011 | 0.847 | 0.33 | 31.966 | 26.554 | 1.556 | Non-cave_Terrestrial |
| AMNH | 125819 | Eurycea quadridigitata | 4.986 | 5.829 | 0.911 | 5.222 | 6.839 | 1.152 | 0.799 | 49.504 | 30.402 | 1.188 | Non-cave_Terrestrial |
| AMNH | 143803 | Eurycea quadridigitata | 4.119 | 5.658 | 0.944 | 3.864 | 6.729 | 1.314 | 0.778 | 40.532 | 28.052 | 1.388 | Non-cave_Terrestrial |
| AMNH | 172401 | Eurycea quadridigitata | 4.296 | 6.037 | 0.928 | 4.229 | 7.842 | 1.166 | 0.909 | 45.762 | 35.188 | 1.385 | Non-cave_Terrestrial |
| AMNH | 172404 | Eurycea quadridigitata | 4.418 | 6.987 | 0.85 | 4.625 | 7.9 | 1.13 | 1.199 | 39.295 | 37.502 | 1.58 | Non-cave_Terrestrial |
| AMNH | 182183 | Eurycea quadridigitata | 3.76 | 4.107 | 1.03 | 4.726 | 6.88 | 1.159 | 0.785 | 40.634 | 31.121 | 1.779 | Non-cave_Terrestrial |
| AMNH | 188213 | Eurycea quadridigitata | 4.443 | 6.428 | 0.945 | 4.113 | 6.708 | 1.218 | 0.926 | 41.036 | 28.112 | 1.213 | Non-cave_Terrestrial |
| AMNH | 188214 | Eurycea quadridigitata | 4.275 | 5.769 | 0.923 | 3.828 | 6.947 | 1.007 | 0.861 | 41.612 | 29.177 | 1.498 | Non-cave_Terrestrial |
| AMNH | 188215 | Eurycea quadridigitata | 4.029 | 5.912 | 0.771 | 4.516 | 7.007 | 0.987 | 1.152 | 40.07 | 27.14 | 1.307 | Non-cave_Terrestrial |
| AMNH | 188216 | Eurycea quadridigitata | 4.889 | 6.481 | 1.113 | 5.582 | 7.747 | 1.231 | 0.704 | 44.672 | 34.063 | 1.325 | Non-cave_Terrestrial |
| AMNH | 2276 | Eurycea rathbuni | 15.178 | 16.763 | 1.585 | 8.817 | 15.741 | 2.083 | 1.228 | 41.758 | 60.874 | 0.788 | Cave_Aquatic |
| AMNH | 2279 | Eurycea rathbuni | 12.573 | 14.845 | 1.436 | 8.167 | 17.606 | 2.811 | 0.998 | 33.12 | 66.526 | 0.503 | Cave_Aquatic |
| AMNH | 2281 | Eurycea rathbuni | 13.107 | 13.681 | 1.613 | 7.797 | 14.035 | 1.943 | 0.73 | 25.253 | 61.258 | 0.694 | Cave_Aquatic |
| AMNH | 2282 | Eurycea rathbuni | 10.741 | 13.084 | 1.253 | 6.995 | 12.778 | 1.444 | 0.852 | 30.563 | 47.601 | 0.337 | Cave_Aquatic |
| AMNH | 2285 | Eurycea rathbuni | 13.017 | 9.768 | 1.288 | 7.297 | 15.52 | 2.5 | 0.755 | 32.738 | 52.65 | 0.77 | Cave_Aquatic |
| AMNH | 2288 | Eurycea rathbuni | 9.153 | 10.799 | 1.359 | 6.135 | 8.997 | 1.112 | 1.684 | 23.054 | 39.645 | 0.409 | Cave_Aquatic |
| AMNH | 22645 | Eurycea rathbuni | 7.242 | 10.727 | 1.083 | 5.569 | 11.492 | 1.169 | 1.576 | 26.419 | 38.502 | 0.528 | Cave_Aquatic |
| AMNH | 22646 | Eurycea rathbuni | 8.963 | 14.441 | 1.592 | 7.132 | 14.458 | 1.799 | 1.591 | 35.454 | 46.041 | 0.419 | Cave_Aquatic |
| AMNH | 22647 | Eurycea rathbuni | 10.968 | 18.098 | 1.271 | 9.007 | 12.671 | 1.28 | 0.661 | 38.891 | 43.941 | 0.619 | Cave_Aquatic |
| AMNH | 51178 | Eurycea rathbuni | 10.868 | 17.498 | 1.548 | 8.488 | 16.589 | 1.427 | 0.944 | 39.659 | 47.826 | | Cave_Aquatic |
| AMNH | 58632 | Eurycea rathbuni | 9.435 | 11.762 | 0.6 | 6.466 | 14.029 | 0.654 | 1.396 | 19.612 | 38.21 | 0.257 | Cave_Aquatic |
| AMNH | 58633 | Eurycea rathbuni | 7.66 | 12.89 | 0.786 | 5.515 | 15.126 | 1.122 | 0.928 | 30.47 | 39.804 | 0.464 | Cave_Aquatic |
| AMNH | 58634 | Eurycea rathbuni | 9.131 | 13.541 | 0.92 | 5.852 | 13.763 | 0.637 | 1.014 | 29.91 | 35.948 | 0.344 | Cave_Aquatic |
| AMNH | 62119 | Eurycea rathbuni | 8.709 | 12.498 | 1.096 | 5.096 | 16.424 | 1.462 | 0.724 | 33.19 | 46.13 | 0.221 | Cave_Aquatic |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 155730 | Eurycea rathbuni | 10.664 | 18.214 | 1.385 | 9.056 | 18.058 | 1.368 | 0.944 | 40.94 | 41.93 | | Cave_Aquatic |
| NMNH | 37051 | Eurycea spelaea | 9.574 | 12.189 | 2.591 | 9.017 | 16.097 | 3.098 | 1.72 | 52.605 | 61.962 | | Cave_Terrestrial |
| NMNH | 37052 | Eurycea spelaea | 9.625 | 10.293 | 2.116 | 8.231 | 9.96 | 2.682 | 1.687 | 50.168 | 56.719 | | Cave_Terrestrial |
| NMNH | 37053 | Eurycea spelaea | 6.453 | 6.154 | 1.587 | 7.301 | 8.96 | 1.541 | 1.02 | 32.217 | 37.626 | 0.951 | Cave_Terrestrial |
| NMNH | 38787 | Eurycea spelaea | 9.179 | 10.222 | 2.434 | 10.263 | 11.7 | 2.648 | 1.74 | 52.528 | 55.343 | 1.729 | Cave_Terrestrial |
| NMNH | 38788 | Eurycea spelaea | 9.66 | 9.351 | 2.696 | 9.217 | 15.377 | 2.141 | 1.861 | 62.835 | 59.135 | 1.83 | Cave_Terrestrial |
| NMNH | 38789 | Eurycea spelaea | 7.831 | 10.05 | 1.966 | 8.129 | 9.351 | 2.558 | 1.413 | 58.32 | 47.7 | 1.474 | Cave_Terrestrial |
| NMNH | 54326 | Eurycea spelaea | 9.686 | 12.845 | 2.832 | 9.181 | 11.203 | 3.326 | 2.12 | 50.602 | 61.288 | 1.359 | Cave_Terrestrial |
| NMNH | 57327 | Eurycea spelaea | 8.797 | 11.938 | 2.456 | 9.19 | 12.274 | 3.03 | 1.83 | 56.315 | 61.181 | 1.082 | Cave_Terrestrial |
| NMNH | 57332 | Eurycea spelaea | 7.277 | 7.659 | 1.93 | 6.726 | 9.963 | 2.455 | 1.31 | 47.23 | 50.025 | 0.821 | Cave_Terrestrial |
| NMNH | 153780 | Eurycea tridentifera | 7.174 | 10.33 | 0.798 | 4.793 | 10.786 | 1.067 | 1.797 | 23.801 | 22.868 | 0.453 | Cave_Aquatic |
| NMNH | 153781 | Eurycea tridentifera | 6.16 | 6.947 | 0.54 | 3.555 | 7.417 | 0.904 | 1.022 | 27.779 | 29.677 | 0.602 | Cave_Aquatic |
| NMNH | 153782 | Eurycea tridentifera | 5.818 | 6.725 | 0.476 | 3.271 | 6.128 | 0.846 | 0.861 | 19.396 | 23.487 | 0.598 | Cave_Aquatic |
| NMNH | 153783 | Eurycea tridentifera | 4.693 | 5.917 | 0.558 | 2.983 | 7.277 | 0.899 | 0.933 | 21.884 | 22.235 | 1.232 | Cave_Aquatic |
| NMNH | 153784 | Eurycea tridentifera | 4.405 | 6.261 | 0.511 | 2.918 | 6.582 | 0.603 | 1.1 | 18.524 | 21.521 | 0.388 | Cave_Aquatic |
| NMNH | 153785 | Eurycea tridentifera | 4.212 | 5.359 | 0.482 | 2.373 | 6.397 | 0.495 | 0.672 | 18.813 | 18.997 | 0.43 | Cave_Aquatic |
| AMNH | 62060 | Eurycea tynerensis | 3.272 | 3.587 | 0.579 | 2.901 | 4.211 | 0.858 | 0.384 | 24.989 | 29.724 | 0.842 | Non-cave_Aquatic |
| AMNH | 62061 | Eurycea tynerensis | 3.972 | 5.091 | 0.717 | 3.054 | 5.646 | 0.708 | 0.745 | 26.345 | 28.529 | 0.892 | Non-cave_Aquatic |
| AMNH | 182184 | Eurycea tynerensis | 3.194 | 3.518 | 0.576 | 3.44 | 4.149 | 0.508 | 0.323 | 28.292 | 29.615 | 1.073 | Non-cave_Aquatic |
| AMNH | 80090 | Eurycea wallacei | 3.289 | 4.603 | 0.386 | 1.848 | 5.433 | 0.394 | 0.449 | 13.785 | 19.953 | 0.135 | Cave_Aquatic |
| AMNH | 80091 | Eurycea wallacei | 4.449 | 6.423 | 0.506 | 2.566 | 6.109 | 0.647 | 0.418 | 17.967 | 23.595 | 0.263 | Cave_Aquatic |
| AMNH | 172387 | Eurycea wallacei | 3.649 | 4.355 | 0.588 | 2.55 | 4.558 | 0.979 | 0.415 | 13.494 | 18.467 | 0.201 | Cave_Aquatic |
| AMNH | 182197 | Eurycea wallacei | 3.494 | 5.107 | 0.396 | 2.437 | 4.743 | 0.521 | 0.635 | 13.077 | 17.619 | 0.415 | Cave_Aquatic |
| AMNH | 99654 | Eurycea wilderae | 6.685 | 8.435 | 1.52 | 5.64 | 9.61 | 1.811 | 1.225 | 51.368 | 39.189 | 1.656 | Non-cave_Terrestrial |
| AMNH | 99655 | Eurycea wilderae | 6.076 | 8.215 | 1.276 | 5.57 | 9.741 | 1.645 | 1.26 | 53.578 | 40.35 | 1.84 | Non-cave_Terrestrial |
| AMNH | 99657 | Eurycea wilderae | 6.238 | 7.245 | 1.514 | 5.413 | 7.949 | 1.674 | 1.747 | 42.287 | 36.512 | 1.832 | Non-cave_Terrestrial |
| AMNH | 99658 | Eurycea wilderae | 5.77 | 7.604 | 1.446 | 4.929 | 8.422 | 1.876 | 1.226 | 50.049 | 38.119 | 1.681 | Non-cave_Terrestrial |
| AMNH | 99659 | Eurycea wilderae | 6.674 | 7.939 | 1.486 | 6.059 | 9.32 | 2.046 | 0.986 | 55.488 | 44.332 | 1.889 | Non-cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 99660 | Eurycea wilderae | 5.948 | 8.373 | 1.423 | 5.155 | 10.014 | 1.602 | 1.269 | 53.004 | 40.795 | 1.831 | Non-cave_Terrestrial |
| AMNH | 115827 | Eurycea wilderae | 4.737 | 6.677 | 1.064 | 5.266 | 10.054 | 1.49 | 1.282 | 50.934 | 37.154 | 1.28 | Non-cave_Terrestrial |
| AMNH | 115829 | Eurycea wilderae | 5.153 | 7.902 | 1.122 | 5.379 | 10.438 | 1.456 | 1.369 | 58.034 | 39.118 | 1.258 | Non-cave_Terrestrial |
| AMNH | 115830 | Eurycea wilderae | 5.653 | 8.4 | 1.168 | 6.114 | 11.581 | 1.726 | 1.581 | 65.387 | 42.22 | 1.501 | Non-cave_Terrestrial |
| AMNH | 115833 | Eurycea wilderae | 6.095 | 7.743 | 1.26 | 6.179 | 10.168 | 1.925 | 1.335 | 58.364 | 42.062 | 1.428 | Non-cave_Terrestrial |
| AMNH | 115834 | Eurycea wilderae | 5.487 | 8.646 | 1.318 | 5.864 | 10.609 | 1.698 | 1.224 | 56.287 | 39.632 | 1.634 | Non-cave_Terrestrial |
| AMNH | 115835 | Eurycea wilderae | 5.054 | 8.263 | 1.179 | 5.253 | 10.484 | 1.56 | 1.329 | 55.29 | 36.557 | 1.301 | Non-cave_Terrestrial |
| AMNH | 115836 | Eurycea wilderae | 4.917 | 6.858 | 1.296 | 4.718 | 10.181 | 1.461 | 1.131 | 50.933 | 35.509 | 1.326 | Non-cave_Terrestrial |
| AMNH | 127036 | Eurycea wilderae | 4.34 | 6.101 | 0.957 | 4.199 | 6.196 | 1.233 | 0.845 | 38.961 | 32.577 | 1.283 | Non-cave_Terrestrial |
| AMNH | 127039 | Eurycea wilderae | 4.167 | 5.667 | 0.895 | 4.281 | 7.187 | 1.283 | 0.885 | 38.919 | 32.419 | 1.264 | Non-cave_Terrestrial |
| AMNH | 155790 | Eurycea wilderae | 4.81 | 7.12 | 1.019 | 4.852 | 9.405 | 1.196 | 0.863 | 30.622 | 32.061 | 1.218 | Non-cave_Terrestrial |
| AMNH | 155791 | Eurycea wilderae | 4.677 | 7.344 | 0.95 | 4.502 | 8.563 | 1.416 | 1.084 | 37.525 | 43.436 | 1.568 | Non-cave_Terrestrial |
| AMNH | 171738 | Eurycea wilderae | 5.59 | 7.081 | 1.242 | 5.084 | 8.506 | 1.307 | 1.323 | 26.146 | 44.497 | 1.663 | Non-cave_Terrestrial |
| AMNH | 171739 | Eurycea wilderae | 4.971 | 5.148 | 1.103 | 4.63 | 6.714 | 1.226 | 0.564 | 38.34 | 37.97 | 1.324 | Non-cave_Terrestrial |
| AMNH | 172392 | Eurycea wilderae | 5.701 | 9.065 | 1.164 | 6.381 | 8.876 | 1.437 | 1.237 | 33.108 | 39.246 | 1.772 | Non-cave_Terrestrial |
| AMNH | 95353 | Gyrinophilus porphyriticus | 9.207 | 8.174 | 2.01 | 10.051 | 8.367 | 2.447 | 1.54 | 40.395 | 55.099 | 1.274 | Cave_Terrestrial |
| AMNH | 137525 | Gyrinophilus porphyriticus | 13.079 | 15.153 | 3.378 | 12.838 | 16.849 | 3.367 | 1.609 | 41.484 | 93.658 | 3.669 | Cave_Terrestrial |
| AMNH | 137533 | Gyrinophilus porphyriticus | 10.641 | 9.615 | 2.438 | 9.989 | 10.624 | 2.641 | 1.263 | 31 | 62.48 | 2.006 | Cave_Terrestrial |
| AMNH | 137538 | Gyrinophilus porphyriticus | 12.043 | 9.944 | 2.701 | 11.31 | 14.977 | 3.082 | 1.758 | 42.143 | 70.606 | 2.928 | Cave_Terrestrial |
| AMNH | 137590 | Gyrinophilus porphyriticus | 10.455 | 9.398 | 2.608 | 9.724 | 9.797 | 2.481 | 1.136 | 40.265 | 57.712 | 1.254 | Cave_Terrestrial |
| AMNH | 137591 | Gyrinophilus porphyriticus | 10.137 | 9.323 | 2.077 | 10.24 | 11.572 | 2.71 | 1.124 | 39.248 | 56.742 | 1.482 | Cave_Terrestrial |
| AMNH | 137593 | Gyrinophilus porphyriticus | 9.552 | 9.827 | 2.517 | 10.382 | 9.118 | 2.533 | 0.998 | 42.651 | 58.975 | 1.427 | Cave_Terrestrial |
| AMNH | 137631 | Gyrinophilus porphyriticus | 12.852 | 11.205 | 3.073 | 11.496 | 16.193 | 3.885 | 2.194 | 45.029 | 78.49 | 2.401 | Cave_Terrestrial |
| AMNH | 137632 | Gyrinophilus porphyriticus | 10.887 | 13.713 | 2.612 | 10.309 | 15.807 | 3.199 | 1.705 | 38.977 | 75.936 | 2.353 | Cave_Terrestrial |
| AMNH | 137653 | Gyrinophilus porphyriticus | 17.023 | 17.691 | 4.719 | 18.167 | 21.134 | 5.143 | 2.991 | 72.221 | 119.842 | 3.954 | Cave_Terrestrial |
| AMNH | 137654 | Gyrinophilus porphyriticus | 16.159 | 18.13 | 3.91 | 17.058 | 21.519 | 5.219 | 2.795 | 76.475 | 113.408 | 3.256 | Cave_Terrestrial |
| AMNH | 157716 | Gyrinophilus porphyriticus | 13.842 | 16.283 | 3.578 | 17.532 | 19.846 | 3.698 | 2.948 | 87.135 | 85.935 | 2.639 | Cave_Terrestrial |
| AMNH | 169622 | Gyrinophilus porphyriticus | 24.362 | 11.092 | 3.201 | 12.077 | 13.516 | 3.345 | 2.658 | 62.303 | 77.954 | 2.542 | Cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 170907 | Gyrinophilus porphyriticus | 8.679 | 9.963 | 2.313 | 9.908 | 13.957 | 3.683 | 2.948 | 46.11 | 68.947 | 3.182 | Cave_Terrestrial |
| AMNH | 182194 | Gyrinophilus porphyriticus | 10.777 | 12.333 | 2.01 | 10.443 | 18.434 | 2.787 | 1.361 | 55.287 | 77.817 | 2.469 | Cave_Terrestrial |
| AMNH | 190416 | Gyrinophilus porphyriticus | 14.652 | 12.795 | 3.46 | 15.776 | 18.803 | 4.277 | 4.27 | 45.85 | 106.782 | 2.717 | Cave_Terrestrial |
| NMNH | 285091 | Hydromantes brunus | 5.902 | 10.953 | 1.13 | 4.501 | 10.857 | 1.397 | 1.667 | 22.557 | 31.64 | 2.593 | Non-cave_Terrestrial |
| NMNH | 285093 | Hydromantes brunus | 5.345 | 6.743 | 0.938 | 5.22 | 6.994 | 1.39 | 1.205 | 17.264 | 23.634 | 1.928 | Non-cave_Terrestrial |
| NMNH | 285094 | Hydromantes brunus | 5.501 | 6.865 | 0.915 | 5.108 | 6.84 | 1.193 | 1.401 | 17.711 | 22.853 | 1.992 | Non-cave_Terrestrial |
| NMNH | 285097 | Hydromantes brunus | 4.432 | 5.9 | 0.667 | 3.315 | 6.102 | 1.494 | 0.985 | 15.192 | 19.85 | 1.761 | Non-cave_Terrestrial |
| NMNH | 321295 | Hydromantes brunus | 11.258 | 14.574 | 2.459 | 9.039 | 16.23 | 3.095 | 2.713 | 49.119 | 55.379 | 3.89 | Non-cave_Terrestrial |
| NMNH | 545723 | Hydromantes brunus | 7.445 | 9.051 | 1.204 | 5.223 | 10.578 | 1.519 | 1.53 | 21.744 | 30.875 | 2.522 | Non-cave_Terrestrial |
| NMNH | 58732 | Hydromantes genei | 7.205 | 9.129 | 1.66 | 5.728 | 9.547 | 1.535 | 1.031 | 22.551 | 33.639 | 2.787 | Cave_Terrestrial |
| NMNH | 58733 | Hydromantes genei | 4.408 | 4.93 | 0.908 | 3.277 | 5.795 | 0.951 | 0.546 | 11.897 | 18.331 | 1.295 | Cave_Terrestrial |
| NMNH | 93878 | Hydromantes genei | 10.681 | 15.11 | 2.485 | 10.033 | 18.979 | 4.292 | 2.169 | 50.849 | 58.325 | 3.44 | Cave_Terrestrial |
| NMNH | 93880 | Hydromantes genei | 10.462 | 16.68 | 2.365 | 8.494 | 22.756 | 2.449 | 2.651 | 49.441 | 59.173 | 3.625 | Cave_Terrestrial |
| NMNH | 100925 | Hydromantes genei | 10.072 | 19.464 | 2.04 | 7.631 | 17.777 | 2.499 | 2.627 | 61.391 | 41.524 | 3.185 | Cave_Terrestrial |
| NMNH | 100930 | Hydromantes genei | 10.379 | 15.683 | 1.836 | 8.09 | 18.378 | 2.704 | 2.574 | 48.151 | 53.573 | 3.297 | Cave_Terrestrial |
| NMNH | 100932 | Hydromantes genei | 9.114 | 16.772 | 1.778 | 8.006 | 14.462 | 2.667 | 1.904 | 37.051 | 52.767 | 2.97 | Cave_Terrestrial |
| AMNH | 23485 | Hydromantes italicus | 8.681 | 12.366 | 2.01 | 7.472 | 12.389 | 2.325 | 2.182 | 27.145 | 52.033 | 2.393 | Cave_Terrestrial |
| AMNH | 23645 | Hydromantes italicus | 9.448 | 13.842 | 1.837 | 7.541 | 10.626 | 2.605 | 1.75 | 32.263 | 50.672 | 2.329 | Cave_Terrestrial |
| AMNH | 24873 | Hydromantes italicus | 8.66 | 10.012 | 2.555 | 7.499 | 10.989 | 1.635 | 1.487 | 34.262 | 47.057 | 3.085 | Cave_Terrestrial |
| AMNH | 24874 | Hydromantes italicus | 9.674 | 15.047 | 2.506 | 8.552 | 13.641 | 2.21 | 2.905 | 41.339 | 53.212 | 2.561 | Cave_Terrestrial |
| AMNH | 34631 | Hydromantes italicus | 9.512 | 13.023 | 2.376 | 8.81 | 12.227 | 2.498 | 3.483 | 32.732 | 51.687 | 2.266 | Cave_Terrestrial |
| AMNH | 34632 | Hydromantes italicus | 8.82 | 15.057 | 2.071 | 7.072 | 16.549 | 1.992 | 1.547 | 37.729 | 54.756 | 2.119 | Cave_Terrestrial |
| AMNH | 34633 | Hydromantes italicus | 9.568 | 14.473 | 2.353 | 7.432 | 15.293 | 3.36 | 1.627 | 38.346 | 55.567 | 2.752 | Cave_Terrestrial |
| AMNH | 52692 | Hydromantes italicus | 9.453 | 11.126 | 1.826 | 7.426 | 11.926 | 2.327 | 1.757 | 34.411 | 49.702 | 2.522 | Cave_Terrestrial |
| AMNH | 54694 | Hydromantes italicus | 10.526 | 18.021 | 2.081 | 10.356 | 17.261 | 2.54 | 1.779 | 33.898 | 65.84 | 2.71 | Cave_Terrestrial |
| AMNH | 54695 | Hydromantes italicus | 10.329 | 14.518 | 2.081 | 8.642 | 14.167 | 3.415 | 1.593 | 38.649 | 59.431 | 2.369 | Cave_Terrestrial |
| AMNH | 54696 | Hydromantes italicus | 11.581 | 16.005 | 2.446 | 9.39 | 15.088 | 3.116 | 1.311 | 45.963 | 62.773 | 2.983 | Cave_Terrestrial |
| AMNH | 65109 | Hydromantes italicus | 9.903 | 13.373 | 2.091 | 7.359 | 14.133 | 2.975 | 1.325 | 35.788 | 58.492 | 2.507 | Cave_Terrestrial |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMNH | 65110 | Hydromantes italicus | 9.685 | 10.16 | 1.946 | 7.604 | 14.598 | 2.421 | 1.193 | 32.221 | 55.705 | 2.459 | Cave_Terrestrial |
| AMNH | 65111 | Hydromantes italicus | 9.043 | 14.669 | 1.826 | 6.554 | 15.533 | 2.071 | 1.375 | 39.774 | 59.958 | 2.668 | Cave_Terrestrial |
| AMNH | 65112 | Hydromantes italicus | 6.749 | 8.192 | 1.145 | 4.972 | 10.269 | 1.448 | 1.042 | 23.099 | 34.535 | 1.968 | Cave_Terrestrial |
| AMNH | 149539 | Hydromantes italicus | 8.539 | 10.594 | 1.441 | 6.36 | 12.542 | 1.761 | 1.643 | 28.134 | 51.758 | 2.327 | Cave_Terrestrial |
| AMNH | 149540 | Hydromantes italicus | 9.067 | 12.844 | 1.662 | 7.85 | 12.903 | 2.338 | 1.863 | 32.22 | 50.977 | 1.839 | Cave_Terrestrial |
| AMNH | 149541 | Hydromantes italicus | 10.479 | 14.251 | 1.94 | 8.703 | 14.55 | 2.22 | 1.45 | 42.806 | 58.289 | 2.422 | Cave_Terrestrial |
| AMNH | 149542 | Hydromantes italicus | 11.107 | 14.12 | 2.255 | 9.779 | 14.971 | 3.408 | 1.104 | 35.204 | 59.952 | 2.587 | Cave_Terrestrial |
| AMNH | 149543 | Hydromantes italicus | 9.818 | 13.192 | 2.059 | 8.985 | 13.341 | 2.82 | 1.473 | 40.591 | 56.153 | 2.3 | Cave_Terrestrial |
| AMNH | 53808 | Hydromantes platycephalus | 8.704 | 12.358 | 1.823 | 8.947 | 15.483 | 2.433 | 1.863 | 35.84 | 56.365 | 2.593 | Non-cave_Terrestrial |
| AMNH | 53809 | Hydromantes platycephalus | 8.541 | 11.476 | 1.554 | 7.626 | 12.031 | 1.659 | 0.984 | 21.746 | 42.696 | 2.359 | Non-cave_Terrestrial |
| AMNH | 53810 | Hydromantes platycephalus | 6.939 | 7.574 | 1.294 | 6.722 | 10.169 | 1.586 | 1.044 | 16.498 | 33.235 | 2.017 | Non-cave_Terrestrial |
| AMNH | 53811 | Hydromantes platycephalus | 6.556 | 7.825 | 1.1 | 5.754 | 9.765 | 1.828 | 1.083 | 20.158 | 32.934 | 1.497 | Non-cave_Terrestrial |
| AMNH | 53812 | Hydromantes platycephalus | 9.477 | 10.711 | 1.816 | 8.55 | 14.447 | 2.272 | 1.168 | 30.359 | 51.079 | 2.062 | Non-cave_Terrestrial |

# CHAPTER 2

## THE PHYLOGEOGRAPHY AND POPULATION GENETICS OF A CAVE-DWELLING SALAMANDER, *EURYCEA LUCIFUGA*

**Abstract**

The evolutionary history and ecology of cave-dwelling species has been driven historically by studies of morphologically adapted cave-restricted species. As a result, our understanding of troglophiles' evolutionary history and ecology is limited to a few studies, which present differing accounts of troglophiles' relationship with the cave habitat, and its impact on population dynamics. We used phylogenetics, demographical statistics, and population genetic methods to study lineage divergence and population structure in the cave salamander *Eurycea lucifuga* across its range, as well as inferring dates of divergence among major clades. Results of phylogenetic and demographic analyses indicate that there are three main lineages within *E. lucifuga* corresponding to the Western, central, and Eastern regions of the range. Divergence among these major regions dates to the Pliocene and Pleistocene, with evidence of recent expansion into present-day localities. Population genetic analyses largely corroborate these results, but indicate isolation within the central clade between populations located in Indiana and those in Kentucky and Tennessee. Genetic diversity as well as a Bayesian analysis of divergence scenario likelihoods suggest that populations may have expanded from the Southern central areas of its range in two steps: first an expansion to the West and then another to the East. Correlation of divergence among regional lineages in *Eurycea lucifuga* with the dramatic climatic and geological shifts of the Pliocene and Pleistocene is similar to what we see in other cave-dwelling species.

**Introduction:**

Examining populations' evolutionary histories in the context of their spatial distribution often gives us a better understanding about the formation of lineages and species, which can then be used in studies of adaptation and morphological evolution. A useful area of study for questions of this nature is the existence of cave-dwelling species, and their divergence from surface-dwelling relatives. The vast majority of research on cave systems has focused on troglobites (cave-restricted morphologically adapted species), which are characterized by a suite of acquired traits known as troglomorphy. These morphological and physiological traits are thought to act both as a mechanism behind subterranean speciation (Barr *et al.* 1968; Culver 1982) and as a constraint upon dispersal and gene flow in troglobites (Porter 2007). For this reason, troglobitic cave-dwellers are often characterized by endemism and limited range (Christman *et al.* 2005; Culver & Holsinger 1992); for example, the majority of US troglobites are endemic to a single county (Culver *et al.* 2000).

Although a great deal of attention is paid to troglobitic and/or troglomorphic species in the literature, there are other classes of cave-dwelling species. Of particular interest here is a class of cave-dwellers known as troglophiles. These species maintain permanent cave populations, but vary in their restrictedness to the cave habitat (Sket 2008). For some taxa, not being restricted to the cave habitat allows for greater dispersal in troglophilic populations than in troglobitic populations. Caccone (1985) studied several species of cave arthropods of varying cave restrictedness and found that while the amount of gene flow between populations and population structure varied tremendously among all species, and were highly dependent on both the characteristics of the cave and

surface environments as well as innate characteristics of the species, troglobites tended to have lower levels of gene flow among caves than troglophiles (Caccone 1985). In contrast, populations of the troglophilic isopod *Androniscus dentiger* exhibited surprisingly high differentiation with no detectable inter-population gene flow (Gentile & Sbordoni 1998). Similarly, very low levels of gene flow and high population structure have been described in troglophilic Sardinian cave salamanders (genus *Hydromantes*), likely due to the inhospitable surface environments that discourage dispersal outside of caves (Chiari *et al.* 2012). These studies suggest that, depending on environmental and species characteristics, troglophiles may experience restriction to caves in the same way that troglobites do, though perhaps for different reasons. However, the relative lack of attention paid to troglophilic species prevents us from understanding how inhabiting the cave environment will impact the population and demographic dynamics of a species where cave association is not mandated by morphological restrictions, as it is in troglobitic species.

*Study system*

We studied the phylogeographic history and population dynamics of a troglophilic salamander in the genus *Eurycea* (Plethodontidae). *Eurycea* is particularly notable for its abundance of troglobitic species. Though surface-dwelling is the ancestral state in this genus, cave-dwelling, and troglomorphy, evolved independently at least five times across the genus (Bonett *et al.* 2014). *Eurycea lucifuga* is a troglophilic salamander commonly found in limestone caves throughout the Southeast and Midwest United States (Figure 1). *Eurycea lucifuga* has a multi-stage life cycle, in which individuals are restricted to aquatic cave habitats in the egg and larval stages before fully metamorphosing into

brightly colored adults (Petranka 1998). There are anecdotal reports of *E. lucifuga* being in the forest matrix surrounding caves (Hutchison 1958; Petranka 1998), though cave habitats are by far the most common environment for *E. lucifuga*, and individuals are often found large distances (>1000m) beyond cave entrances (H.E. pers. obs.).

Despite the frequency with which *Eurycea lucifuga* is found across its wide range and its occupancy of a charismatic and conservationally important niche, very little is known about its evolutionary history or for how long it has inhabited caves. Understanding these important attributes is a critical component of examining other aspects of its ecology and life history. We investigated the phylogeography of *Eurycea lucifuga* using phylogenetic, population genetic, and demographic statistical methods in order to better understand its evolutionary history. First, we used a tree-building approach to reconstruct the phylogeographic history of populations across the range of *E. lucifuga* and estimated the timing of divergence between populations in different geographic regions. Second, we used a classical population genetics approach to assess how genetic variation is structured across the range, and to infer a reconstruction of historic lineage splitting.

**Methods:**

*Data collection*

Between 2012 and 2013 we collected tail clips from between one and nine individuals from populations of *Eurycea lucifuga* throughout its range (Figure 1). A population is defined here as a group of individuals collected from inside an entrance to a cave. This definition can be problematic in cases where cave entrances are part of a larger cave system; however, to the best of our knowledge the majority of cave entrances

in this study are unconnected from other entrances. Animals were released at their capture site following tissue extraction.

Tissue samples were stored in 95% ethanol at room temperature at the University of Virginia (Charlottesville, VA). We extracted total genomic DNA from each sample using either a phenol-chloroform method (Sambrook *et al.* 1989) or Chelex resin, using a 5-10% slurry of Chelex and an incubation time of 180 minutes at 95°C. We then amplified three gene fragments commonly used in salamander systematics using polymerase chain reaction (PCR): two mitochondrial loci, NADH dehydrogenase 2 (ND2) and cytochrome b (cyt*b*), and one nuclear locus, proopiomelanocortin (POMC). Primer information and thermocycling conditions are shown in Table S1 (Supporting information). We cleaned the PCR products using ExoSAP-IT (Affymetrix) and then sequenced them in both directions using Sanger sequencing on an ABI 3730*xl* at GENEWIZ (South Plainfield, NJ). We used the software program Geneious v 6.1.6 (Biomatters, Aukland, New Zealand) to edit the resulting sequence, then used the Geneious alignment option in Geneious to create multiple alignments for each locus. Alignments were unambiguous, and there were no gaps in the sequence, nor heterozygous sites in the nuclear locus. We included sequence data from *E. longicauda* and *Pseudotriton ruber* as outgroups. We collapsed identical haplotypes using the web-based software program ALTER (Glez-Peña *et al.* 2010). All sequences are deposited in GenBank (For collection records including accession numbers see Table S2, Supporting information). We used Xia's Index of Sequence Saturation in the software package DAMBE (Xia & Lemey 2009; Xia *et al.* 2003) to test whether each locus was phylogenetically informative.

We also genotyped each individual at 19 microsatellite loci designed specifically for *E. lucifuga* (Appendix 1). Loci were amplified in multiplexed reactions using PCR, and products were sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT) for fragment analysis using a 3730*xl* 96-Capillary Genetic Analyzer, with the DS-33 dye set. Finally, we used GeneMarker software (SoftGenetics LLC, State College, PA) to call alleles at each marker locus for each individual.

*Phylogenetic analyses*

We analyzed the sequence data from cyt*b*, ND2, and POMC individually and as a combined, partitioned dataset under maximum likelihood (ML) and Bayesian inference (BI) using RAxML 7.2.6 (Stamatakis 2006) and MrBayes 3.1.2 (Ronquist *et al.* 2012). Individuals with missing data were excluded from any reconstructions where the loci were included in a combined analysis. Appropriate models of sequence evolution were inferred for each locus using both the Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and Decision Theory Performance-Based Selection (DT) in the program jModelTest v 2.1.4 (Posada 2008). We first performed ML phylogenetic analysis using the ML + thorough bootstrap analysis option with 2 independent runs and 1000 nonparametric bootstraps. We then used Bayes Factors implemented in MrBayes to compare the heuristic mean likelihood of two BI analyses, in which one model specified partitioning among codons and one model specified a simpler partitioning scheme with only partitioning among the gene loci. This comparison indicated that the simpler partitioning model was more likely (individual codon partitioning: likelihood = -6000.91; combined codon partitioning: likelihood = -5788.31). Upon incorporating the correct partitioning model as well as appropriate substitution rates, each MrBayes analysis was

run for 50,000,000 generations and trees were sampled every 2,500 generations, with 3 heated chains and one unheated chain. After analyzing Bayesian results for convergence as well as comparing individual searches using Tracer (Drummond *et al.* 2012), the first 20% of the generations were discarded as a burn-in period. We used TreeAnnotator (Drummond *et al.* 2012) to sample the trees and infer a single tree for each analysis using Maximum Clade Credibility. We visualized trees using the program FigTree v 1.4.2 (Drummond *et al.* 2012), including ML bootstrapping and BI posterior probability information on the resulting BI tree at each well-supported major clade.

We also used the program BEAST v1.8.1 (Drummond *et al.* 2012) to recreate a species tree using the three partitioned sequence datasets. We used a stepping-stone analysis comparing the use of a relaxed molecular clock with a strict clock model implemented in MrBayes for 50 steps, each with 19,500 generations, which indicated that the strict clock model had a higher mean marginal likelihood (strict: Marginal likelihood (ln) = -6288.50, relaxed: Marginal likelihood (ln) = -6334.81). Accordingly, we used a published rate to calibrate the clock for cytb and nd2 (0.0062 substitutions/my and 0.0037 substitutions/my respectively; (Mueller 2006)), and estimated the rates of POMC with reference to those rates. We ran the analysis two times independently for 500 million generations, sampling trees every 20,000 generations under a Yule model with piecewise linear population size and a constant root. After using Tracer (Drummond *et al.* 2012) to compare the independent runs and test for convergence we discarded the first 20% of the resulting trees as a burn-in, and inferred a single tree with TreeAnnotator (Drummond *et al.* 2012). This tree was visualized using Figtree v 1.4.2 (Drummond *et al.* 2012). We also performed mismatch analysis and calculated Tajima's *D* and Fu's *F* statistics in

Arlequin v.305 (Excoffier & Lischer 2010) to test for evidence of recent population expansions or bottlenecks for each of the major divergence events predicted by our phylogenetic analyses as a comparison with divergence times predicted by BEAST. Using methods outlined by Holsinger (2008), we used the estimates of tau produced by mismatch analysis to calculate the time of the predicted expansion for each geographic group using the published rates for both cytb and nd2 (Mueller 2006).

*Population genetic analyses*

We performed a cluster analysis of the microsatellite genotype data using the program STRUCTURE v.2.3.4 (Pritchard *et al.* 2000). Following the methods of Coulon *et al.* (2008), we first ran the analysis with K=2, then analyzed each partition separately using a range of K=1-10. The most appropriate number of clusters was found by analyzing the results using the Evanno method (Evanno *et al.* 2005) in the web based software Structure Harvester (Earl 2012). We then visualized the clustering in distruct (Rosenberg 2003). To test whether the pattern of variation could be explained by isolation by distance, we used the software program Genepop (Raymond & Rousset 1995; Rousset 2008) to examine the correlation between genetic and geographic distances with Mantel tests. We performed a separate analysis within each of the main clusters found by STRUCTURE using 1000 permutations of the Mantel tests. We examined genetic diversity within each region by comparing estimations of heterozygosity and the inbreeding coefficients directly. To estimate how variation is partitioned among- and within-groups and populations, and to estimate Wright's *F* statistics, we used Analysis of Molecular Variance (AMOVA) in the program GenoDive (Meirmans & Van Tienderen 2004). Finally, to compare the topologies predicted by the

Likelihood and Bayesian trees with those predicted by the BEAST species reconstruction and the microsatellite structuring data, we used Approximate Bayesian Computation in the program DIYABC (Cornuet *et al.* 2014) to infer the likelihood of different divergence scenarios. We tested four scenarios (Figure 2), one in which a radiation suggests an equal age for each major clade, and three more with each combination of divergences. We kept the default priors for each analysis, and ran the simulation for 400,000 iterations. Following the simulation, we performed model selection using both a direct estimation of the most likely scenario with 500 iterations as well as a logistical regression using 4000 iterations. Model checking was performed to compare axes of variation within the summary statistics produced from the simulation to those predicted by the posterior predicted distribution of the best-fitting model.

**Results**

Fragments of cyt*b*, ND2 and POMC (1582bp total) from 93 individuals from 27 populations were included in our phylogenetic reconstructions and demographic statistical analyses. jModelTest (Posada 2008) predicted the most appropriate model of evolution for each locus to be TPM2uf+I+G (cyt*b*), TIM3+I (ND2), and TIM2+G (POMC). Since these models are not available options in most phylogenetic reconstruction programs, we used the slightly overparameterized models GTR+I+G, GTR+I, and HKY+G, respectively during the analyses in MrBayes and RaxML, and BEAST. None of the loci exhibited significant saturation (cytb: $T=49.061$, $Df=463$, $p<0.0001$; nd2: $T=62.6957$, $Df=549$, $p<0.0001$; POMC: $T=122.5041$, $Df=465$, $p<0.0001$), indicating that our sequence data contain useful phylogenetic information.

We genotyped 233 individuals from 53 populations at 19 microsatellite loci. Summary statistics from the genotyped dataset can be found in Appendix A. The number of alleles per locus ranged from 2-7. With very few exceptions, all loci were in Hardy-Weinberg equilibrium within populations (Appendix A, Table 4).

*Phylogeographic analyses*

The ML and Bayesian concatenated trees (Figure 3) both feature two well-supported major clades organized by geographic region; the topologies are very similar with only minor rearrangements within major clades. The central clade contains populations in western Tennessee, Kentucky, and Indiana, and the Eastern/Western clade contains the populations within Virginia, West Virginia, Eastern Tennessee, Oklahoma, and Missouri. The Western populations form a monophyletic clade within the Eastern populations. Haplotypes were shared among populations within the major geographic regions; however, support at the tips was very low. Relationships among the clades differ from simple geographic expectations: the Western and Eastern regions are more closely related to each other than either is to the central clade. Gene trees of individual loci (Figs S1-3, Supporting Information) generally reflect these relationships, although the nuclear locus POMC did not have a large influence on the topology, and as a result the combined trees generally represent the mitochondrial history of the species. However, the topology of the tree produced using species tree methods in BEAST indicate that all three geographic regions form distinct clades, and that the central and Eastern populations are more closely related to each other than they are to the Western populations (Figure 4). While branching leading to the Western clade is well-supported, with posterior probabilities of 1, the relationships among populations in the Eastern and central clades

are not as well supported. This is reflective of the fact that BEAST occasionally reconstructed a different topology for the tree, similar to that of the MrBayes and RaxML predictions with the Eastern and Western clades forming a monophyly and the central clade less closely related. Though the inconsistencies between the two reconstruction methods indicate some ambiguity in the DNA sequence data, the improved performance of species tree methods in general (Degnan & Rosenberg 2006; Heled & Drummond 2010; Liu & Pearl 2007) suggests to us that the BEAST reconstruction is the more accurate scenario. This is supported by results of the divergence dating and population genetic analyses we performed.

*Divergence dating*

The chronogram produced in BEAST (Figure 4) dates divergence from *Eurycea longicauda* at approximately 4 million years ago (mya), and between the two major clades (Eastern/Western and Central regions) at almost 2 mya. Divergence within each region was dated at more recently than a million years ago. Estimates of Tajima's D and Fu's F statistics corroborate this evidence of recent population expansion within each major clade, with significantly negative values of Fu's F, Tajima's D, or both parameters for each expansion event (Table 1). Mismatch analysis did not indicate a departure from the null hypothesis of recent expansion in any group either with a significant SSD value or significant values for Harpending's raggedness index. Using Holsinger's (2008) methods for inferring timing of divergence from an estimate of *tau*, we found that expansion of the Western populations following divergence from the Central populations may have occurred in the range of one to three million years ago, the Eastern clade approximately 1-2.5 mya, and the Central populations experiencing expansion much

more recently, on the order of tens of thousands of years ago (Table 1). This timing, and the topology of the species tree reconstructed using BEAST are supported by the results of an analysis comparing different divergence scenarios using DIYABC, which predicted that the most likely scenario includes a primary splitting of the Western populations, followed by a subsequent divergence between the central and Eastern populations (Table 2, Figure 2).

*Population genetic analyses*

The STRUCTURE analysis indicates that the model with the highest likelihood is K=4 with a mean likelihood of -4198.9. Assignment to these genetic clusters partitions the samples into clear geographic groups: Western, Indiana, South-central (Kentucky and Tennessee), and Eastern populations (Figure 5). We did not find significant evidence of isolation by distance within the Western or Eastern populations; however, correlations between genetic and geographic distances were significant among populations in the northern Central region (r=0.05, p=0.023) and in the Southern central region (r=0.07, p=0.043). When compared directly, the central regions contain significantly more heterozygosity than the Western or Eastern regions (Table 3), so there may have been greater power to detect subtle patterns of isolation by distance. AMOVA results indicate significant population structure among sampling localities within the Eastern, Central and Western regions ($F_{SC}$=0.093, p=0.001) and strong population genetic divergence among regions ($F_{CT}$=0.331, p=0.001). 33% of genetic variance is partitioned among regions, and 6% among localities within each region. A substantial amount of variation is partitioned among individuals within localities (13%, $F_{IS}$=0.208, p=0.001) (Table 4).

**Discussion**

Results of the phylogenetic analyses and demographic statistics suggest a pattern of ancient lineage divergences within *Eurycea lucifuga*, followed by more recent population expansion within each region. The timing of divergence among the clades estimated in BEAST (1.7-2.7mya) is similar to lineage splitting in other cave-dwelling species (Finston *et al.* 2007; Lefébure *et al.* 2006; Leys *et al.* 2003; Strecker *et al.* 2004; Zakšek *et al.* 2007), which lends support to Wessel's (Wessel *et al.* 2007) finding that the age of a cave-dwelling lineage is not correlated with its morphological adaptation to the caves. This age places divergence within *E. lucifuga* during the late Pliocene or early Pleistocene, in the midst of climatic and geological change, but well before the last glacial advance. The sharing of haplotypes among localities in concert with the results of Tajima's D, Fu's F, and mismatch analyses suggest that within each major region expansion has been recent; however, our estimations of the timing of that expansion indicate that it was well before the retreat of the last glacial ice sheet at the end of the Pleistocene. As the last 700,000 years brought about particularly extreme climatic oscillations (Hewitt 1996; Webb & Bartlein 1992), the coincidence of expansion with that time suggests that the recent expansion we estimate was a result of that period of global change. It is probable that oscillating climatic conditions caused a series of expansions and contractions in the range of *E. lucifuga*; however, signatures of subsequent expansions would be overwhelmed by the initial expansion (Rogers 1995).

Population genetic analyses reveal that the majority of genetic variation across the range of *Eurycea lucifuga* is partitioned among the major geographic regions, and populations structure within each region is quite low ($R_{ST}$=0.038). This low structure

particularly contrasts with closely related cave-restricted species (*E. pterophila/E. nana/E. neotenes*: $F_{ST}$=0.249-0.922; (Lucas *et al.* 2009)) and with other Plethodontids studied using protein sequences, in which estimates of $F_{ST}$ ranged from 0.13 (*Plethodon cinereus*) to 0.80 (*Plethodon dorsalis dorsalis*) (Larson *et al.* 1984). However, population structure is similar to estimates seen at smaller scales in *P. cinereus* ($F_{ST}$=0.019; Cabe *et al.* (2007)) and *Ambystoma maculatum* ($F_{ST}$=0.041; Purrenhage *et al.* (2009)). While low population structure could be a signature of the recent expansion predicted by our demographic statistical analysis, it also suggests a pattern of persistent gene flow among populations. Interestingly, there are distinct differences in population genetic structure and diversity among the main geographic clades of *E. lucifuga*, with the central populations exhibiting both greater observed and expected heterozygosity and more population structure than the Western and Eastern populations. Although we expect that central populations will have greater heterozygosity than peripheral populations, this is usually accompanied by higher population structure in peripheral populations (Eckert *et al.* 2008). We speculate that in this case, because movement between caves is often dependent on the characteristics of the surface environment (Caccone 1985; Chiari *et al.* 2012), it is possible that the variations in climate and landscape in the different regions impacts this species' dispersal ability, causing variation in population structure.

In reconstructing the phylogeographic history of a species it is important to keep in mind the geological and climatic processes that may have influenced a species' present distribution. The divergence dates estimated using BEAST and demographic statistics indicate that *Eurycea lucifuga*'s population dynamics have mainly been influenced by the

late Pleiocene and early Pleistocene eras, which was a time of great climatic and geological change. The earth was shifting from a warming period during the Miocene (~24-5mya) to a cooling period from the Pliocene (~5-2mya) until glacial retreat at the end of the Pleistocene (~0.01mya). Throughout the Pliocene and Pleistocene glacial advance and retreat oscillated a number of times, and increased rainfall in non-glaciated areas benefited the species that took refuge there (Levin 2009). At its most extensive time, the Laurentide ice cap reached as far South as forty degrees latitude (Stearn *et al.* 1979). Fluctuations in temperature and ice cover caused corresponding oscillations in species' ranges, which often led to a series of bottleneck at range edges, resulting in reduced genetic diversity among those edge populations (Hewitt 1996). These expansions and contractions applied equally to warm-adapted species during the onset of glaciation, and cold-adapted species during inter-glacial periods (Hewitt 1996). During glaciation, many species retreated to unglaciated areas known as glacial refugia. Often the use of multiple glacial refugia was a cause of divergence among lineages, (Bossu *et al.* 2013; Phillips 1994; Zamudio & Savage 2003), and many species show signs of intraspecific divergence dating to periods of glaciation (Avise 2000; Kozak *et al.* 2006; Niemiller *et al.* 2008), although pre-glaciation divergence has been estimated in other species (Burbrink *et al.* 2008; Hoffman & Blouin 2004; Jones *et al.* 2006). Refugia have been predicted in areas both East and West of the Mississippi River in the central basin, as well as in the Southern Appalachians (Bossu *et al.* 2013; Hoffman & Blouin 2004; Rissler 2010; Taylor *et al.* 2007; Zamudio & Savage 2003).

Following glacial retreat, species' dispersal from glacial refugia and expansion into the present-day ranges involved certain patterns repeated across taxa. Range

expansions led to a significant clustering of amphibian taxa in areas with very high species richness: primarily in the Appalachian Mountains and throughout Alabama (Rissler 2010). Many taxa exhibit consistent phylogenetic breaks across five 'barrier zones' across the United States: between the Atlantic and Gulf coasts, the Appalachian Mountains, and across the Appalachicola River (SE United States), the Tombigbee River (Alabama), and the Mississippi River (Soltis *et al.* 2006). There are three alternative scenarios we could predict given these trends and the results of our analyses:

1) The clear divisions among geographic lineages across the range could indicate that each clade expanded from a different glacial refugium, located to the West of the Mississippi River, East of the Mississippi in the central lowlands, and/or in the Southern Appalachians. This would be a similar history to that of *Rana pipiens* (Hoffman & Blouin 2004) or *Ambystoma maculatum* (Zamudio & Savage 2003).

2) Given the difference in genetic diversity and heterozygosity between the central clade and the peripheral clades, an alternative scenario is that of a single refugium in the central basin to the East of the Mississippi. Following expansion northward, the Western and Eastern clades may have formed as a result of separate migration events, and their low diversity could then be attributed to founder effects. This would explain the inclusion of Eastern and Western alleles within the central populations.

3) It could be predicted that range expansion in *Eurycea lucifuga* was closely tied to geomorphological history throughout this time in addition to climatic fluctuations due to its cave inhabitancy. It has been suggested that the caves served as refugia for cold-adapted species during the warming of interglacial periods (Barr & Holsinger 1985), which could be the case in this species, particularly given the timing of expansion in each

region. While cave formations are particularly ancient in the Ozarks (ranging from Cambrian to Mississippian- 530 to 330 mya) (Elliott & Ashley 2005), cave ages in the Appalachians, specifically the Cumberland, ranged from 5.68-0.02mya (Anthony & Granger 2007). Mammoth Cave was formed between 2.4-2.3mya, but experienced instability until around 0.7mya (Granger *et al.* 2001). If *Eurycea lucifuga* retreated into the cave habitat in order to avoid the warming climate of an interglacial period, it is plausible that this would have occurred earlier in the Western region where caves had been stable for millions of years, whereas establishment in caves in the central and Eastern regions would not have occurred until those formations were structurally stable.

The results of species tree reconstructions, genetic clustering using STRUCTURE, and results of DIYABC, in addition to the increased diversity within the central clade, suggest that the most likely scenario is that the Western clade diverged first, followed by a split between the central and Eastern populations. Sequential dispersal from the central region to the West and East would explain our observation that the central clade contains the most genetic diversity as well as the highest heterozygosity of the clades.

While we cannot distinguish among these hypotheses at present, our results indicate that *Eurycea lucifuga* is a species of early Pliocene origin, and that its distribution and ecology have been largely influenced by the climatic and geological events of the late Pliocene and early Pleistocene. While we may have expected expansion into present cave localities to have occurred recently, following the last glacial retreat, the timing of divergence and regional population expansion is similar to that of other cave species. However, evidence of recent gene flow among populations is

suggestive that the ecology of this species differs from most cave-dwelling taxa, which show limited gene flow. Incorporating this information with that of other systems will benefit our understanding of how species have changed with past earth events, and predict what their future evolutionary and ecological trajectories may be.

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## Figures

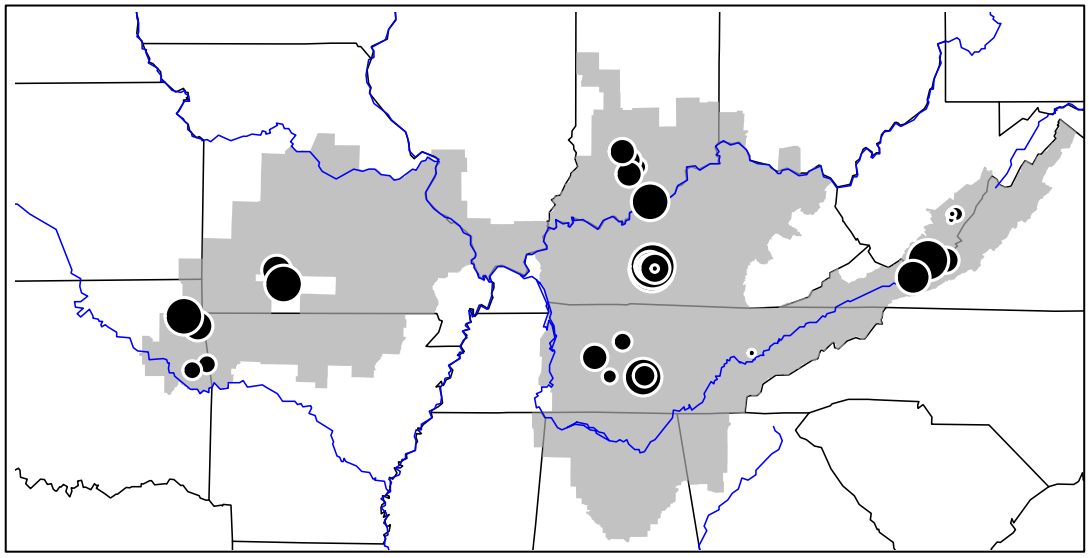

Figure 1. Depiction of the range of *Eurycea lucifuga* (shown in grey), and collection locations (black circles). Size of the location marker indicates number of samples collected, which ranged from 1-11.

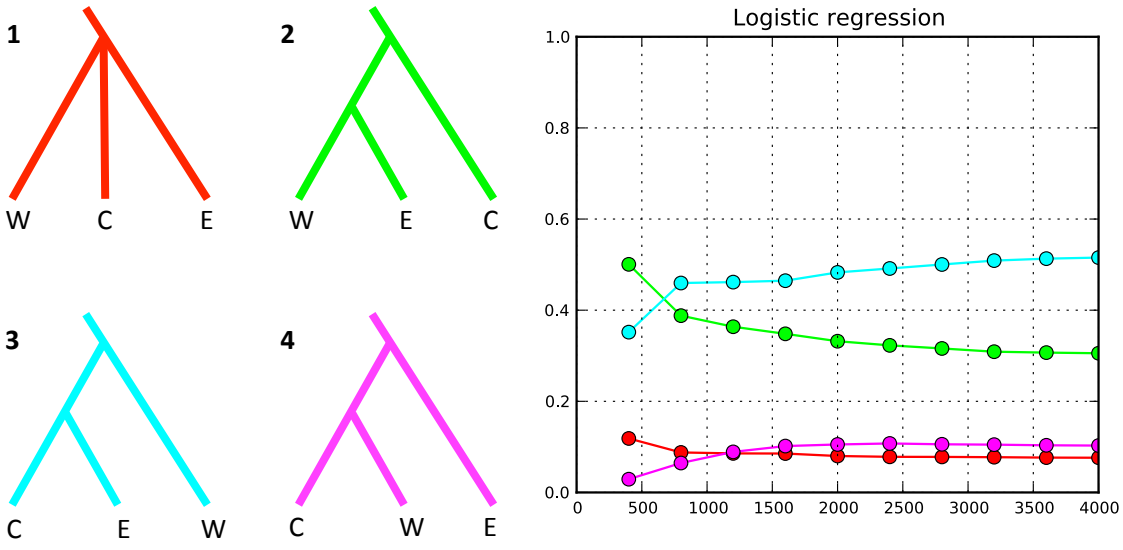


Figure 2. The four scenarios tested in the DIYABC model, depicting hypothetical relationships among the Eastern, central, and Western populations. On the right the results of a logistical model comparing the posterior probability of each scenario with the number of simulations used to calculate it.

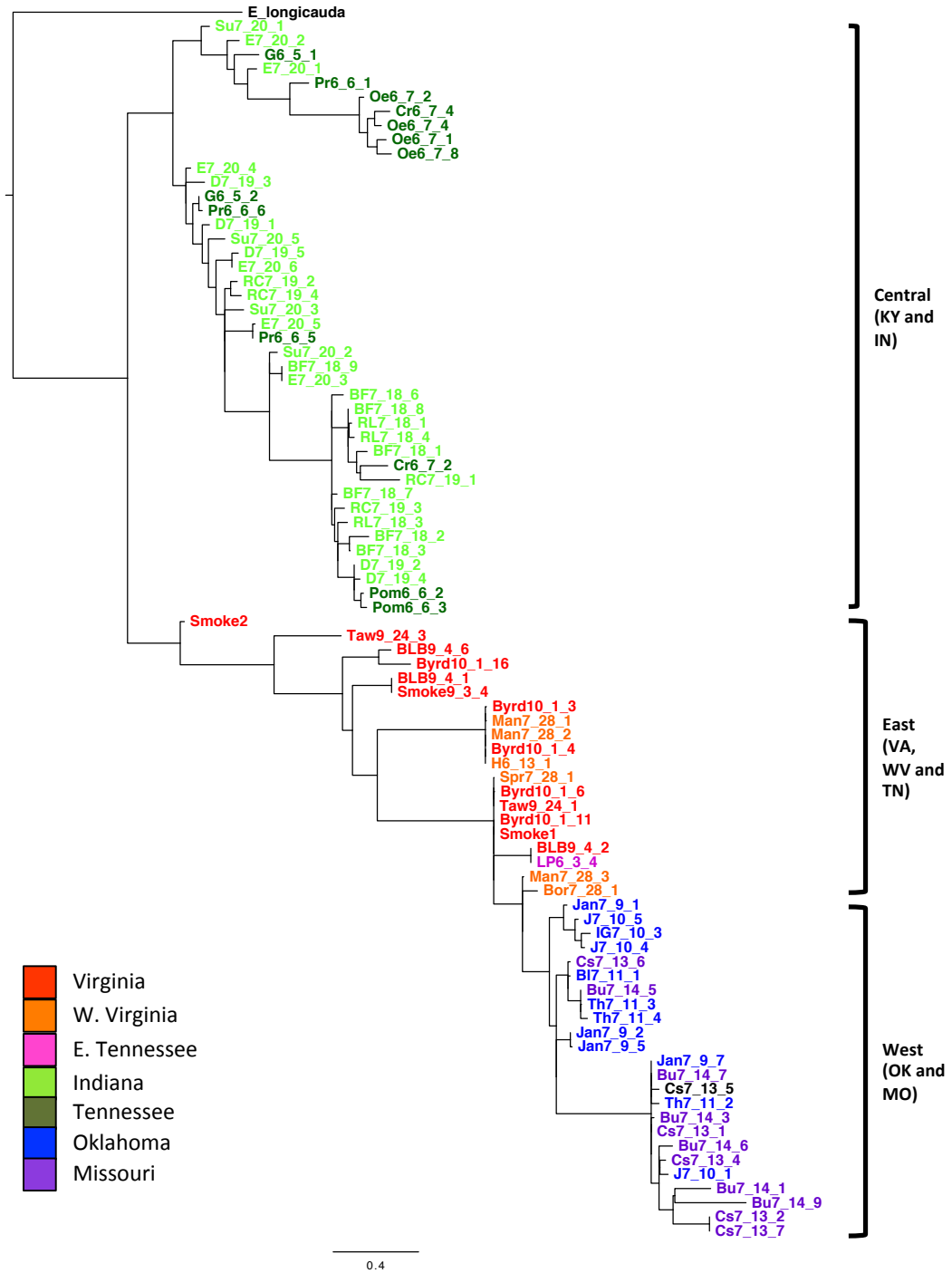


Figure 3. Bayesian phylogeny inferred from the concatenated sequence data of cyt*b*, ND2 and POMC using MrBayes. *Pseudotriton ruber* was included as an outgroup, but is not shown here. Posterior support values above 0.5 are shown above, and maximum likelihood bootstraps above 50 are shown below branches. Colors correspond to the regions from which samples were collected, as indicated in the legend.

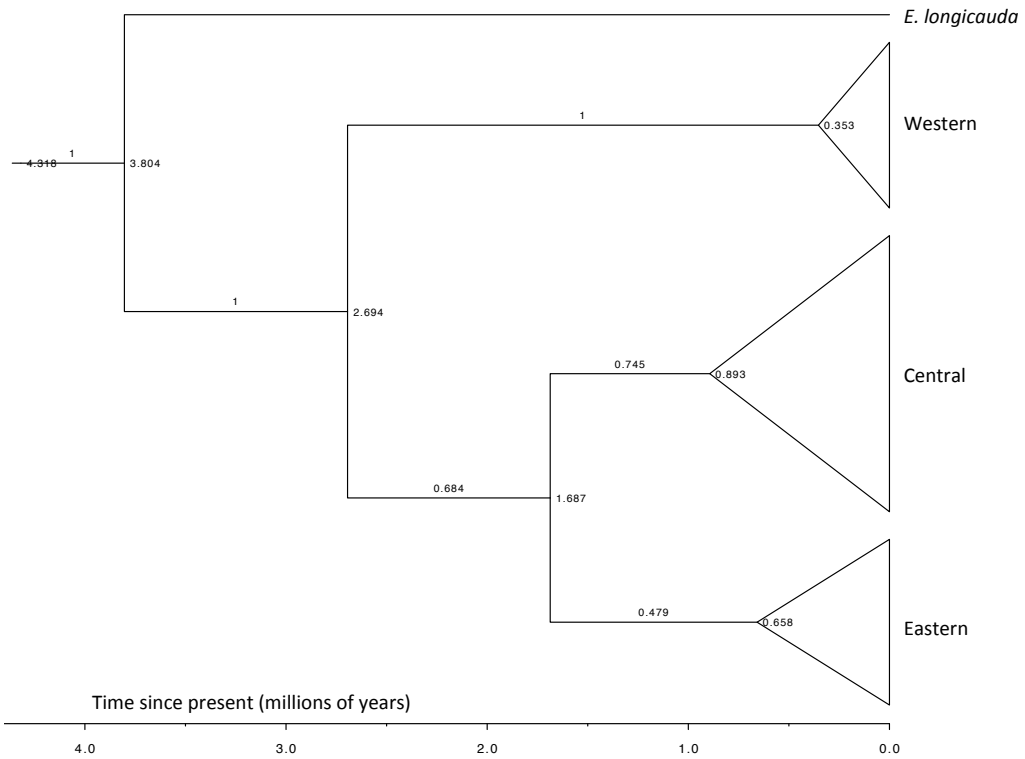


Figure 4. Dates of divergence, inferred using the species tree method in BEAST with a strict clock, indicating divergence of the major lineages during the late Pliocene and early Pleistocene. Node labels reflect the estimates ages of divergence, and branch labels represent the posterior probabilities.

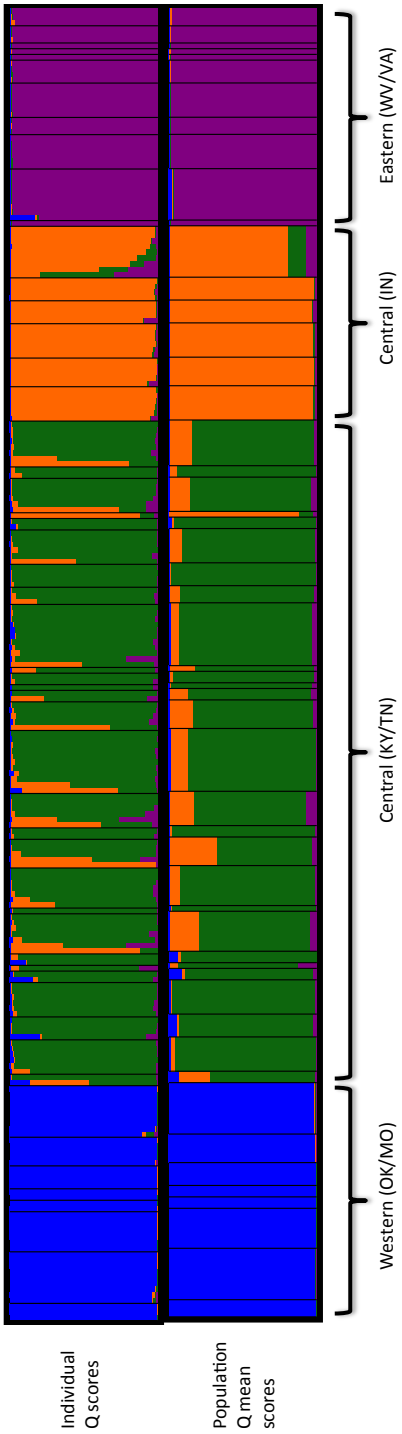


Figure 5. The geographic clustering of diversity, estimated using STRUCTURE, indicates four major clades across the range of *Eurycea lucifuga*. While the Eastern and Western clades are distinct from each other entirely, they both contribute to the diversity found within the two central clades, indicating either asymmetric gene flow or past expansion from the central region.

## Tables

Table 1. Results of demographic analysis using Tajima's D and Fu's F statistics, as well as Mismatch analysis, indicate that recent expansion is likely within each region, on a time scale that supports divergence dates found in BEAST species tree inference.

| **Major divergence:** | **Western** | **Central** | **Eastern and Western** |
|---|---|---|---|
| **Tajima's D** | -1.85* | -0.67 | -2.07* |
| **Fu's F** | -19.70* | -24.07* | -1.43 |
| | | | |
| **Tau** | 23.37 / 5.02 | 0.24 / 0.13 | 6.35 / 10.54 |
| **$Theta_0$** | 0 / 0 | 13.89/ 0 | 0 / 0 |
| **$Theta_1$** | 3.17 / 2.42 | 99,999.00 / 99,999.00 | 6.88 / 12.69 |
| | | | |
| **SSD** | 0.015 | 0.006 | 0.044 |
| **R** | 0.017 | 0.003 | 0.059 |
| **Evidence for Recent Expansion?** | Yes | Yes | Yes |
| **Estimated time since expansion:** | 3.3my/1.2my | 34,000y/33,000y | 909,600y/2.58my |

Table 2. A comparison of the posterior probabilities of each of four divergence scenarios analyzed in DIYABC calculated using 4000 simulations, presented with confidence intervals. The highest probability, that of scenario three, is in bold.

| | Posterior Probability | 95% CI |
|---|---|---|
| Scenario 1 | 0.3055 | 0.2241,0.3869 |
| Scenario 2 | 0.0762 | 0.0055,0.1469 |
| Scenario 3 | **0.5154** | 0.4494,0.5815 |
| Scenario 4 | 0.1029 | 0.0313,0.1745 |

Table 3. Comparisons of heterozygosity, inbreeding values, and distance statistics among the four main lineages in *Eurycea lucifuga*. Heterozygosity is significantly greater in the central regions than the East or West, supporting a hypothesis of expansion from the central region.

| **Statistic** | **Western** | **Central-KY and TN** | **Central-IN** | **Eastern** | **OSx** | **P-value** |
|---|---|---|---|---|---|---|
| Ho | 0.165 | 0.217 | 0.183 | 0.114 | 0.149 | **0.003** |
| Hs | 0.163 | 0.269 | 0.226 | 0.163 | 0.18 | **0.003** |
| Gis | -0.014 | 0.195 | 0.192 | 0.303 | 0.459 | **0.012** |
| Gst | 0.063 | 0.062 | 0.016 | -0.089 | 0.247 | 0.641 |
| D_est | 0.015 | 0.025 | 0.006 | -0.018 | 0.063 | 0.929 |

Table 4. AMOVAs across the range as well as within each region indicate that the majority of genetic variation is partitioned among regions, and that regions differ widely in the amount of population structure they exhibit.

| **Range-wide** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Source | Nested in | %var | F-stat | F-value | Std.Dev. | c.i.2.5% | c.i.97.5% | P-value |
| Within Individual | -- | 0.613 | R_it | 0.387 | 0.26 | 0.164 | 0.705 | -- |
| Among Individual | Population | 0.014 | R_is | 0.022 | 0.233 | -0.111 | 0.435 | 0.279 |
| Among Population | Series_A | 0.038 | R_sc | 0.057 | 0.03 | 0.009 | 0.074 | **0.001** |
| Among Region | -- | 0.335 | R_ct | 0.335 | 0.188 | 0.16 | 0.593 | **0.001** |
| **Western** | | | | | | | | |
| Source | Nested in | %var | F-stat | F-value | Std.Dev. | c.i.2.5% | c.i.97.5% | P-value |
| Within Individual | -- | 1.184 | R_it | -0.184 | 0.487 | -0.333 | 0.609 | -- |
| Among Individual | Population | -0.201 | R_is | -0.204 | 0.511 | -0.368 | 0.619 | 0.942 |
| Among Population | -- | 0.017 | R_st | 0.017 | 0.023 | -0.043 | 0.06 | 0.249 |
| **South-Central** | | | | | | | | |
| Source | Nested in | %var | F-stat | F-value | Std.Dev. | c.i.2.5% | c.i.97.5% | P-value |
| Within Individual | -- | 0.829 | R_it | 0.171 | 0.094 | 0.056 | 0.432 | -- |
| Among Individual | Population | 0.087 | R_is | 0.095 | 0.144 | -0.023 | 0.427 | **0.039** |
| Among Population | -- | 0.084 | R_st | 0.084 | 0.06 | 0.004 | 0.127 | **0.001** |
| **Indiana** | | | | | | | | |
| Source | Nested in | %var | F-stat | F-value | Std.Dev. | c.i.2.5% | c.i.97.5% | P-value |
| Within Individual | -- | 1.037 | R_it | -0.037 | 0.311 | -0.216 | 0.454 | -- |
| Among Individual | Population | -0.092 | R_is | -0.098 | 0.337 | -0.292 | 0.421 | 0.842 |
| Among Population | -- | 0.055 | R_st | 0.055 | 0.018 | -0.001 | 0.1 | **0.046** |
| **Eastern** | | | | | | | | |
| Source | Nested in | %var | F-stat | F-value | Std.Dev. | c.i.2.5% | c.i.97.5% | P-value |
| Within Individual | -- | 0.859 | R_it | 0.141 | 0.289 | 0.02 | 0.679 | -- |
| Among Individual | Population | 0.147 | R_is | 0.146 | 0.264 | 0.044 | 0.681 | 0.123 |
| Among Population | -- | -0.006 | R_st | -0.006 | 0.042 | -0.036 | 0.057 | 0.534 |

## Supplementary Figures

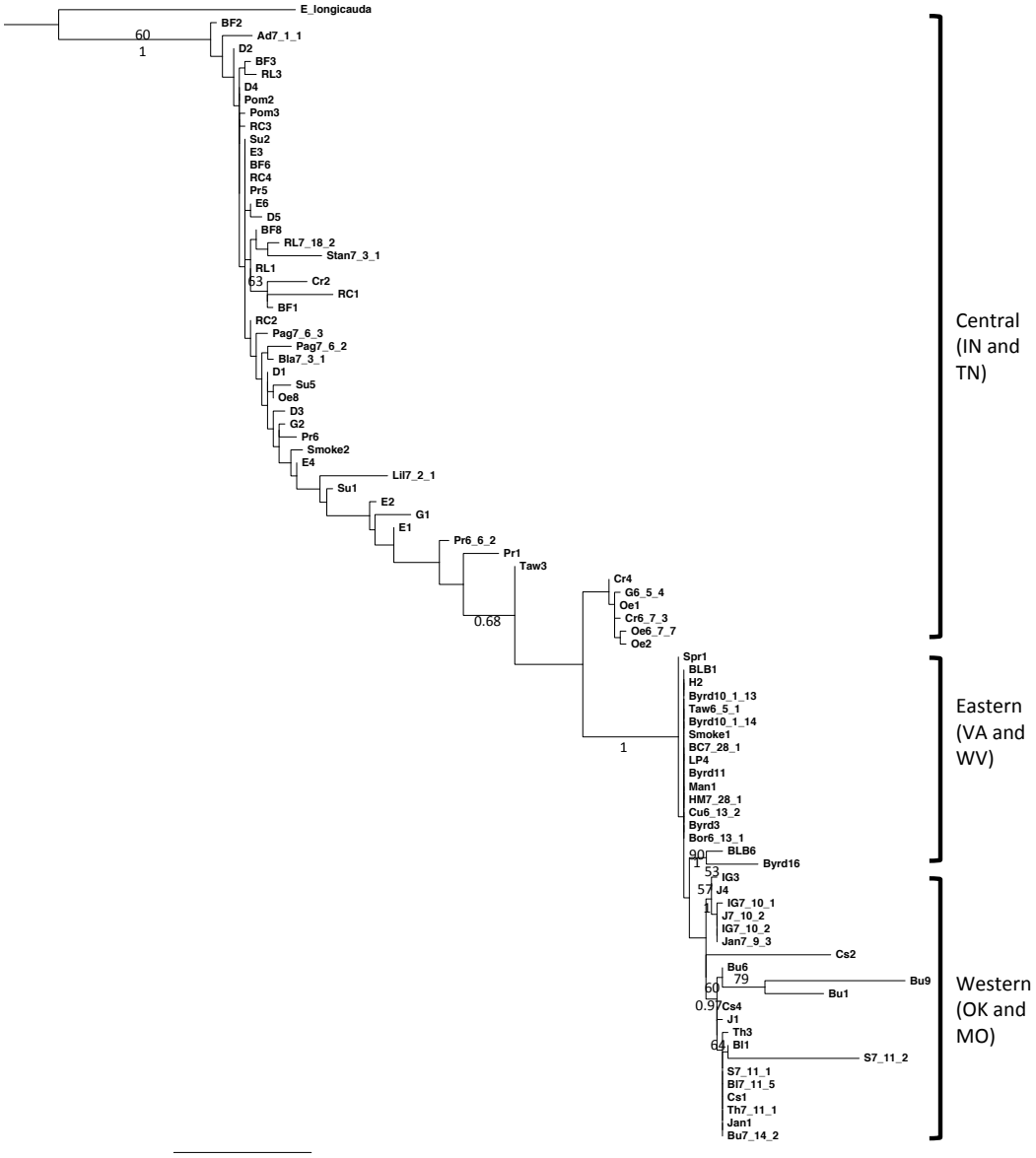


Fig. S1. Maximum likelihood tree of cytb, on which the major geographic regions are labeled. *Pseudotriton ruber* was included as an outgroup, but is not shown here. Branches are labeled with bootstraps above 50. Bayesian reconstructions supported major regional relationships, and posterior support values above 0.5 from the Bayesian reconstruction are included below branches where appropriate.

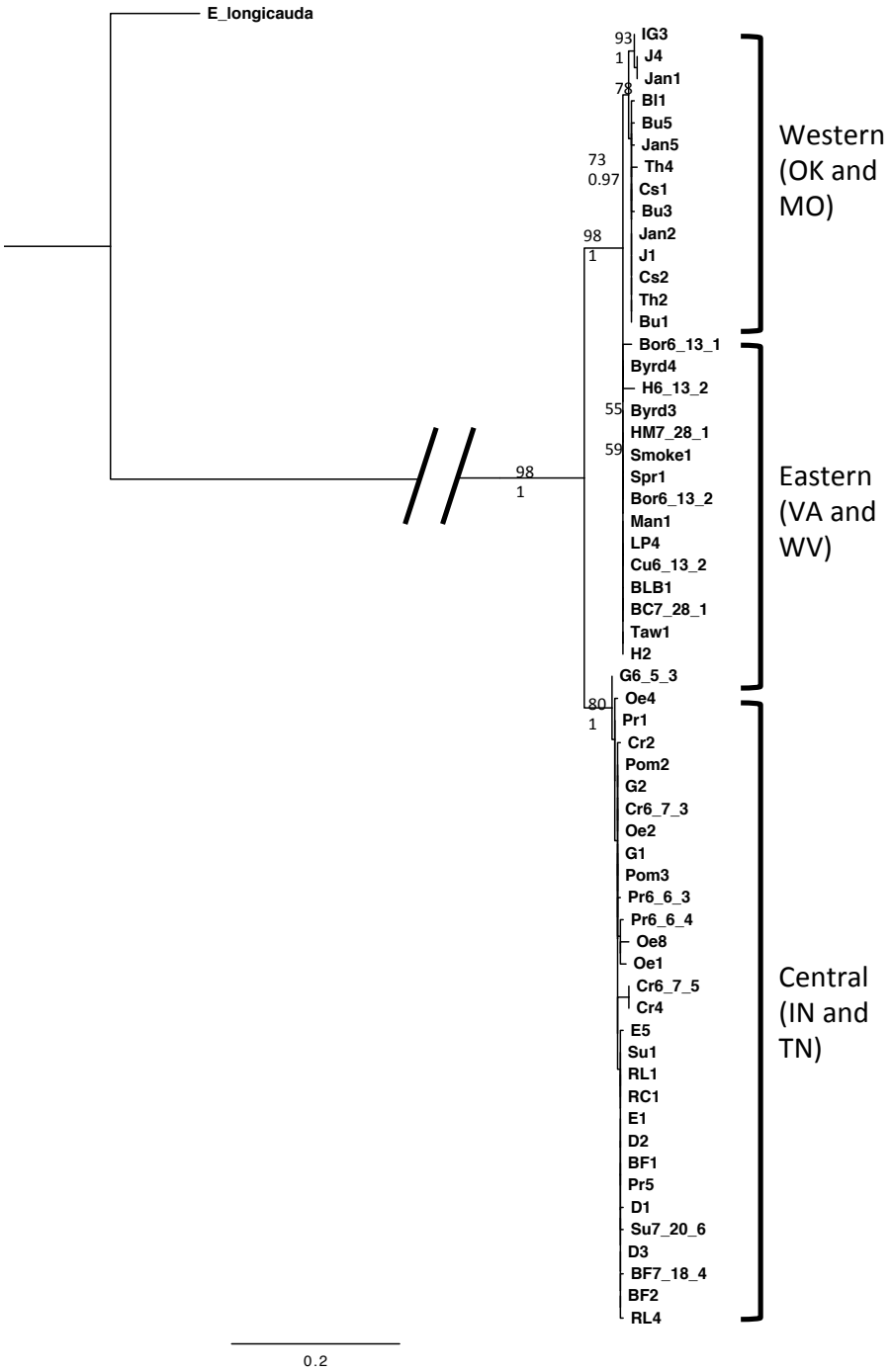


Fig. S2. Maximum likelihood tree of Nd2, on which the major geographic regions are labeled. *Pseudotriton ruber* was included as an outgroup, but is not shown here. Branches are labeled with bootstraps above 50. Bayesian reconstructions supported major regional relationships, and posterior support values above 0.5 from the Bayesian reconstruction are included below branches where appropriate.

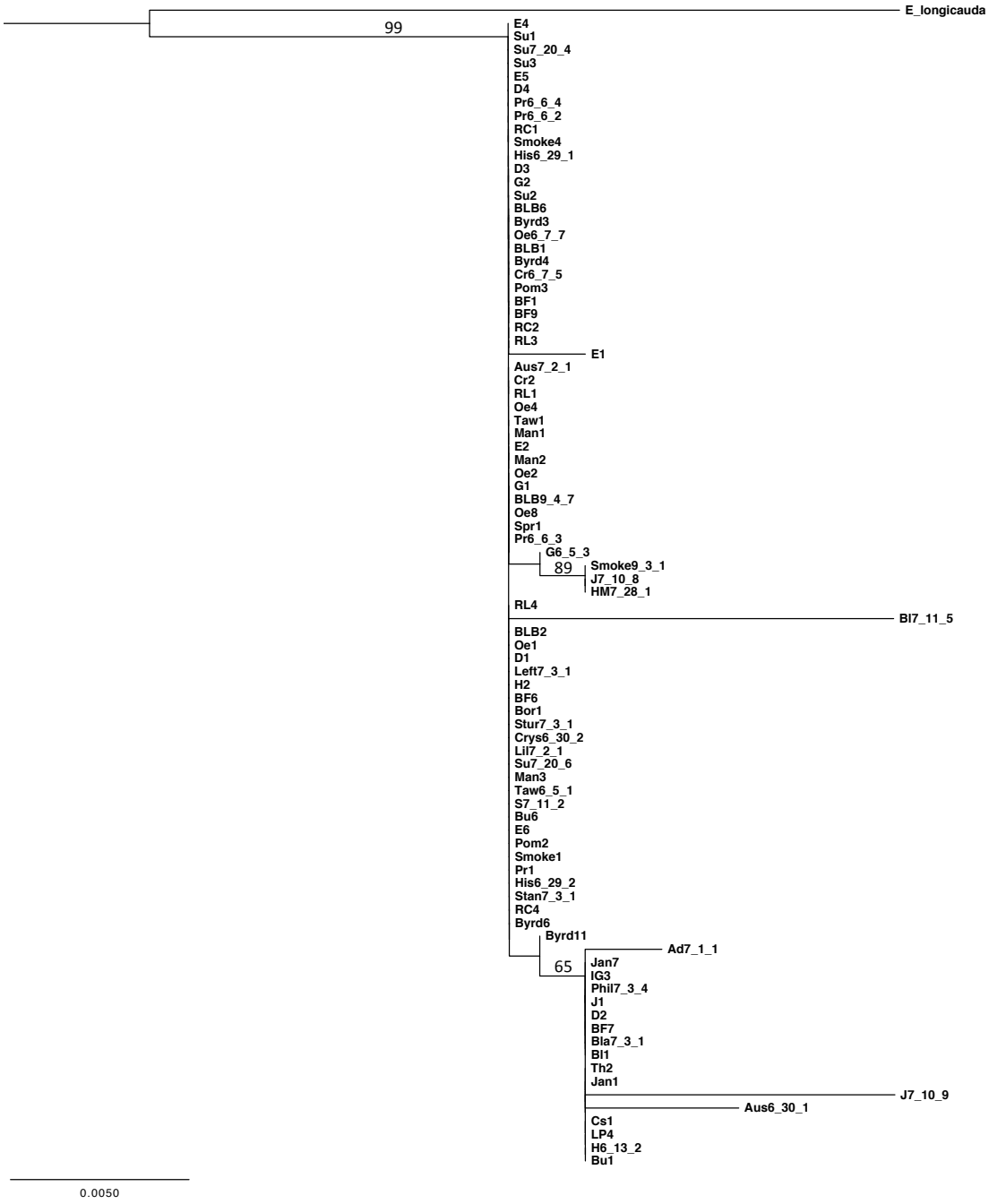


Fig. S3. Maximum likelihood tree of POMC. *Pseudotriton ruber* was included as an outgroup, but is not shown here. Major regional groups were recovered from POMC data in neither the Maximum Likelihood nor the Bayesian tree. Branches are labeled with bootstraps above 50.

## Supplementary Tables

Table S1. Primer sources and sequence for both PCR and sequencing steps, as well as thermocycler conditions for each primer set.

| Primer | Source | Sequence | Cycling conditions |
|---|---|---|---|
| cyt*b*F | Harlan and Zigler (2009) | AAGATTATTAATAACTCCTTTATTGA | Annealing temp. of 50C, 35 cycles |
| cyt*b*R | | AAAATGCTTGTCCAATTTCAAT | |
| ND2F | This study | TACAAGCCTCAGCATCTGCC | Annealing temp. of 59.4C, 30 cycles |
| ND2R | | ATCCAGAGGTTGGTGGGAGT | |
| POMCF | Lamb *et al.* (2012) | ATATGTCATGAGCCATTTTCGCTGGAA | Annealing temp. of 58C, 45 cycles |
| POMCR | | GGCATTTTTGAAAAGAGTCATTAGAGG | |

Table S2.: Collection efforts/results

| Sample ID | Species | State | Cave Location | Date |
|---|---|---|---|---|
| Ad7.1.1 | *Eurycea lucifuga* | Kentucky | Adwell | 7/1/13 |
| BF7.18.1 | *Eurycea lucifuga* | Indiana | Bankley/Fairground | 7/18/12 |
| BF7.18.2 | *Eurycea lucifuga* | Indiana | Bankley/Fairground | 7/18/12 |
| BF7.18.3 | *Eurycea lucifuga* | Indiana | Bankley/Fairground | 7/18/12 |
| BF7.18.6 | *Eurycea lucifuga* | Indiana | Bankley/Fairground | 7/18/12 |
| BF7.18.7 | *Eurycea lucifuga* | Indiana | Bankley/Fairground | 7/18/12 |
| BF7.18.8 | *Eurycea lucifuga* | Indiana | Bankley/Fairground | 7/18/12 |
| BF7.18.9 | *Eurycea lucifuga* | Indiana | Bankley/Fairground | 7/18/12 |
| Bl7.11.1 | *Eurycea lucifuga* | Oklahoma | Blue Moon | 7/11/12 |
| Bla7.3.1 | *Eurycea lucifuga* | Kentucky | Black Rock | 7/3/13 |
| BLB9.4.1 | *Eurycea lucifuga* | Virginia | Blankenship Blowhole | 9/4/11 |
| BLB9.4.2 | *Eurycea lucifuga* | Virginia | Blankenship Blowhole | 9/4/11 |
| BLB9.4.6 | *Eurycea lucifuga* | Virginia | Blankenship Blowhole | 9/4/11 |
| Bor7.28.1 | *Eurycea lucifuga* | West Virginia | Boarhole | 7/29/12 |
| Bu7.14.1 | *Eurycea lucifuga* | Missouri | Bull Creek | 7/14/12 |
| Bu7.14.3 | *Eurycea lucifuga* | Missouri | Bull Creek | 7/14/12 |
| Bu7.14.5 | *Eurycea lucifuga* | Missouri | Bull Creek | 7/14/12 |
| Bu7.14.6 | *Eurycea lucifuga* | Missouri | Bull Creek | 7/14/12 |
| Bu7.14.7 | *Eurycea lucifuga* | Missouri | Bull Creek | 7/14/12 |
| Bu7.14.9 | *Eurycea lucifuga* | Missouri | Bull Creek | 7/14/12 |
| Byrd10.1.11 | *Eurycea lucifuga* | Virginia | Byrd's water | 10/1/11 |
| Byrd10.1.16 | *Eurycea lucifuga* | Virginia | Byrd's water | 10/1/11 |
| Byrd10.1.3 | *Eurycea lucifuga* | Virginia | Byrd's water | 10/1/11 |
| Byrd10.1.6 | *Eurycea lucifuga* | Virginia | Byrd's water | 10/1/11 |
| Cr6.7.2 | *Eurycea lucifuga* | Tennessee | Crews | 6/7/12 |
| Cr6.7.4 | *Eurycea lucifuga* | Tennessee | Crews | 6/7/12 |

| | | | | |
|---|---|---|---|---|
| Cr7.13.1 | *Eurycea lucifuga* | Missouri | Crighton Spring | 7/13/12 |
| Cr7.13.2 | *Eurycea lucifuga* | Missouri | Crighton Spring | 7/13/12 |
| Cr7.13.4 | *Eurycea lucifuga* | Missouri | Crighton Spring | 7/13/12 |
| Cr7.13.5 | *Eurycea lucifuga* | Missouri | Crighton Spring | 7/13/12 |
| D7.19.1 | *Eurycea lucifuga* | Indiana | Donnehue | 7/19/12 |
| D7.19.2 | *Eurycea lucifuga* | Indiana | Donnehue | 7/19/12 |
| D7.19.3 | *Eurycea lucifuga* | Indiana | Donnehue | 7/19/12 |
| D7.19.4 | *Eurycea lucifuga* | Indiana | Donnehue | 7/19/12 |
| D7.19.5 | *Eurycea lucifuga* | Indiana | Donnehue | 7/19/12 |
| E7.20.1 | *Eurycea lucifuga* | Indiana | Lost Cave | 7/20/12 |
| E7.20.2 | *Eurycea lucifuga* | Indiana | Lost Cave | 7/20/12 |
| E7.20.3 | *Eurycea lucifuga* | Indiana | Lost Cave | 7/20/12 |
| E7.20.4 | *Eurycea lucifuga* | Indiana | Lost Cave | 7/20/12 |
| E7.20.5 | *Eurycea lucifuga* | Indiana | Lost Cave | 7/20/12 |
| E7.20.6 | *Eurycea lucifuga* | Indiana | Lost Cave | 7/20/12 |
| G6.5.1 | *Eurycea lucifuga* | Tennessee | Gillespie | 6/5/12 |
| G6.5.2 | *Eurycea lucifuga* | Tennessee | Gillespie | 6/5/12 |
| H6.13.1 | *Eurycea lucifuga* | West Virginia | Higganbotham | 6/13/13 |
| IG7.10.3 | *Eurycea lucifuga* | Oklahoma | Iron Gate | 7/10/12 |
| J7.10.1 | *Eurycea lucifuga* | Oklahoma | Jail | 7/10/12 |
| J7.10.4 | *Eurycea lucifuga* | Oklahoma | Jail | 7/10/12 |
| J7.10.5 | *Eurycea lucifuga* | Oklahoma | Jail | 7/10/12 |
| Jan7.9.1 | *Eurycea lucifuga* | Oklahoma | January-Stansbury | 7/9/12 |
| Jan7.9.2 | *Eurycea lucifuga* | Oklahoma | January-Stansbury | 7/9/12 |
| Jan7.9.5 | *Eurycea lucifuga* | Oklahoma | January-Stansbury | 7/9/12 |
| Jan7.9.7 | *Eurycea lucifuga* | Oklahoma | January-Stansbury | 7/9/12 |
| LP6.3.4 | *Eurycea lucifuga* | Tennessee | Lost Puddle | 6/3/12 |
| Man7.28.1 | *Eurycea lucifuga* | West Virginia | Mann | 7/29/12 |
| Man7.28.2 | *Eurycea lucifuga* | West Virginia | Mann | 7/29/12 |
| Man7.28.3 | *Eurycea lucifuga* | West Virginia | Mann | 7/29/12 |
| Oe6.7.1 | *Eurycea lucifuga* | Tennessee | Eoff | 6/7/12 |
| Oe6.7.2 | *Eurycea lucifuga* | Tennessee | Eoff | 6/7/12 |
| Oe6.7.4 | *Eurycea lucifuga* | Tennessee | Eoff | 6/7/12 |
| Oe6.7.8 | *Eurycea lucifuga* | Tennessee | Eoff | 6/7/12 |
| Pom6.6.2 | *Eurycea lucifuga* | Tennessee | Pompie | 6/6/12 |
| Pom6.6.3 | *Eurycea lucifuga* | Tennessee | Pompie | 6/6/12 |
| Pr6.6.1 | *Eurycea lucifuga* | Tennessee | Prowell | 6/6/12 |
| Pr6.6.5 | *Eurycea lucifuga* | Tennessee | Prowell | 6/6/12 |
| Pr6.6.6 | *Eurycea lucifuga* | Tennessee | Prowell | 6/6/12 |
| RC7.19.1 | *Eurycea lucifuga* | Indiana | Roberts | 7/19/12 |
| RC7.19.2 | *Eurycea lucifuga* | Indiana | Roberts | 7/19/12 |
| RC7.19.3 | *Eurycea lucifuga* | Indiana | Roberts | 7/19/12 |
| RC7.19.4 | *Eurycea lucifuga* | Indiana | Roberts | 7/19/12 |

| | | | | |
|---|---|---|---|---|
| RL7.18.1 | *Eurycea lucifuga* | Indiana | Robinson Ladder | 7/18/12 |
| RL7.18.2 | *Eurycea lucifuga* | Indiana | Robinson Ladder | 7/18/12 |
| RL7.18.3 | *Eurycea lucifuga* | Indiana | Robinson Ladder | 7/18/12 |
| S7.11.1 | *Eurycea lucifuga* | Oklahoma | Survivalist | 7/11/12 |
| S7.11.2 | *Eurycea lucifuga* | Oklahoma | Survivalist | 7/11/12 |
| Smoke1 | *Eurycea lucifuga* | Virginia | Smokehole | 9/3/11 |
| Smoke2 | *Eurycea lucifuga* | Virginia | Smokehole | 9/3/11 |
| Smoke9.3.4 | *Eurycea lucifuga* | Virginia | Smokehole | 9/3/11 |
| Spr7.28.1 | *Eurycea lucifuga* | West Virginia | Spring | 7/29/12 |
| Stan7.2.1 | *Eurycea lucifuga* | Kentucky | Stan's Well | 7/2/13 |
| Su7.20.1 | *Eurycea lucifuga* | Indiana | Sullivan | 7/20/12 |
| Su7.20.2 | *Eurycea lucifuga* | Indiana | Sullivan | 7/20/12 |
| Su7.20.3 | *Eurycea lucifuga* | Indiana | Sullivan | 7/20/12 |
| Su7.20.4 | *Eurycea lucifuga* | Indiana | Sullivan | 7/20/12 |
| Su7.20.5 | *Eurycea lucifuga* | Indiana | Sullivan | 7/20/12 |
| Su7.20.6 | *Eurycea lucifuga* | Indiana | Sullivan | 7/20/12 |
| Taw9.24.1 | *Eurycea lucifuga* | Virginia | Tawney's | 9/24/11 |
| Taw9.24.2 | *Eurycea lucifuga* | Virginia | Tawney's | 9/24/11 |
| Taw9.24.3 | *Eurycea lucifuga* | Virginia | Tawney's | 9/24/11 |
| Th7.11.1 | *Eurycea lucifuga* | Oklahoma | Third | 7/11/12 |
| Th7.11.2 | *Eurycea lucifuga* | Oklahoma | Third | 7/11/12 |
| Th7.11.3 | *Eurycea lucifuga* | Oklahoma | Third | 7/11/12 |
| Th7.11.4 | *Eurycea lucifuga* | Oklahoma | Third | 7/11/12 |

# CHAPTER 3

## POTENTIAL SELECTIVE MAINTENANCE OF COLOR THROUGH NON-CAVE DISPERSAL IN THE CAVE SALAMANDER, *EURYCEA LUCIFUGA* (PLETHODONTIDAE)

**Abstract**

Coloration is important in vertebrate physiological function and communication. The evolution of color in amphibians is thought to be driven largely by predator pressure, which has resulted in bright red and orange patterning in aposematic species and their mimics. In lineages that colonized caves coloring is often lost as a result of relaxed selection, presumably due to lack of light. However, some cave-dwelling salamanders that are not restricted to the cave environment have retained their coloration. We tested for evidence that natural selection maintains the coloration of a brightly colored cave-dwelling salamander, *Eurycea lucifuga,* on different geographic scales: across its range and within a single large cave system. We found through a comparison of phenotypic and neutral genetic differentiation that color variation exhibits different spatial structuring than neutral loci, suggesting that it has been influenced by non-neutral processes. Furthermore, genetic analyses suggest that dispersal among caves occurs predominantly over the surface habitat, which may contribute to selective maintenance of visual traits such as coloration. These results indicate that occasional exposure to environmental selection pressures may influence trait evolution, and also emphasize the importance of choosing appropriate or multiple spatial scales at which to examine evolutionary and ecological dynamics.

**Introduction**

Color patterning has been extensively studied because of its variability and also its clear functional importance to many taxa. Amphibian systems have been the focus of many such studies due to the diversity of coloration even among closely related taxa, and the dynamic selection regimes that characterize coloration evolution (Rudh & Qvarnström 2013). Among amphibian systems, main functions of coloration include thermoregulation, UV protection, communication to mates, and avoidance of predation through crypsis, active defense behaviors, or aposematism (Rudh & Qvarnström 2013). The last of these, aposematism, is thought to be the driver behind some of the most dramatic displays of coloration, and has been the inspiration of research into the proximate and ultimate mechanisms of this type of predator avoidance. Aposematic coloration advertises an animal's toxicity to predators conspicuously, including colors, odors, and behavior (Lindström 1999; Poulton 1890).

Warning coloration is an important strategy for amphibians, since they have small bodies and lack protective structures, and therefore are vulnerable to predation (Rudh & Qvarnström 2013). Although coloration displays are variable, typical warning colors are red, orange, yellow, and white (Lindström 1999). These colors are produced by three types of specialized pigment-containing epidermal cells called chromatophores: xanthophores, which produce yellow and red pigmentation, iridophores, which contain reflecting platelets, and melanophores, which are responsible for darker pigmentations (Rudh & Qvarnström 2013).

Although salamanders tend to be largely nocturnal, it is not uncommon to observe diurnal activity in most species (Brandon & Huheey 1975), and they represent valuable

prey for a wide variety of predators (Petranka 1998). However, the combination of aposematism and a tendency to hide in dry weather make salamanders infrequent prey for some birds, such as the Hermit thrush and barred and screech owls (Jaeger 1981). Nonetheless, the risk of predation is common enough for both aposematism and mimicry of aposematic species to have developed. A common aposematism/mimicry paradigm is that of the 'red salamanders' found in many regions of North America. For example, the brightly colored species *Notophthalmus viridescens*, which is highly toxic, lives in sympatry with two Mullerian mimics which benefit from its warning coloration, *Pseudotriton ruber* (Brandon *et al.* 1979; Brodie 1981; Howard & Brodie 1971) and the red erythristic phase of *Plethodon cinereus* (Tilley *et al.* 1982). Similarly, *Taricha* newts in the western United States have influenced the mimetic coloration of *Ensatina eschscholtzii xanthoptica* (Kuchta & Reeder 2005). Bird predators are able to discriminate between red aposematic or mimic species and their cryptic counterparts (Kraemer & Adams 2014), and red individuals still experience significantly less predation than other morphs (Brodie Jr & Brodie III 1980; Tilley *et al.* 1982). Mimicry in salamanders also requires less precision than has been found in butterfly or wasp-fly systems (Howard & Brodie 1973).

The variety of color patterns exhibited by many salamander species makes the lack of pigmentation in cave-restricted species stand out in stark contrast. There are approximately twelve species and subspecies of cave-obligate salamanders in the contiguous United States, most within the Plethodontid genus *Eurycea* (Culver *et al.* 2000). Cave obligates are well known for the parallel evolution of a suite of traits known as troglomorphy, which include both regressive features such as depigmentation and eye

reduction and constructive features such as enhanced extra-optic sensory structures (Porter 2007). The evolutionary mechanisms responsible for these traits have been well characterized in *Astyanax* cave fish. While some troglomorphic traits such as eye reduction and sensory structures arise through directional selection (Jeffery *et al.* 2003; Jeffery 2009; Yamamoto *et al.* 2004), depigmentation is apparently the result of relaxed selection and subsequent accumulation of loss of function mutations (Protas *et al.* 2007). Despite the repeated evolution of troglomorphy in cave-dwelling taxa (Jones *et al.* 1992), there remains a class of cave species that has not acquired troglomorphic traits. These species, known as troglophiles, maintain permanent populations in caves but anecdotal reports suggest they can visit niches outside of caves in the surface environment (Sket 2008).

Hypotheses regarding why some species become morphologically troglomorphic while others do not include the amount of time a species has inhabited the cave environment, a tendency of troglophiles to live closer to cave entrances where presumably they would be exposed to light conditions, and developmental constraints on troglomorphic features (Pipan & Culver 2012). It has also been suggested that inbreeding depression prevents the formation of viable troglomorphic population because of their isolation and small population numbers (Avise & Selander 1972). Some of these hypotheses are not well supported: for example, the prevalence of young troglomorphic populations (e.g. *Astyanax* fish (Strecker *et al.* 2004), *Cixiidae* plant hoppers (Wessel *et al.* 2007), *Nesticella* spiders (Zhang & Li 2013)) leads to the conclusion that a cave lineage's age is not correlated with its degree of troglomorphy (Wessel *et al.* 2007). Exposure to a photic environment such as in the entrances of caves does seem like a

likely contributing factor to an avoidance of troglomorphy, though troglophilic salamanders show a preference for deeper, darker, more humid caves (Ficetola *et al.* 2012, 2013; Lunghi *et al.* 2014). Exposure to light may be more common in troglophiles due to their tendency to move among caves more than troglomorphic cave species (Caccone 1985). Though troglomorphic species do occasionally leave the caves, particularly at night (Schlegel *et al.* 2009), common use of the surface habitat during dispersal by troglophiles may impose selection pressure on coloration as a predator defense strategy, leading to its persistence in troglophilic populations.

In this study, we explore the maintenance of pigmentation in troglophiles. We tested whether selection influences coloration by comparing phenotypic differentiation and neutral genetic variation in a troglophilic salamander, *Eurycea lucifuga* at both broad and fine spatial scales. In order to determine whether exposure to the surface environment plays a part in the selective maintenance of color, we also characterized dispersal within a large cave system, focusing on whether gene flow occurs across the surface habitat as opposed to through subterranean routes.

**Methods**

*Study system*

*Eurycea lucifuga*, the Cave Salamander, is a troglophilic salamander commonly found in caves throughout the Central and Southeast United States. It belongs to a genus in which cave-dwelling and troglomorphy have evolved independently multiple times (Bonett *et al.* 2014). The coloration of *E. lucifuga* as adults is very similar to that of other 'red salamanders': the body is bright orange, with black spots covering the entire surface (Fig. 1). It is unknown whether, like most members of Plethodontinae (Brodie

1977), *E. lucifuga* is noxious to predators; however, the larvae are palatable to certain fish predators (Kats *et al.* 1988). In addition to color patterning, *E. lucifuga* has a characteristic anti-predator posture similar to that of many Plethodontids, in which the animals contort their torso into a circular posture while pointing their tail straight up, and undulating the tail. This behavior is effective in deterring predation by shrews by directing them to the tail, which when bitten exposes the predator to the noxious secretions and is autotomizable (Brodie *et al.* 1979). Though *E. lucifuga* spends the majority of its adult life in the crevices of cave floors and walls, and is obligately associated with caves during the egg and larval stages, anecdotal reports suggest it is occasionally found outside of caves (Hutchison 1958; Petranka 1998).

*Sample collection and data compilation*

Over the months of May through July during 2012-2014 we sampled 1-12 individuals of *Eurycea lucifuga* from 27 populations located in Tennessee, Kentucky, Oklahoma, Missouri, and Indiana for a total of 153 individuals (Fig. 2; Table S1). We first collected tail clips to genotype each individual. We also photographed each individual with a Pentax Optio WG-2 digital camera from a distance of twelve inches with the flash on. In each photograph we included as a color standard a paint sample card (color BHG511).

Tail tissues were stored at room temperature in 95% ethanol until total genomic DNA was extracted from each using either a phenol-chloroform (Sambrook *et al.* 1989) or chelex (using a 5-10% slurry of Chelex and an incubation time of 180 minutes at 95°C) extraction method (Walsh *et al.* 1991). We genotyped each individual at 19 microsatellite loci developed for this species (Appendix A) by amplification in

multiplexed reactions using the Polymerase Chain Reaction (PCR), and products were sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT) for fragment analysis using a 3730*xl* 96-capillary Genetic Analyzer with the DS-33 dye set. We used the software GeneMarker (SoftGenetics LLC, State College, PA) to call alleles at each locus for each individual.

We digitally analyzed the photographs using the software program ImageJ (Rasband 1997-2014) First we measured values of Red, Green and Blue (RGB) from an area in the orange background skin, from a black spot, and from the color standard in the photograph. Color measurements on each salamander were made on the dorsum between the fore- and hindlimbs; care was taken to avoid light fluctuations such as shine spots. All RGB measurements were converted to Hue, Saturation, and Value (HSV) using the function rgb2hsv() in the R statistical package rgDevices (R Core Team), in R v3.1.2 (R Development Core Team, 2008) interfaced with RStudio v.00.98.1091(RStudio, Inc.). We regressed HSV for orange background and black spot onto the corresponding standard trait using the function lm() in the R statistical package *stats* (R Core Team) to obtain the residuals for further analysis. Following the example of Zhou *et al.* (2014) we perfromed Principal Components Analysis (PCA) on hue, saturation, and value for both the orange background and black spots in order to examine a trait for each representing the greatest amount of variation. We used the function prcomp() in the R package *stats*. Next, a rectangular area was selected on the dorsum of each salamander midway between the forelimb and hind limb junctions, approximately as wide as the salamander. We measured the area of all of the spots within that rectangle as well as the total area of the selection, and divided spot area by the total selection area to produce a black spot:orange

background ratio trait. Thus, the three traits we examine in the subsequent analyses were the first principal component (PC1) of the orange background ("orange PC1"), PC1 of the black spots ("black PC1"), and the ratio of spot area to total area ("black:orange ratio"). The majority of variance (86%) in orange PC1 is represented by strong negative loadings of saturation and value, with little variation seen in the hue of the orange background. The first principal component representing black PC1 comprises 65% of the variance, and represents strong positive loadings of all color components, hue, saturation, and value (Table 1).

To complement our range-wide sampling scheme with fine-scale sampling, we extensively sampled *E. lucifuga* within one local area, the Mammoth Cave National Park (Edmonson, KY). We collected from 13 cave entrances, with the goal of capturing individuals from caves connected to the main Mammoth Cave system as well as those isolated from the system (Fig. 3). We obtained information about the subterranean connectedness of the cave entrances from park officials, and recorded whether or not they are separated by the Green River, which may present a potential barrier to above-ground dispersal. As with the range-wide data, we performed PCA to obtain traits representing the majority of variation in both the orange background and black spots. The first principal component representing variation in the orange background trait ("orange PC1") accounts for 96% of variance, and is largely representative of strong positive loadings on saturation and value. PC1 for the black spot trait ("black PC1") accounts for 59% of variation, and represents equally strong loadings for each color component (Table 1).

*Analyses of genetic and phenotypic variance*

To determine population genetic structure, we used the program GenoDive (Meirmans & Van Tienderen 2004) to produce a table of pairwise $F_{ST}$ values for each population in the dataset, replacing any negative values with zero. Additionally, we used the function earth.dist() in the R statistical package fossil (Vavrek 2011) to produce a matrix of pairwise geographic distances.

We followed the methods of Antoniazza *et al.* (2010), Manier *et al.* (2007), and Berardi *et al.* (In review) to calculate values of $P_{ST}$ as estimations of phenotypic differentiation analogous to $F_{ST}$. This method is an adaptation of the $F_{ST}$-$Q_{ST}$ comparison, in which differentiation in neutral genetic variation is compared with a representation of among-population variation in a standardized quantitative trait (Spitze 1993). However, because calculations of $Q_{ST}$ require estimates of additive genetic variance that are often unavailable in wild populations, the phenotypic equivalent, $P_{ST}$, is used in substitution (Sæther *et al.* 2007). For each color trait we used the results of a linear model to populate two matrices containing among- and within-population pairwise mean squares. We divided the difference between these matrices by the weighted number of populations, which resulted in a matrix of between-population variance ($\sigma^2b$). These $\sigma^2b$ values were used with the within-population variances ($\sigma^2w$, approximated by the within-population mean squares) to produce pairwise estimates of phenotypic structure, $P_{ST,}$ using the following equation:

$$P_{ST} = \frac{g\,\sigma^2 b}{(g\sigma^2 b) + (2h^2\,\sigma^2 w)}$$

where g is approximated by among-population genetic variance and $h^2$ is heritability. $P_{ST}$ is commonly used to detect evidence of local adaptation or stabilizing selection on

phenotypic traits (Antoniazza *et al.* 2010; Berardi *et al.* In review; Sæther *et al.* 2007; Spitze 1993).

As neither g nor $h^2$ have been estimated for this species, we looked to the literature for guidance on estimates to use in our calculations. Although heritability of color traits has not been estimated in any close relatives, the prediction that heritability in color traits in anurans is predicted to be high (Hoffman & Blouin 2004) led us to follow the example of other $F_{ST}$-$P_{ST}$ comparisons in conducting our analyses with a heritability of 0.5 (Merilä & Crnokrak 2001; Storz 2002; Wilson *et al.* 2013). As other studies comparing phenotypic and neutral genetic structure have used additive genetic variances of 1 (Alho *et al.* 2010; Antoniazza *et al.* 2010; Leinonen *et al.* 2006; Manier *et al.* 2007), and this is within the range observed in Hansen (2011). Thus, we used this estimation of g=1. We therefore proceeded with a heritability of 0.5 and additive genetic variance of 1 for our traits, but interpretation of these results must take into account that much higher or much lower heritability and additive genetic variance could affect the results of our analyses.

To understand the implications of these assumptions on our results, we performed sensitivity analyses of our $P_{ST}$ calculations by permuting them using three values of g (0.01, 0.1, and 1) and five values of $h^2$ (0.1, 0.25, 0.5, 0.75, and 1) (Antoniazza *et al.* 2010; Berardi *et al.* In review; Manier *et al.* 2007). This gives us an understanding of how varying these parameters will impact our analyses.

*Statistical Analyses: Gene flow across the range*

First we examined how phenotype and population genetic structure vary across geographic distance. We used the standardized color traits to test whether color varies

across the range of *Eurycea lucifuga*. We used a nested linear model with the R function lm() to test whether traits varied among populations and among the major genetically differentiated clades in this species (see Chapter 2). Type II tests were performed with the Anova() function in the package *car* (Fox & Weisberg 2011). We also tested whether phenotypic differentiation varies across the range based on geographic distance with a Mantel test, including pairwise $P_{ST}$ as the dependent variable and geographic distance as the independent variable. We used the mantel() function in the R statistical package *vegan* (Oksanen *et al.* 2014) to perform Mantel tests, using the Pearson method with 1000 permutations. We next tested for isolation by distance by comparing pairwise $F_{ST}$ with geographic distance using a linear model with the function lm(), as well as including $F_{ST}$ and geographic distance in a Mantel test with the function mantel().

To compare phenotypic variation with neutral genetic variation, we compared estimates of pairwise differentiation across the range. For each color trait we compared phenotypic and genetic variation while controlling for geographic distance using a partial Mantel test with the function mantel(). In each Mantel model we included pairwise $P_{ST}$ as the x distance variable, pairwise $F_{ST}$ as the y distance variable, and pairwise geographic distance as the z distance variable. By testing for correlations between $P_{ST}$ and $F_{ST}$ while controlling for geographic distance we were able to infer whether or not each color trait exhibits differentiation that is consistent with what we expect based on neutral evolutionary processes. If pairwise $P_{ST}$ and pairwise $F_{ST}$ are significantly correlated, we infer that similar neutral processes have influenced the evolution of both; however, a lack of significant correlation suggests that the pattern of differentiation in the color trait has been influenced by either diversifying or stabilizing selection depending on

the relationship of $P_{ST}$ and $F_{ST}$ (Antoniazza *et al.* 2010; Whitlock 2008). We also directly compared global $P_{ST}$ and $F_{ST}$ using student's t-tests with the function t.test() in the R package to test whether overall there is greater phenotypic or neutral genetic divergence across the range.

Following this, we ran the same analyses on a smaller-scale dataset containing only populations from Mammoth Cave National Park to test whether the relationship between phenotypic and genetic differentiation changed at different spatial scales. The Mammoth analyses used the same methods as we described previously.

*Statistical analyses: Gene flow at small scales*

We next used comparisons among genetic distance, geographic distance, and subterranean and surface connectivity to infer whether dispersal and/or gene flow occurs primarily over the surface or underground in *Eurycea lucifuga*. We used only the pairwise $F_{ST}$ and pairwise geographic distances from the populations within Mammoth Cave National Park (MCNP). We then manually populated a binary 'cave connection' matrix, indicating whether intersecting populations were connected or unconnected with each other via the main Mammoth Cave system. Lastly, we manually populated a binary 'river connection' matrix, indicating whether populations are on the same side ('connected') or opposite sides ('unconnected') of the Green River, which runs through the park.

We first established whether our analyses may be biased by the arrangement of the cave entrances (e.g. if all cave entrances with subterranean connections were spatially clustered), so we tested the data for differences in pairwise geographic distance among connected and unconnected caves in a linear model. A second linear model tested the

effect of the two connectivity parameters (river and cave connectivity) on genetic distance, including geographic distance as a covariate. Both linear models were constructed in the function lm(), and Type II tests were implemented using the Anova() function. We then used Analysis of Molecular Variance in the software GenoDive with 999 permutations to examine how genetic variation was partitioned among the Mammoth Cave National Park populations. One model was run including each population's position relative to the Green River (North or South) as regional groups, and a second model included whether each population was connected to the Mammoth Cave system or not as groups. Finally, to better visualize relatedness among populations in the context of their connectedness below- and above-ground, we performed a Principal Coordinate Analysis using the function pco() in the R package labdsv (Roberts 2013). This analysis projects measures of distance (in this case, genetic distance) in multiple dimensions, then reduces the dimensionality using Principal Components Analysis on the projection. The result is a visualization of genetic distance among cave entrance populations.

**Results**

*Range-wide analysis*

A visual summary of the means and standard errors of each principal component among the populations indicates that variation is greater in black spot PC1 than in either the orange background PC1 or the black:orange ratio (Figure 4). Most of this variation seems to exist within the central region, both in among- and within-population variance.

Results of an ANOVA testing for differences in color traits among regions indicate that there are no significant differences in orange PC1 or the black:orange ratio across the range of *Eurycea lucifuga*, but that black PC1 does vary significantly among

regions (Table 2). Mantel tests indicate that there is no significant correlation between pairwise geographic distance and orange PC1 (Mantel's r=0.0335, p=0.335), black PC1 (Mantel's r=0.062, p=0.238), or black:orange ratio (Mantel's r=0.038, p=0.299). In contrast, we did find significant isolation by distance in a Mantel test including pairwise $F_{ST}$ and geographic distance (Mantel's r=0.933, p<0.001).

We found no significant correlations between pairwise $P_{ST}$ and pairwise $F_{ST}$ when controlling for pairwise geographic distance in partial Mantel tests including orange PC1 or black PC1, which suggests that variation in these traits has not been structured by the neutral processes influencing neutral loci. Differentiation in orange PC1 increases faster across geographic distance than does $F_{ST}$, while differentiation in black PC1 increases slower than $F_{ST}$. However, the partial Mantel test for correlation between $P_{ST}$ and $F_{ST}$ including the black:orange ratio indicate a significant correlation between phenotypic and neutral genetic differentiation (Table 3; Figure 5). Significant differences between the global means of $P_{ST}$ and $F_{ST}$ for both orange PC1 and black PC1 but not black:orange ratio are consistent with the results of the Mantel tests (Table 4). A difference in the spatial structuring of variation in phenotypic traits and neutral genetic variation suggests that selection may be acting on both orange PC1 and black PC1. In contrast, these results suggest that color patterning, represented by the black:orange ratio, has been largely influenced by neutral processes.

*Mammoth Cave National Park $P_{ST}$-$F_{ST}$ comparisons*

ANOVA results at this much smaller scale (0.35-10.69km) indicate that both black PC1 and black:orange ratio differed significantly among Mammoth populations (Table 2). Tests for correlations between color trait differentiation and pairwise

geographic distance using Mantel tests were nonsignificant for orange PC1 (Mantel's r=-0.020, p=0.524), black PC1 (Mantel's r=-0.138, p=0.799), and black:orange ratio (Mantel's r=-0.057, p=0.629). Likewise, there was no evidence of genetic isolation by distance among the Mammoth populations (Mantel's r=-0.142, p=0.791).

When we performed partial Mantel tests to examine correlation between $P_{ST}$ and $F_{ST}$ while accounting for geographic distance, we found that there were no significant correlations for any of the three traits, although the relationship between black:orange ratio and $F_{ST}$ was approaching significance (Table 3). However, t-tests comparing the means of $P_{ST}$ and $F_{ST}$ for each color trait indicated that all three traits differed significantly from mean neutral genetic differentiation, with $P_{ST}$ greater than $F_{ST}$ in each case (Table 4).

*Sensitivity analyses*

Our sensitivity analyses indicate that heritability only moderately affects the estimation of $P_{ST}$. However, different values of *g* (the additive genetic variation among populations) strongly influence the magnitude of $P_{ST}$ and therefore its relationship with $F_{ST}$ (Fig. 6, Table S2). It is possible that if g is smaller than the estimate we chose to work with, $P_{ST}$ may be significantly lower than $F_{ST}$ in all traits and at all spatial scales. This would suggest that stabilizing selection has acted on color. Because of our inability to accurately predict the correct estimates of $h^2$ and g, we are able to make better predictions about the difference in spatial structuring of phenotypic and neutral genetic variation than about direct comparisons between the two estimates of differentiation.

*Mammoth Cave National Park genetic and geological comparisons*

There was no significant difference in geographic distance among caves connected as part of the Mammoth Cave System versus unconnected caves ($F=1.814$; $DF=1,180$; $p=0.18$), indicating that genetic differentiation among these two groups of caves was not due to differences in the spatial distribution of cave entrances. Whether or not two cave entrances are connected within the Mammoth Cave system had a significant impact on the genetic distance between those populations ($F=11.391$; $DF=1,179$; $p<0.001$). Unexpectedly, populations connected underground through the Mammoth Cave complex exhibited greater genetic differentiation than populations outside the Mammoth complex, indicating that underground connections present a barrier to gene flow relative to surface distance. Finally, populations on the same side of the Green River were significantly less genetically differentiated than those on opposing sides of the river ($F=4.131$, $DF=1,178$, $p=0.044$), demonstrating the Green River has been a significant migratory barrier for this species (Figure 7).

Estimates of population structure indicate that the vast majority of variance in Mammoth Cave National Park is contained within individuals (approximately 84%), and most of the rest of the genetic variance is partitioned among individuals within populations (approximately 13%). Very little variance was partitioned among groups when we included either connectedness with the Mammoth cave system or via the Green River in the AMOVA model (Table 5). These patterns of genetic isolation can be detected visually using Principal Coordinate Analysis to cluster populations (Figure 8). Populations on either side of the Green River were similarly genetically variable (i.e. they spanned a similar broad range of both PCo Axes), but for populations south of the Green

River the locations along the two PCo Axes co-varied in a positive way whereas the opposite was true for populations North of the Green River which likely accounts for the genetic differentiation between the two groups. South of the Green River, populations collected from Mammoth Cave spanned the entire range of genetic variation on both axes, whereas populations not connected to the Mammoth Cave complex had less genetic variance along the first PCo Axis. (Figure 8).

Taken together, significant isolation by distance at larger spatial scales, genetic differentiation across the Green River, and genetic differentiation among cave populations known to be interconnected by subterranean aquatic corridors suggest migration is achieved primarily by surface terrestrial routes in this species.

**Discussion**

We compared differentiation in color traits with that of neutral genetic variation in *Eurycea lucifuga* at different spatial scales in order to assess the selective maintenance of warning coloration in a cave-dwelling salamander. We found that most of the variance in the orange background among salamanders is accounted for by variation in the saturation and value of the orange, and not the hue itself. However, hue, saturation, and value all contributed equally to the variance seen in the black spot trait. We found significant differences in black spot traits across the range, but not the orange background nor the ratio of spot to background. Additionally, there were several indications that non-neutral processes have affected some of these traits: First, we saw that differentiation in the orange and black traits exhibited different spatial structuring than neutral genetic differentiation. Second, while the black spot trait exhibited no isolation by distance within the Mammoth Cave National Park, and differentiation was not correlated with that

of neutral genetic variation, there were significant differences in black spots among Mammoth populations, which suggests that the structure of variation is influenced by processes other than those influencing the neutral genetic differentiation. Third, our comparison of genetic differentiation among Mammoth populations with subterranean and surface barriers provided a potential explanation for non-neutral influences on color traits: that dispersal most likely occurs across the surface habitat in this species rather than through cave conduits. However, it is important to note that our interpretations of whether color is under diversifying or stabilizing selection depends on the additive genetic variance in these traits, which is unknown.

We also found differences in the spatial structure of the different color traits, suggesting that they are influenced by different selective or neutral forces. Patterns of trait variation differ between the orange background and black spot traits and the black to orange ratio, with the orange background and black to orange ratio exhibiting smaller differences among means as well as smaller standard errors than the black spots. Variation among populations in black spots is especially notable among central populations, which is the most variable of the regions. Comparisons of the spatial structuring of variation between these color traits and neutral genetic differentiation suggests that both the black and orange color traits have been influenced by non-neutral processes, but that the ratio of black to orange may have experienced a relaxation of selection and has been influenced by neutral processes.

Sensitivity analyses indicate that our estimates of $P_{ST}$ depend on the amount of additive genetic variance we included in our models for each trait. While we would not expect to see a change in the spatial relationships between $F_{ST}$ and $P_{ST}$ over distance, a

change in additive genetic variance in color traits would alter the magnitude of $P_{ST}$ for each trait. This would alter our interpretations of how selection may be acting on these traits. For example, though phenotypic differentiation in the orange hue is greater at all scales than neutral genetic differentiation, as described above, if additive genetic variance plays a smaller role in the variance of that trait it may change the relationship and suggest a neutral process or purifying selection. While our results support a role of possible selective maintenance of color traits, without a better estimation of both heritability and additive genetic variance we are better able to make inferences about the differences in spatial structure between $P_{ST}$ and $F_{ST}$ than about the role of different selective forces.

The results of this study reinforce a suggestion made by others that the scale at which a study is conducted has important effects on its results and implications in studies of both population and ecological dynamics as well as selection analyses (Addicott *et al.* 1987; Barton & Slatkin 1986; Levene 1953; Svensson & Sinervo 2004). For example, Svensson and Sinervo (2004) found that variation in local selective regimes was undetected when data were analyzed on a global scale, which suggests that not only should scale be an important parameter when designing studies, taking into account aspects of a species' ecology such as dispersal and predation, but that analysis at multiple scales is a valuable tool for understanding selection regimes. In our case, had we restricted our comparison of phenotypic and neutral genetic variation to a local scale we would have observed that phenotypic differentiation is great than neutral genetic differentiation and hypothesized that this was due to local adaptation, but ignored the important role that spatial structuring plays in variation of color traits in this species.

However, by examining color traits and neutral genetic loci at both range-wide and local scales, we were able to capture the complicated relationships among their variation.

There are many possible explanations for why and how spatial structuring of color variation may differ from neutral expectations in this system. First, our finding that fine-scale genetic differentiation is influenced more by surface features than cave features suggests that movement across the surface may expose individuals to environmental and community dynamics that make particular color patterns beneficial as visual cues. A potential role for the bright coloration in *Eurycea lucifuga* is aposemetism or mimicry, as is seen in other 'red salamander' systems (Howard & Brodie 1971; Kuchta & Reeder 2005; Tilley *et al.* 1982). Salamanders are generally profitable prey for animals like birds, mammals, snakes, fishes, turtles, frogs, crayfish, predatory insects, and other salamanders (Petranka 1998), and the selective pressure imposed by predators could account for the lack of variation in the orange background trait at both rangewide and small scales, as well as its differential spatial relationship with neutral genetic variation. It is particularly suggestive that the hue of the orange background is particularly invariant compared to the saturation and value components of background color. Predation pressure during dispersal across the surface habitat plausibly explains this lack of variation. Though we cannot address specific correlations between the variation we found in color traits and the ability of predators to perceive those differences, it has been shown in birds that red coloration promotes predator avoidance (Brodie Jr & Brodie III 1980; Tilley *et al.* 1982), but that patterning does not impact a predator's ability to learn avoidance (Aronsson & Gamberale-Stille 2008). This may explain why we see potentially evidence of selection on orange color but not spot patterning.

Selection from predator pressure is by no means the only potential explanation for maintenance of color in *Eurycea lucifuga*. Color has also been found to play physiological roles that may drive spatial variation, as in the thermoregulatory properties underlying feather color in bearded vultures *Gypaetus barbatus barbatus* (Margalida *et al.* 2008). In many species coloration is reflective of individual condition. For example, different color morphs in the wall lizard, *Podarcis muralis*, exhibit correlated differences in body size, parasite prevalence and infection intensity, running stamina, and immune function (Calsbeek *et al.* 2010). Similarly, color variation has been found to be an honest signal of parasitism, which influences mate choice in fence lizards, *Sceloperus occidentalis* (Ressel & Schall 1989), and of general body condition in house finches, *Carpodacus mexicanus* (Hill 2000). Color pattern variation rather than color variation can also indicate poor condition, as in the correlation between asymmetrical spot numbers and body asymmetricality in the spotted salamander, *Ambystoma maculatum* (Davis & Maerz 07). Coloration has also been shown to be an important component of mate attraction and courtship, especially in species with a visual courtship display such as red-spotted newts, *Notophthalmus v. viridescens* (Davis & Grayson 2008) and the ruff, *Philomachus pugnax* (Widemo 1998). There can also be large effects of ontogeny and development on coloration and color patterning, as McClureand McCune (2003) found in zebrafish, in which pigment patterning was influenced by changes in size and shape during growth. Arizona tiger salamanders, *Ambystoma tigrinum*, experience change in coloration over time, growing paler as they age (Fernandez & Collins 1988). Finally, neutral genetic processes could explain the differences in spatial structure of phenotype and genotype we see, through processes such as bottleneck events during range

expansion, or a homogenizing effect of local differentiation across regions. Direct measurements of selection in this species will help us better understand which processes are influencing color traits in *Eurycea lucifuga*.

The results of our examination of dispersal within Mammoth Cave National Park suggest migration occurs primarily via surface dispersal, but the patterns of dispersal are likely to be more complex. The greater genetic distance between caves connected by the Mammoth Cave system is primarily the result of three populations (Historical, New Discovery and Violet City) that span nearly all the range of genetic divergence across PCoA1 and PCoA2 despite being relatively close geographically (Figure 8). We hypothesize that the historical dispersal among these cave entrances are likely to be a complex mix of colonization, extinction and founder effects. These populations are also embedded within a ridge and valley system that may drive patterns of land-based dispersal.

Finally, our results offer a striking contrast with a similar European terrestrial troglophilic salamander genus, *Hydromantes*, which exhibits very little dispersal among caves, and for whom the surface habitat is clearly a barrier (Chiari *et al.* 2012). Troglophilic cricket populations also exhibit an avoidance of surface habitats, and restrictedness to cave habitats (Caccone & Sbordoni 1987). Invertebrate troglophiles have also shown variance in their dispersal capabilities, resulting from both environmental variation as well as intrinsic phenotypic traits (Caccone 1985). Terrestriality has not proved to be a barrier to subterranean dispersal in other cave-dwelling species in the Mammoth Cave system. The cave beetle, *Ptomophagus hirtus*, for example, exhibits frequent dispersal across the Green River, which has led to

hypotheses that dispersal occurs via hydrological conduits underground despite this species' typical avoidance of aquatic habitats (Laing *et al.* 1976). High genetic uniformity among populations has also been found in other invertebrate species that typically avoid the surface habitat, the cave cricket *Hadenoecus subterraneus* (Caccone & Sbordoni 1987) and the Kentucky cave beetle, *Neaphaenops tellkampfii* (Giuseffi et al. 1978). However, dispersal abilities of the vertebrate inhabitants of Mammoth Cave have not been examined. Many of the unique aspects of *Eurycea lucifuga's* biology are likely the result of the fact that despite being a cave inhabitant, subterranean aquatic habitats rather than surface habitats present the most significant barriers to dispersal. It is interesting to note that many of our generalities about adaptation to the cave environment stem from aquatic species and invertebrates that might follow a very different course of dispersal and adaptive evolution after moving underground.

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**Figures**:

Figure 1. Adult *Eurycea lucifuga*, on its most common substrate, the rock wall of caves.

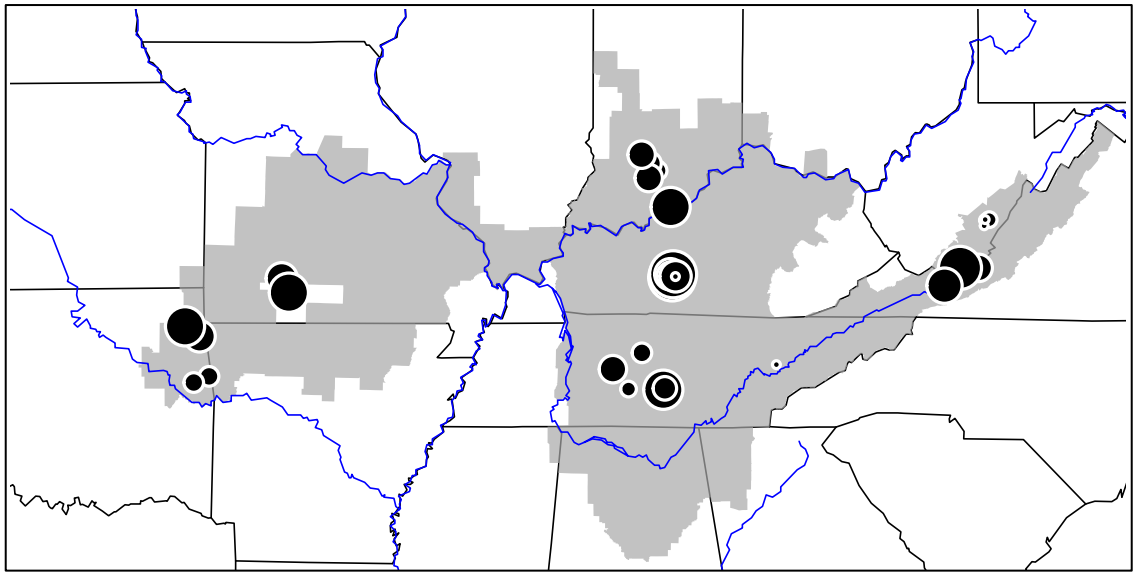

Figure 2. Collection locations (black markers) superimposed over the range of *Eurycea lucifuga* (grey). Size of locality markers is representative of the number of samples collected from that locality (ranging from 1 to 11).

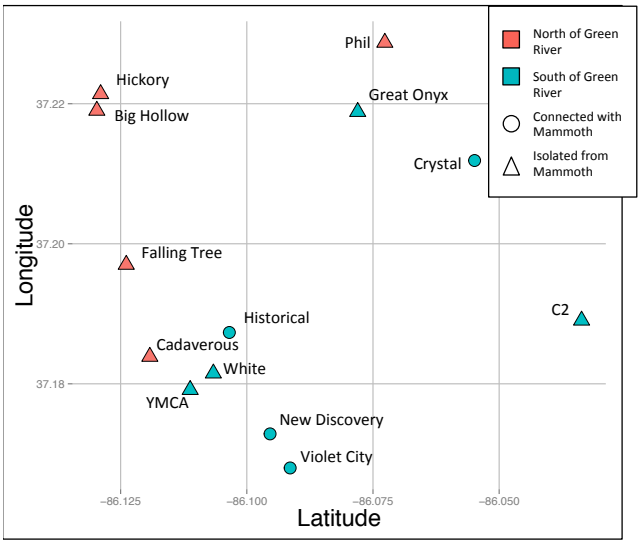


Figure 3. Geographic distribution of collecting localities within the local area of Mammoth Cave National Park. Colors and shapes represent connection underground via the Mammoth Cave system, and aboveground in relation to the Green River, as shown in the legend.

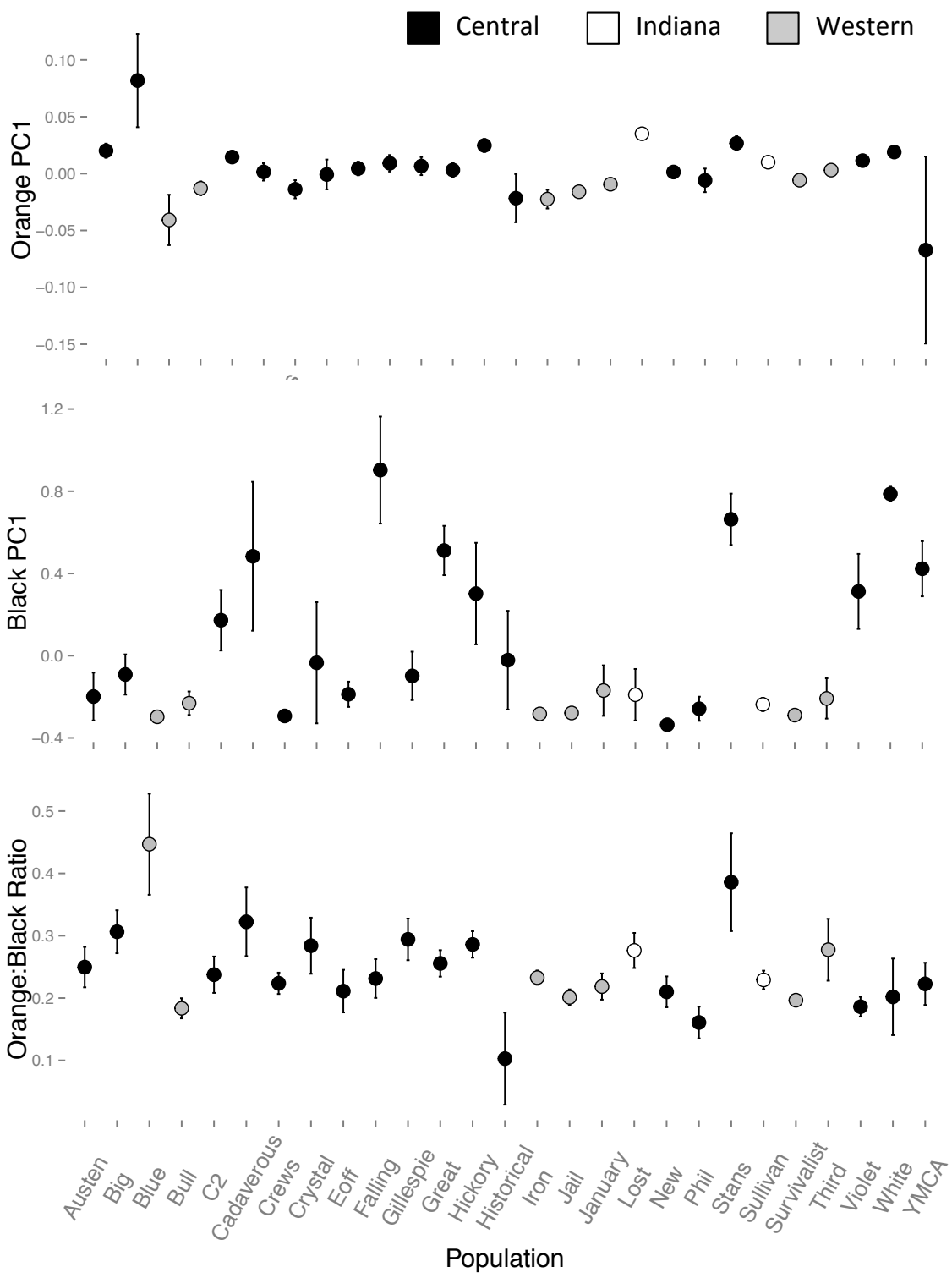


Figure 4. Means and standard errors of each color trait within each population across the range. Shades indicate regions within the range of *Eurycea lucifuga*- black=central, white=Indiana, grey=Western.

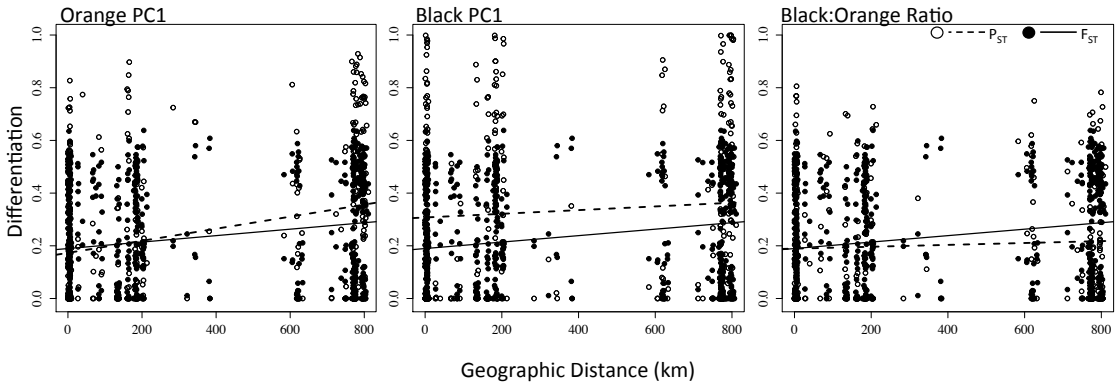


Figure 5. Pairwise $P_{ST}$ values (dotted line, open circles) for orange background PC1, black spot PC1, and black:orange ratio, each in comparison with pairwise $F_{ST}$ (black line, filled circles), plotted against pairwise geographic distance in kilometers.

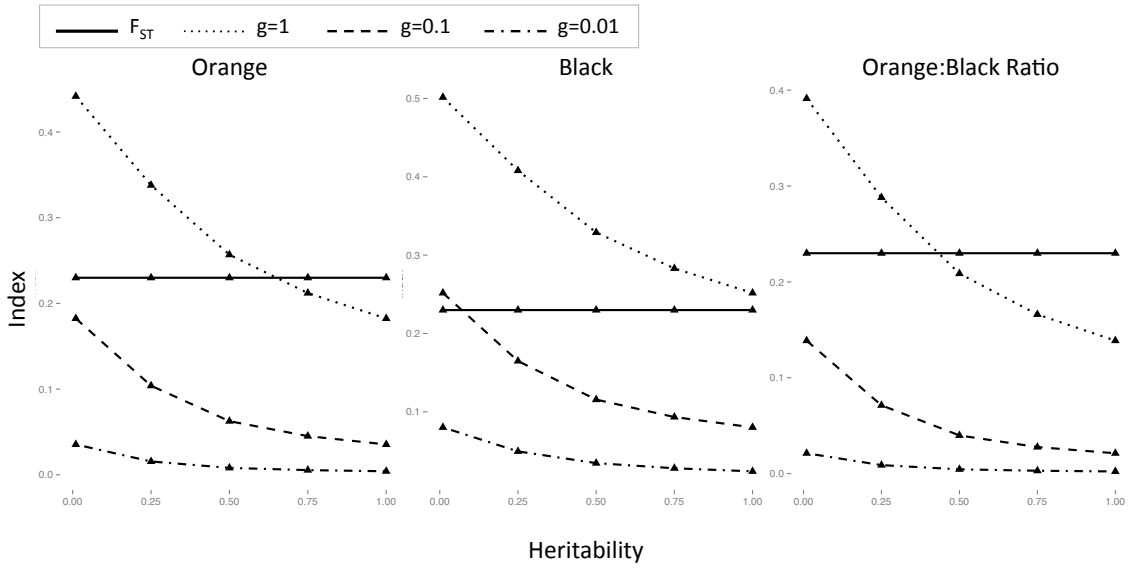


Figure 6. Results of a sensitivity analysis in which mean $P_{ST}$ was estimated for each color trait using multiple values of heritability (0, 0.25, 0.5, 0.75, and 1) and additive genetic variance (0.01, 0.1, and 1). These are plotted in comparison with $F_{ST}$ (black line).

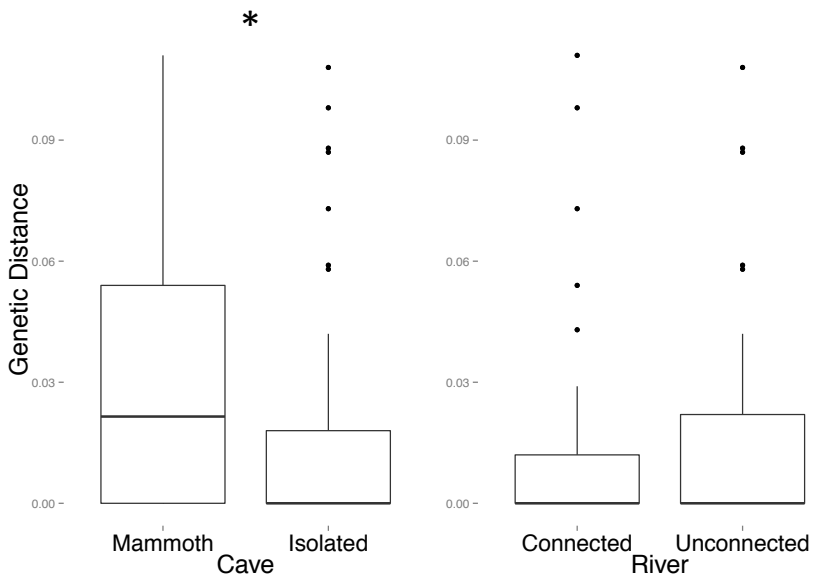


Figure 7. A comparison of pairwise $F_{ST}$ between caves which are either connected or disconnected in the context of subterranean paths (Cave) and the Green River (River). Bars represent 95% confidence intervals.

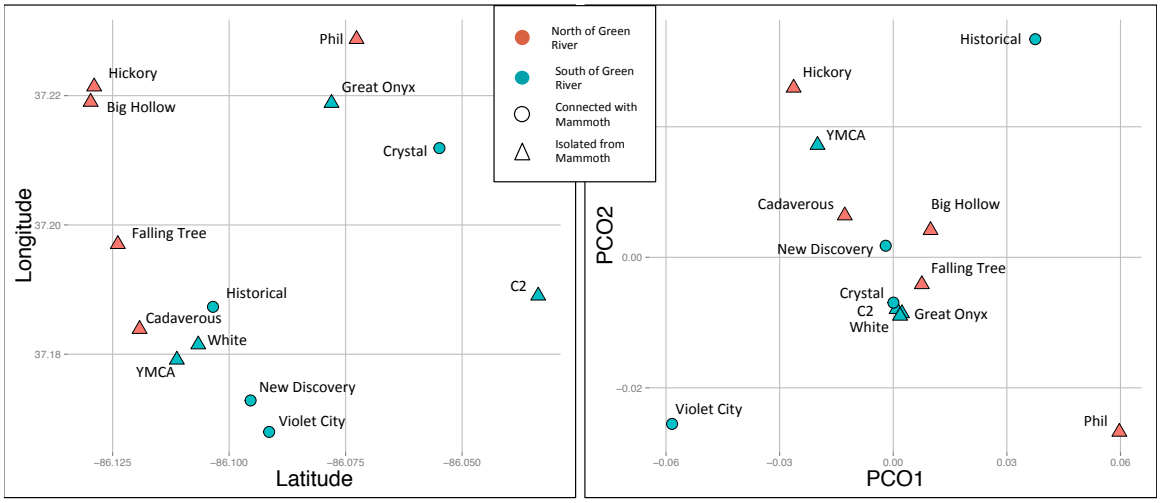


Figure 8. A comparison of cave entrances plotted according to their latitude and longitude (left), and according to their genetic similarity as estimated using the first two axes of a Principal Coordinate Analysis (PCoA; right).

## Tables:

Table 1. Results of Principal Components Analyses performed on the rangewide data and the Mammoth Cave National Park data using hue, saturation, and value for both the orange background and black spot traits.

| **Rangewide** | | **Orange Background** | | |
|---|---|---|---|---|
| | | **PC1** | **PC2** | **PC3** |
| | **Standard Deviation** | 0.064 | 0.021 | 0.009 |
| | **Prop. of Variance** | 0.886 | 0.098 | 0.016 |
| | **Cumulative Variance** | 0.886 | 0.984 | 1.000 |
| | **Hue** | -0.113 | -0.928 | 0.355 |
| | **Saturation** | -0.505 | -0.254 | -0.825 |
| | **Value** | -0.856 | 0.272 | 0.440 |
| | | **Black Spot** | | |
| | | **PC1** | **PC2** | **PC3** |
| | **Standard Deviation** | 0.444 | 0.284 | 0.159 |
| | **Prop. of Variance** | 0.651 | 0.266 | 0.083 |
| | **Cumulative Variance** | 0.651 | 0.917 | 1.000 |
| | **Hue** | 0.520 | 0.786 | 0.334 |
| | **Saturation** | 0.623 | -0.082 | -0.778 |
| | **Value** | 0.584 | -0.612 | 0.533 |
| **Mammoth** | | **Orange Background** | | |
| | | **PC1** | **PC2** | **PC3** |
| | **Standard Deviation** | 0.091 | 0.015 | 0.010 |
| | **Prop. of Variance** | 0.962 | 0.026 | 0.012 |
| | **Cumulative Variance** | 0.962 | 0.988 | 1.000 |
| | **Hue** | 0.058 | 0.955 | -0.291 |
| | **Saturation** | 0.486 | 0.228 | 0.844 |
| | **Value** | 0.872 | -0.190 | -0.451 |
| | | **Black Spot** | | |
| | | **PC1** | **PC2** | **PC3** |
| | **Standard Deviation** | 0.508 | 0.385 | 0.184 |
| | **Prop. of Variance** | 0.587 | 0.337 | 0.077 |
| | **Cumulative Variance** | 0.587 | 0.923 | 1.000 |
| | **Hue** | 0.514 | -0.810 | 0.283 |
| | **Saturation** | 0.627 | 0.130 | -0.768 |
| | **Value** | 0.585 | 0.572 | 0.574 |

Table 2. ANOVA results depicting color trait differences among geographic regions across the range including Region as a fixed effect and Population nested within region as a random effect, and among

populations within Mammoth Cave National Park including Population as the fixed effect. Significant p values are in bold.

| | Trait | Chi-square | DF | p | |
|---|---|---|---|---|---|
| Range-wide | Orange PC1 | 4.185 | 2, 147 | 0.123 | |
| | Black PC1 | 10.844 | 2, 147 | **0.004** | |
| | Black:Orange Ratio | 0.143 | 2, 147 | 0.931 | |
| | **Trait** | **SS** | **DF** | **F** | **p** |
| Mammoth | Orange PC1 | 0.087 | 12, 55 | 0.866 | 0.585 |
| | Black PC1 | 7.486 | 12, 55 | 3.495 | **0.001** |
| | Black:Orange Ratio | 0.173 | 12, 55 | 2.398 | **0.014** |

Table 3. Mantel tests, conducted using 999 permutations, included a comparison of range-wide Pst by geographic distance and $P_{ST}$-$F_{ST}$ by geographic distance. Significant correlations are in bold.

| | Range-wide | | Mammoth | |
|---|---|---|---|---|
| **Trait** | **Mantel's r** | **p** | **Mantel's r** | **p** |
| Orange PC1 | 0.01 | 0.449 | 0.19 | 0.123 |
| Black PC1 | -0.108 | 0.872 | 0.07 | 0.333 |
| Black:Orange Ratio | 0.258 | **0.005** | 0.223 | 0.073 |

Table 4. Results of independent and pairwise student's t-tests comparing rangewide $P_{ST}$ of each color trait to pairwise $F_{ST}$, which had a mean of 0.2299.

| | | t | DF | p |
|---|---|---|---|---|
| Range-wide | Orange PC1 | 2.076 | 1292 | **0.038** |
| | Black PC1 | 6.847 | 1176 | **<0.0001** |
| | Black:Orange Ratio | -1.813 | 1389 | 0.07 |
| Mammoth | Orange PC1 | 8.577 | 159 | **<0.0001** |
| | Black PC1 | 11.496 | 157 | **<0.0001** |
| | Black:Orange Ratio | 10.099 | 158 | **<0.0001** |

Table 5. Results of two Analyses of Molecular Variance, including both the side of the Green River that a cave entrance is located, and whether or not it is connected with the main Mammoth Cave system as regional partitions. Significant p-values are in bold.

| Source of Variation | Nested in | %var | F-stat | F-value | p |
|---|---|---|---|---|---|
| Within Individual | -- | 0.832 | R_it | 0.168 | -- |
| Among Individual | Population | 0.124 | R_is | 0.129 | **0.018** |
| Among Population | N or S of River | 0.028 | R_sc | 0.029 | 0.126 |
| Among River Groups | -- | 0.016 | R_ct | 0.016 | 0.181 |

| Source of Variation | Nested in | %var | F-stat | F-value | p |
|---|---|---|---|---|---|
| Within Individual | -- | 0.841 | R_it | 0.159 | -- |
| Among Individual | Population | 0.13 | R_is | 0.134 | **0.012** |
| Among Population | Cave/Isolated | 0.046 | R_sc | 0.045 | **0.048** |
| Among Cave/Isolated | -- | -0.017 | R_ct | -0.017 | 0.789 |

**Supplementary Tables:**

S1: Collecting information and raw color values for Hue, Saturation, and Value of each individual's orange background, black spots, and the standard to which each was compared. Spot Ratio is the ratio of spot area to background area.

| | | | | Orange Background | | | Black Spot | | | Standard | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Region** | **ID** | **Population** | **Hue** | **Saturation** | **Value** | **Hue** | **Saturation** | **Value** | **Hue** | **Saturation** | **Value** | **Spot Ratio** |
| **1** | Central | Aus6.30.1 | Austen | 0.0669 | 0.0587 | 0.0357 | 0.4941 | 0.0211 | 0.0124 | 0.0939 | 0.0705 | 0.0568 | 0.2821 |
| **2** | Central | Aus7.2.1 | Austen | 0.0357 | 0.0542 | 0.0675 | 0.0124 | 0.0301 | 0.0382 | 0.0568 | 0.0784 | 0.0869 | 0.2173 |
| **3** | Central | Big7.3.1 | Big Hollow | 0.0371 | 0.0406 | 0.0350 | 0.0101 | 0.0097 | 0.0131 | 0.0422 | 0.0457 | 0.0500 | 0.1960 |
| **4** | Central | Big7.3.2 | Big Hollow | 0.0350 | 0.0268 | 0.0251 | 0.0131 | 0.5012 | 0.4963 | 0.0500 | 0.0482 | 0.0494 | 0.4184 |
| **5** | Central | Big7.3.3 | Big Hollow | 0.0251 | 0.0352 | 0.0474 | 0.4963 | 0.0102 | 0.0140 | 0.0494 | 0.0519 | 0.0476 | 0.2885 |
| **6** | Central | Big7.3.4 | Big Hollow | 0.0474 | 0.0407 | 0.0336 | 0.0140 | 0.0119 | 0.0098 | 0.0476 | 0.0421 | 0.0419 | 0.3961 |
| **7** | Central | Big7.3.5 | Big Hollow | 0.0336 | 0.0298 | 0.0281 | 0.0098 | 0.0065 | 0.4894 | 0.0419 | 0.0469 | 0.0493 | 0.3768 |
| **8** | Central | Big7.3.6 | Big Hollow | 0.0281 | 0.0268 | 0.0250 | 0.4894 | 0.4932 | 0.0096 | 0.0493 | 0.0482 | 0.0475 | 0.2632 |
| **9** | Central | Big7.3.7 | Big Hollow | 0.0250 | 0.0396 | 0.0486 | 0.0096 | 0.0184 | 0.4756 | 0.0475 | 0.5171 | 0.9896 | 0.2058 |
| **10** | Central | C27.1.1 | Frank's Cave | 0.0209 | 0.0326 | 0.0492 | 0.4965 | 0.4924 | 0.4938 | 0.0454 | 0.0484 | 0.0516 | 0.1779 |
| **11** | Central | C27.1.2 | Frank's Cave | 0.0492 | 0.0458 | 0.0423 | 0.4938 | 0.0057 | 0.0071 | 0.0516 | 0.0504 | 0.0491 | 0.1832 |
| **12** | Central | C27.1.3 | Frank's Cave | 0.0423 | 0.0371 | 0.0516 | 0.0071 | 0.4832 | 0.4881 | 0.0491 | 0.0479 | 0.0685 | 0.3511 |
| **13** | Central | C27.1.4 | Frank's Cave | 0.0305 | 0.0727 | 0.0650 | 0.9590 | 0.0172 | 0.0044 | 0.0465 | 0.0905 | 0.0941 | 0.3452 |
| **14** | Central | C27.2.1 | Frank's Cave | 0.0689 | 0.0666 | 0.0663 | 0.0108 | 0.0051 | 0.0074 | 0.0923 | 0.0782 | 0.0571 | 0.1896 |
| **15** | Central | C27.2.2 | Frank's Cave | 0.0663 | 0.0688 | 0.0671 | 0.0074 | 0.0142 | 0.4843 | 0.0571 | 0.0692 | 0.0794 | 0.2266 |
| **16** | Central | C27.2.3 | Frank's Cave | 0.0732 | 0.0610 | 0.0617 | 0.0194 | 0.9492 | 0.9799 | 0.0865 | 0.0723 | 0.0915 | 0.1887 |
| **17** | Central | Cad6.30.1 | Cadaverous | 0.0613 | 0.0588 | 0.0551 | 0.9646 | 0.9684 | 0.9603 | 0.0819 | 0.0749 | 0.0685 | 0.2759 |
| **18** | Central | Cad6.30.2 | Cadaverous | 0.0551 | 0.0536 | 0.0573 | 0.9603 | 0.9483 | 0.9626 | 0.0685 | 0.0694 | 0.0657 | 0.2965 |
| **19** | Central | Cad6.30.3 | Cadaverous | 0.0573 | 0.0560 | 0.0595 | 0.9626 | 0.4967 | 0.0053 | 0.0657 | 0.0664 | 0.0660 | 0.2568 |
| **20** | Central | Cad6.30.4 | Cadaverous | 0.0595 | 0.0820 | 0.0952 | 0.0053 | 0.0171 | 0.0226 | 0.0660 | 0.0866 | 0.0886 | 0.2428 |
| **21** | Central | Cad6.30.5 | Cadaverous | 0.0952 | 0.0753 | 0.0614 | 0.0226 | 0.0366 | 0.0445 | 0.0886 | 0.0655 | 0.0637 | 0.5398 |
| **22** | Central | Cr6.7.1 | Crews | 0.0614 | 0.0610 | 0.0566 | 0.0445 | 0.0357 | 0.0304 | 0.0637 | 0.0672 | 0.0722 | 0.2750 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **23** | Central | Cr6.7.2 | Crews | 0.0566 | 0.0637 | 0.0645 | 0.0304 | 0.0316 | 0.0390 | 0.0722 | 0.0770 | 0.0763 | 0.1541 |
| **24** | Central | Cr6.7.3 | Crews | 0.0645 | 0.0549 | 0.0502 | 0.0390 | 0.0506 | 0.0450 | 0.0763 | 0.0649 | 0.0552 | 0.2039 |
| **25** | Central | Cr6.7.4 | Crews | 0.0502 | 0.0483 | 0.0508 | 0.0450 | 0.0343 | 0.0346 | 0.0552 | 0.0667 | 0.0737 | 0.3622 |
| **26** | Central | Cr6.7.5 | Crews | 0.0508 | 0.0732 | 0.0981 | 0.0346 | 0.0388 | 0.0450 | 0.0737 | 0.0650 | 0.0711 | 0.2527 |
| **27** | Central | Cr7.13.1 | Crews | 0.0981 | 0.0939 | 0.0849 | 0.0450 | 0.0432 | 0.0353 | 0.0711 | 0.0827 | 0.0807 | 0.1670 |
| **28** | Central | Cr7.13.2 | Crews | 0.0849 | 0.0837 | 0.0774 | 0.0353 | 0.0253 | 0.0356 | 0.0807 | 0.0849 | 0.0850 | 0.1644 |
| **29** | Central | Cr7.13.3 | Crews | 0.0774 | 0.0876 | 0.0935 | 0.0356 | 0.0371 | 0.0264 | 0.0850 | 0.0713 | 0.0482 | 0.1646 |
| **30** | Central | Cr7.13.4 | Crews | 0.0935 | 0.0850 | 0.0838 | 0.0264 | 0.0299 | 0.0386 | 0.0482 | 0.0555 | 0.0813 | 0.2358 |
| **31** | Central | Cr7.13.5 | Crews | 0.0838 | 0.1069 | 0.1290 | 0.0386 | 0.0635 | 0.0828 | 0.0813 | 0.0845 | 0.0855 | 0.2324 |
| **32** | Central | Cr7.13.6 | Crews | 0.1290 | 0.0967 | 0.0605 | 0.0828 | 0.0547 | 0.0198 | 0.0855 | 0.0824 | 0.0621 | 0.2414 |
| **33** | Central | Cr7.13.8 | Crews | 0.0605 | 0.0577 | 0.0634 | 0.0198 | 0.0138 | 0.0208 | 0.0621 | 0.0544 | 0.0700 | 0.2308 |
| **34** | Central | Crys6.30.1 | Crystal | 0.0634 | 0.0663 | 0.0573 | 0.0208 | 0.0222 | 0.0188 | 0.0700 | 0.0841 | 0.0812 | 0.3290 |
| **35** | Central | Crys6.30.2 | Crystal | 0.0573 | 0.0562 | 0.0736 | 0.0188 | 0.4953 | 0.4960 | 0.0812 | 0.0548 | 0.0250 | 0.2390 |
| **36** | Central | Oe6.7.1 | Eoff | 0.0490 | 0.0623 | 0.0590 | 0.0311 | 0.0379 | 0.0386 | 0.0400 | 0.0608 | 0.0690 | 0.1963 |
| **37** | Central | Oe6.7.2 | Eoff | 0.0590 | 0.0605 | 0.0754 | 0.0386 | 0.0501 | 0.0683 | 0.0690 | 0.0626 | 0.0618 | 0.1661 |
| **38** | Central | Oe6.7.3 | Eoff | 0.0754 | 0.0616 | 0.0523 | 0.0683 | 0.0536 | 0.0398 | 0.0618 | 0.0680 | 0.0787 | 0.1506 |
| **39** | Central | Oe6.7.4 | Eoff | 0.0523 | 0.0803 | 0.1013 | 0.0398 | 0.0627 | 0.0736 | 0.0787 | 0.0704 | 0.0626 | 0.3119 |
| **40** | Central | Oe6.7.5 | Eoff | 0.1013 | 0.0708 | 0.0487 | 0.0736 | 0.0524 | 0.0424 | 0.0626 | 0.0602 | 0.0623 | 0.0567 |
| **41** | Central | Oe6.7.6 | Eoff | 0.0418 | 0.0555 | 0.0478 | 0.0425 | 0.0423 | 0.0237 | 0.0541 | 0.0705 | 0.0626 | 0.2972 |
| **42** | Central | Oe6.7.7 | Eoff | 0.0517 | 0.0516 | 0.0557 | 0.0330 | 0.0350 | 0.0467 | 0.0666 | 0.0657 | 0.0662 | 0.3904 |
| **43** | Central | Oe6.7.8 | Eoff | 0.0557 | 0.0662 | 0.0625 | 0.0467 | 0.0480 | 0.4781 | 0.0662 | 0.0633 | 0.0672 | 0.1813 |
| **44** | Central | Oe6.7.9 | Eoff | 0.0766 | 0.0485 | 0.0533 | 0.0489 | 0.9072 | 0.0242 | 0.0630 | 0.0713 | 0.0474 | 0.1495 |
| **45** | Central | Fall6.30.1 | Falling Tree | 0.0579 | 0.0602 | 0.0589 | 0.9960 | 0.9763 | 0.9497 | 0.0685 | 0.0687 | 0.0710 | 0.2150 |
| **46** | Central | Fall6.30.2 | Falling Tree | 0.0589 | 0.0657 | 0.0716 | 0.9497 | 0.9661 | 0.9773 | 0.0710 | 0.0544 | 0.0641 | 0.2954 |
| **47** | Central | Fall6.30.3 | Falling Tree | 0.0716 | 0.0512 | 0.0369 | 0.9773 | 0.4820 | 0.4856 | 0.0641 | 0.0689 | 0.0563 | 0.2626 |
| **48** | Central | Fall7.3.1 | Falling Tree | 0.0369 | 0.0502 | 0.0689 | 0.4856 | 0.5012 | 0.0426 | 0.0563 | 0.0579 | 0.0553 | 0.1523 |
| **49** | Central | G6.5.1 | Gillespie | 0.0596 | 0.0781 | 0.0766 | 0.0316 | 0.0536 | 0.0552 | 0.0549 | 0.0556 | 0.0665 | 0.3103 |
| **50** | Central | G6.5.2 | Gillespie | 0.0773 | 0.0605 | 0.0439 | 0.0544 | 0.0406 | 0.0279 | 0.0611 | 0.0623 | 0.0565 | 0.2161 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **51** | Central | G6.5.3 | Gillespie | 0.0439 | 0.0423 | 0.0606 | 0.0279 | 0.0161 | 0.4605 | 0.0565 | 0.0590 | 0.0689 | 0.2744 |
| **52** | Central | G6.5.4 | Gillespie | 0.0411 | 0.0800 | 0.0570 | 0.0023 | 0.9187 | 0.0135 | 0.0630 | 0.0748 | 0.0843 | 0.3757 |
| **53** | Central | GrOn6.29.1 | Great Onyx | 0.0812 | 0.0642 | 0.0667 | 0.9558 | 0.5039 | 0.0092 | 0.0733 | 0.0853 | 0.0833 | 0.3258 |
| **54** | Central | GrOn6.29.10 | Great Onyx | 0.0612 | 0.0728 | 0.0758 | 0.0113 | 0.5008 | 0.4974 | 0.0858 | 0.0886 | 0.0830 | 0.2671 |
| **55** | Central | GrOn6.29.11 | Great Onyx | 0.0758 | 0.0769 | 0.0770 | 0.4974 | 0.4976 | 0.9936 | 0.0830 | 0.0739 | 0.0790 | 0.2096 |
| **56** | Central | GrOn6.29.2 | Great Onyx | 0.0696 | 0.0555 | 0.0525 | 0.5019 | 0.4739 | 0.4740 | 0.0828 | 0.0802 | 0.0702 | 0.2866 |
| **57** | Central | GrOn6.29.3 | Great Onyx | 0.0525 | 0.0612 | 0.0677 | 0.4740 | 0.4977 | 0.4943 | 0.0702 | 0.0645 | 0.0760 | 0.3531 |
| **58** | Central | GrOn6.29.4 | Great Onyx | 0.0677 | 0.0669 | 0.0593 | 0.4943 | 0.4991 | 0.9910 | 0.0760 | 0.0793 | 0.0762 | 0.2765 |
| **59** | Central | GrOn6.29.5 | Great Onyx | 0.0593 | 0.0622 | 0.0650 | 0.9910 | 0.5052 | 0.0135 | 0.0762 | 0.0696 | 0.0647 | 0.1092 |
| **60** | Central | GrOn6.29.6 | Great Onyx | 0.0650 | 0.0486 | 0.0473 | 0.0135 | 0.4917 | 0.9845 | 0.0647 | 0.0601 | 0.0712 | 0.3248 |
| **61** | Central | GrOn6.29.7 | Great Onyx | 0.0473 | 0.0542 | 0.0506 | 0.9845 | 0.9737 | 0.9418 | 0.0712 | 0.0814 | 0.0817 | 0.2001 |
| **62** | Central | GrOn6.29.8 | Great Onyx | 0.0506 | 0.0568 | 0.0645 | 0.9418 | 0.4731 | 0.0150 | 0.0817 | 0.0814 | 0.0757 | 0.2504 |
| **63** | Central | GrOn6.29.9 | Great Onyx | 0.0645 | 0.0892 | 0.1094 | 0.0150 | 0.0166 | 0.0190 | 0.0757 | 0.0779 | 0.0800 | 0.2084 |
| **64** | Central | Hick7.3.3 | Hickory | 0.0387 | 0.0514 | 0.0363 | 0.0088 | 0.9739 | 0.9944 | 0.0641 | 0.0799 | 0.0773 | 0.2791 |
| **65** | Central | Hick7.3.4 | Hickory | 0.0438 | 0.0502 | 0.0658 | 0.9841 | 0.5085 | 0.0126 | 0.0786 | 0.0787 | 0.0842 | 0.2646 |
| **66** | Central | Hick7.3.5 | Hickory | 0.0640 | 0.0675 | 0.0544 | 0.0226 | 0.0026 | 0.0104 | 0.0801 | 0.0884 | 0.0855 | 0.2529 |
| **67** | Central | Hick7.3.6 | Hickory | 0.0610 | 0.0460 | 0.0488 | 0.0065 | 0.5023 | 0.5213 | 0.0870 | 0.0830 | 0.0743 | 0.3476 |
| **68** | Central | His6.29.1 | Historical | 0.0375 | 0.0601 | 0.0798 | 0.9941 | 0.0484 | 0.0586 | 0.0806 | 0.0681 | 0.0882 | 0.0289 |
| **69** | Central | His6.29.2 | Historical | 0.0700 | 0.0918 | 0.1026 | 0.0535 | 0.0613 | 0.0595 | 0.0781 | 0.0786 | 0.0726 | 0.1767 |
| **70** | Central | New6.29.1 | New Discovery | 0.0681 | 0.0626 | 0.0652 | 0.0134 | 0.0058 | 0.0058 | 0.0767 | 0.0750 | 0.0718 | 0.2868 |
| **71** | Central | New6.29.2 | New Discovery | 0.0660 | 0.0643 | 0.0811 | 0.0017 | 0.0098 | 0.0070 | 0.0762 | 0.0673 | 0.0819 | 0.2073 |
| **72** | Central | New6.29.3 | New Discovery | 0.0727 | 0.0755 | 0.0619 | 0.0084 | 0.0085 | 0.0117 | 0.0746 | 0.0821 | 0.0772 | 0.1524 |
| **73** | Central | New6.29.4 | New Discovery | 0.0619 | 0.0618 | 0.0688 | 0.0117 | 0.0172 | 0.0208 | 0.0772 | 0.0684 | 0.0708 | 0.1634 |
| **74** | Central | New6.29.5 | New Discovery | 0.0688 | 0.0563 | 0.0490 | 0.0208 | 0.0264 | 0.0311 | 0.0708 | 0.0557 | 0.0400 | 0.2399 |
| **75** | Central | Phil7.3.1 | Phil | 0.0509 | 0.0548 | 0.0522 | 0.4657 | 0.0265 | 0.0258 | 0.0594 | 0.0682 | 0.0768 | 0.1838 |
| **76** | Central | Phil7.3.2 | Phil | 0.0522 | 0.0629 | 0.0773 | 0.0258 | 0.0156 | 0.0120 | 0.0768 | 0.0736 | 0.0789 | 0.1349 |
| **77** | Central | Phil7.3.3 | Phil | 0.0773 | 0.0750 | 0.0794 | 0.0120 | 0.0164 | 0.0322 | 0.0789 | 0.0745 | 0.0796 | 0.1047 |
| **78** | Central | Phil7.3.4 | Phil | 0.0731 | 0.0856 | 0.0781 | 0.0170 | 0.0474 | 0.0536 | 0.0739 | 0.0852 | 0.0387 | 0.2196 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **79** | Central | Stan7.2.1 | Stans Well | 0.0614 | 0.0458 | 0.0407 | 0.0091 | 0.4997 | 0.9931 | 0.0546 | 0.0722 | 0.0696 | 0.4643 |
| **80** | Central | Stan7.3.1 | Stans Well | 0.0279 | 0.0535 | 0.0448 | 0.9872 | 0.9989 | 0.0058 | 0.0866 | 0.0526 | 0.0439 | 0.3074 |
| **81** | Central | Vici.29.5 | Violet City | 0.0592 | 0.0490 | 0.0467 | 0.0131 | 0.0078 | 0.4991 | 0.0847 | 0.0783 | 0.0752 | 0.1651 |
| **82** | Central | Vici.29.6 | Violet City | 0.0467 | 0.0690 | 0.0709 | 0.4991 | 0.9920 | 0.5040 | 0.0752 | 0.0831 | 0.0832 | 0.1597 |
| **83** | Central | Vici29.4 | Violet City | 0.0847 | 0.0572 | 0.0677 | 0.9885 | 0.0194 | 0.9984 | 0.0813 | 0.0851 | 0.0856 | 0.2009 |
| **84** | Central | Vici6.29.1 | Violet City | 0.0625 | 0.0682 | 0.0649 | 0.5089 | 0.5164 | 0.0294 | 0.0853 | 0.0856 | 0.0930 | 0.1784 |
| **85** | Central | Vici6.29.2 | Violet City | 0.0649 | 0.0635 | 0.0651 | 0.0294 | 0.0178 | 0.0211 | 0.0930 | 0.0911 | 0.0812 | 0.2621 |
| **86** | Central | Vici6.29.3 | Violet City | 0.0651 | 0.0742 | 0.0582 | 0.0211 | 0.0240 | 0.4966 | 0.0812 | 0.0830 | 0.0622 | 0.1307 |
| **87** | Central | Vici6.29.4 | Violet City | 0.0838 | 0.0325 | 0.0531 | 0.0168 | 0.9763 | 0.9911 | 0.0853 | 0.0390 | 0.0728 | 0.2056 |
| **88** | Central | Whi7.1.1 | White | 0.0428 | 0.0526 | 0.0516 | 0.9837 | 0.5053 | 0.4875 | 0.0559 | 0.0777 | 0.0718 | 0.1404 |
| **89** | Central | Whi7.1.2 | White | 0.0522 | 0.0511 | 0.0543 | 0.0194 | 0.9555 | 0.9787 | 0.0826 | 0.0609 | 0.0553 | 0.2634 |
| **90** | Central | YMCA7.4.1 | YMCA | 0.0527 | 0.0577 | 0.0676 | 0.9671 | 0.9773 | 0.4948 | 0.0581 | 0.0714 | 0.0862 | 0.1409 |
| **91** | Central | YMCA7.4.2 | YMCA | 0.0676 | 0.0562 | 0.0587 | 0.4948 | 0.0133 | 0.0200 | 0.0862 | 0.0698 | 0.0731 | 0.2396 |
| **92** | Central | YMCA7.4.3 | YMCA | 0.0587 | 0.0665 | 0.0600 | 0.0200 | 0.5067 | 0.4965 | 0.0731 | 0.0791 | 0.0685 | 0.2109 |
| **93** | Central | YMCA7.4.4 | YMCA | 0.0600 | 0.0540 | 0.0403 | 0.4965 | 0.4781 | 0.9652 | 0.0685 | 0.0669 | 0.0682 | 0.1619 |
| **94** | Central | YMCA7.4.5 | YMCA | 0.0403 | 0.0537 | 0.0498 | 0.9652 | 0.5063 | 0.0199 | 0.0682 | 0.0812 | 0.0693 | 0.4043 |
| **95** | Central | YMCA7.4.6 | YMCA | 0.0498 | 0.0396 | 0.0549 | 0.0199 | 0.4707 | 0.9653 | 0.0693 | 0.0563 | 0.0696 | 0.0961 |
| **96** | Central | YMCA7.4.7 | YMCA | 0.0549 | 0.0683 | 0.0777 | 0.9653 | 0.5092 | 0.0300 | 0.0696 | 0.0882 | 0.0978 | 0.2867 |
| **97** | Central | YMCA7.4.8 | YMCA | 0.0777 | 0.4387 | 0.7776 | 0.0300 | 0.3099 | 0.4536 | 0.0978 | 0.2911 | 0.5629 | 0.2420 |
| **98** | Indiana | E7.20.1 | Lost River | 0.0410 | 0.0406 | 0.0409 | 0.0195 | 0.0054 | 0.0118 | 0.0353 | 0.0283 | 0.0392 | 0.3338 |
| **99** | Indiana | E7.20.1c | Lost River | 0.0405 | 0.0414 | 0.0354 | 0.0049 | 0.0186 | 0.0120 | 0.0321 | 0.0464 | 0.0480 | 0.3269 |
| **100** | Indiana | E7.20.2 | Lost River | 0.0414 | 0.0354 | 0.0322 | 0.0186 | 0.0120 | 0.0217 | 0.0464 | 0.0480 | 0.0476 | 0.1835 |
| **101** | Indiana | E7.20.3 | Lost River | 0.0354 | 0.0322 | 0.0328 | 0.0120 | 0.0217 | 0.0146 | 0.0480 | 0.0476 | 0.0495 | 0.2013 |
| **102** | Indiana | E7.20.4 | Lost River | 0.0325 | 0.0348 | 0.0336 | 0.0182 | 0.0159 | 0.0205 | 0.0486 | 0.0496 | 0.0486 | 0.2070 |
| **103** | Indiana | E7.20.5 | Lost River | 0.0367 | 0.0305 | 0.0300 | 0.0172 | 0.0237 | 0.0278 | 0.0496 | 0.0477 | 0.0463 | 0.3375 |
| **104** | Indiana | E7.20.6 | Lost River | 0.0302 | 0.0428 | 0.0579 | 0.0258 | 0.5129 | 0.9960 | 0.0470 | 0.0605 | 0.0685 | 0.3444 |
| **105** | Indiana | Su7.20.1 | Sullivan | 0.0478 | 0.0442 | 0.0457 | 0.0151 | 0.0299 | 0.0388 | 0.0456 | 0.0460 | 0.0491 | 0.2921 |
| **106** | Indiana | Su7.20.2 | Sullivan | 0.0457 | 0.0509 | 0.0544 | 0.0388 | 0.0387 | 0.0453 | 0.0491 | 0.0535 | 0.0556 | 0.2213 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **107** | Indiana | Su7.20.3 | Sullivan | 0.0544 | 0.0517 | 0.0552 | 0.0453 | 0.0547 | 0.0651 | 0.0556 | 0.0548 | 0.0530 | 0.2236 |
| **108** | Indiana | Su7.20.4 | Sullivan | 0.0471 | 0.0633 | 0.0637 | 0.0584 | 0.0719 | 0.0401 | 0.0555 | 0.0506 | 0.0532 | 0.2459 |
| **109** | Indiana | Su7.20.5 | Sullivan | 0.0635 | 0.0596 | 0.0590 | 0.0560 | 0.0347 | 0.0411 | 0.0519 | 0.0353 | 0.0325 | 0.1919 |
| **110** | Indiana | Su7.20.6 | Sullivan | 0.0590 | 0.0624 | 0.0596 | 0.0411 | 0.0396 | 0.3973 | 0.0325 | 0.0683 | 0.0943 | 0.1999 |
| **111** | Western | BI7.11.1 | Blue Moon | 0.0667 | 0.1013 | 0.1195 | 0.0260 | 0.0377 | 0.0400 | 0.0831 | 0.0948 | 0.0965 | 0.1804 |
| **112** | Western | BI7.11.2 | Blue Moon | 0.1195 | 0.1132 | 0.1228 | 0.0400 | 0.0426 | 0.0402 | 0.0965 | 0.0798 | 0.0678 | 0.6128 |
| **113** | Western | BI7.11.3 | Blue Moon | 0.1228 | 0.1404 | 0.1627 | 0.0402 | 0.0469 | 0.0728 | 0.0678 | 0.0804 | 0.0986 | 0.5278 |
| **114** | Western | BI7.11.4 | Blue Moon | 0.1627 | 0.1275 | 0.0597 | 0.0728 | 0.0654 | 0.0275 | 0.0986 | 0.1008 | 0.0771 | 0.5666 |
| **115** | Western | BI7.11.5 | Blue Moon | 0.0681 | 0.0669 | 0.0665 | 0.0531 | 0.0233 | 0.0287 | 0.0903 | 0.0835 | 0.0828 | 0.5873 |
| **116** | Western | BI7.11.5 | Blue Moon | 0.0883 | 0.0311 | 0.0364 | 0.0504 | 0.0046 | 0.0111 | 0.1031 | 0.0510 | 0.0428 | 0.2059 |
| **117** | Western | Bu17.14.4 | Bull Creek | 0.0862 | 0.0865 | 0.0813 | 0.0368 | 0.0487 | 0.0476 | 0.0954 | 0.0840 | 0.0857 | 0.1430 |
| **118** | Western | Bu17.14.5 | Bull Creek | 0.0839 | 0.0751 | 0.0716 | 0.0482 | 0.0362 | 0.0410 | 0.0849 | 0.0813 | 0.0822 | 0.1707 |
| **119** | Western | Bu17.14.6 | Bull Creek | 0.0716 | 0.0771 | 0.0761 | 0.0410 | 0.0523 | 0.0368 | 0.0822 | 0.0807 | 0.0758 | 0.2856 |
| **120** | Western | Bu17.14.7 | Bull Creek | 0.0761 | 0.0724 | 0.0646 | 0.0368 | 0.0261 | 0.0321 | 0.0758 | 0.0778 | 0.0638 | 0.1985 |
| **121** | Western | Bu17.14.8 | Bull Creek | 0.0646 | 0.0739 | 0.0931 | 0.0321 | 0.0438 | 0.0590 | 0.0638 | 0.0640 | 0.0807 | 0.2530 |
| **122** | Western | Bu17.14.9 | Bull Creek | 0.0931 | 0.0772 | 0.0803 | 0.0590 | 0.0554 | 0.0439 | 0.0807 | 0.0770 | 0.0847 | 0.1372 |
| **123** | Western | Bu7.14.1 | Bull Creek | 0.0983 | 0.0752 | 0.0715 | 0.0436 | 0.0416 | 0.0416 | 0.0840 | 0.0582 | 0.0592 | 0.1474 |
| **124** | Western | Bu7.14.12 | Bull Creek | 0.0591 | 0.1014 | 0.0952 | 0.0424 | 0.0454 | 0.0418 | 0.0706 | 0.0988 | 0.0691 | 0.1864 |
| **125** | Western | Bu7.14.2 | Bull Creek | 0.0553 | 0.0877 | 0.0901 | 0.0414 | 0.0418 | 0.0471 | 0.0473 | 0.0710 | 0.0874 | 0.1831 |
| **126** | Western | Bu7.14.3 | Bull Creek | 0.0889 | 0.0574 | 0.0209 | 0.0445 | 0.5192 | 0.4965 | 0.0792 | 0.0665 | 0.0454 | 0.1303 |
| **127** | Western | IG7.10.1 | Iron Gate | 0.1026 | 0.1058 | 0.1017 | 0.0595 | 0.0546 | 0.0592 | 0.0726 | 0.0889 | 0.0976 | 0.2656 |
| **128** | Western | IG7.10.2 | Iron Gate | 0.1017 | 0.0890 | 0.0842 | 0.0592 | 0.0570 | 0.0423 | 0.0976 | 0.0900 | 0.0867 | 0.2312 |
| **129** | Western | Ig7.10.3 | Iron Gate | 0.0850 | 0.0835 | 0.0711 | 0.0498 | 0.0348 | 0.0505 | 0.0864 | 0.0870 | 0.0762 | 0.2164 |
| **130** | Western | IG7.10.3 | Iron Gate | 0.0835 | 0.0711 | 0.0783 | 0.0348 | 0.0505 | 0.0402 | 0.0870 | 0.0762 | 0.0895 | 0.2179 |
| **131** | Western | J7.10.1 | Jail | 0.0747 | 0.0791 | 0.0761 | 0.0454 | 0.0433 | 0.0472 | 0.0829 | 0.0849 | 0.0803 | 0.2343 |
| **132** | Western | J7.10.2 | Jail | 0.0800 | 0.0722 | 0.0923 | 0.0464 | 0.0480 | 0.0646 | 0.0802 | 0.0804 | 0.0753 | 0.1887 |
| **133** | Western | J7.10.3 | Jail | 0.0822 | 0.0807 | 0.0794 | 0.0563 | 0.0631 | 0.0565 | 0.0779 | 0.0749 | 0.0826 | 0.2492 |
| **134** | Western | J7.10.4 | Jail | 0.0794 | 0.0807 | 0.0866 | 0.0565 | 0.0494 | 0.0617 | 0.0826 | 0.0973 | 0.1007 | 0.2292 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **135** | Western | J7.10.5 | Jail | 0.0717 | 0.1015 | 0.1058 | 0.0474 | 0.0760 | 0.0657 | 0.1039 | 0.0976 | 0.1158 | 0.2024 |
| **136** | Western | J7.10.6 | Jail | 0.1036 | 0.0937 | 0.0719 | 0.0709 | 0.0632 | 0.0528 | 0.1067 | 0.1007 | 0.0771 | 0.1681 |
| **137** | Western | J7.10.7 | Jail | 0.0719 | 0.0673 | 0.0870 | 0.0528 | 0.0356 | 0.0407 | 0.0771 | 0.0740 | 0.0919 | 0.2315 |
| **138** | Western | J7.10.8 | Jail | 0.0724 | 0.1017 | 0.0693 | 0.0262 | 0.0552 | 0.0411 | 0.0795 | 0.1044 | 0.0938 | 0.1318 |
| **139** | Western | J7.10.9 | Jail | 0.0855 | 0.0650 | 0.0667 | 0.0482 | 0.0490 | 0.0568 | 0.0991 | 0.0855 | 0.0819 | 0.1752 |
| **140** | Western | Jan7.9.1 | January-Stansbury | 0.0667 | 0.0674 | 0.0612 | 0.0568 | 0.0449 | 0.0340 | 0.0819 | 0.0758 | 0.0709 | 0.2129 |
| **141** | Western | Jan7.9.2 | January-Stansbury | 0.0612 | 0.0737 | 0.0781 | 0.0340 | 0.0378 | 0.0477 | 0.0709 | 0.0772 | 0.0828 | 0.1522 |
| **142** | Western | Jan7.9.3 | January-Stansbury | 0.0781 | 0.0720 | 0.0724 | 0.0477 | 0.0486 | 0.0363 | 0.0828 | 0.0786 | 0.0684 | 0.1609 |
| **143** | Western | Jan7.9.4 | January-Stansbury | 0.0724 | 0.0761 | 0.0874 | 0.0363 | 0.0269 | 0.0310 | 0.0684 | 0.0828 | 0.0906 | 0.2712 |
| **144** | Western | Jan7.9.5 | January-Stansbury | 0.0874 | 0.0813 | 0.0794 | 0.0310 | 0.0396 | 0.0415 | 0.0906 | 0.0752 | 0.0693 | 0.2174 |
| **145** | Western | Jan7.9.6 | January-Stansbury | 0.0794 | 0.0767 | 0.0770 | 0.0415 | 0.0518 | 0.0360 | 0.0693 | 0.0700 | 0.0470 | 0.3079 |
| **146** | Western | Jan7.9.7 | January-Stansbury | 0.0770 | 0.0693 | 0.0458 | 0.0360 | 0.5040 | 0.9925 | 0.0470 | 0.0430 | 0.0560 | 0.2071 |
| **147** | Western | S7.11.1 | Survivalist | 0.0819 | 0.0750 | 0.0686 | 0.0505 | 0.0378 | 0.0426 | 0.0620 | 0.0616 | 0.0622 | 0.1940 |
| **148** | Western | S7.11.2 | Survivalist | 0.0686 | 0.0622 | 0.0614 | 0.0426 | 0.0346 | 0.0091 | 0.0622 | 0.0456 | 0.0546 | 0.1991 |
| **149** | Western | Th7.11.1 | Third | 0.0596 | 0.0637 | 0.0678 | 0.3973 | 0.3939 | 0.0117 | 0.0943 | 0.0971 | 0.0949 | 0.3339 |
| **150** | Western | Th7.11.2 | Third | 0.0678 | 0.0662 | 0.0675 | 0.0117 | 0.0207 | 0.0436 | 0.0949 | 0.0848 | 0.0786 | 0.3829 |
| **151** | Western | Th7.11.3 | Third | 0.0675 | 0.0754 | 0.0784 | 0.0436 | 0.0558 | 0.0515 | 0.0786 | 0.0876 | 0.0843 | 0.2297 |
| **152** | Western | Th7.11.4 | Third | 0.0784 | 0.0672 | 0.0592 | 0.0515 | 0.0273 | 0.0131 | 0.0843 | 0.0772 | 0.0847 | 0.1635 |

Table S2. Average range-wide $P_{ST}$ values produced from a sensitivity analysis using a range of additive genetic variance and heritabilities.

| **Orange PC1** | **g=1** | **g=.1** | **g=.01** |
|---|---|---|---|
| **h=1** | 0.182 | 0.035 | 0.004 |
| **h=0.75** | 0.212 | 0.045 | 0.005 |
| **h=.5** | 0.257 | 0.063 | 0.008 |
| **h=0.25** | 0.338 | 0.104 | 0.016 |
| **h=.1** | 0.442 | 0.182 | 0.035 |
| **Spot PC1** | **g=1** | **g=.1** | **g=.01** |
| **h=1** | 0.252 | 0.080 | 0.024 |
| **h=0.75** | 0.283 | 0.093 | 0.028 |
| **h=.5** | 0.329 | 0.116 | 0.034 |
| **h=0.25** | 0.408 | 0.165 | 0.049 |
| **h=.1** | 0.502 | 0.252 | 0.080 |
| **Ratio** | **g=1** | **g=.1** | **g=.01** |
| **h=1** | 0.139 | 0.021 | 0.002 |
| **h=0.75** | 0.166 | 0.028 | 0.003 |
| **h=.5** | 0.209 | 0.040 | 0.005 |
| **h=0.25** | 0.288 | 0.071 | 0.009 |
| **h=.1** | 0.391 | 0.139 | 0.021 |

# APPENDIX A

## DEVELOPMENT AND CHARACTERIZATION OF 19 MICROSATELLITE LOCI IN THE CAVE SALAMANDER, *EURYCEA LUCIFUGA (PLETHODONTIDAE)*

**Abstract**

Subterranean environments are home to a multitude of species of conservation concern, since most cave-restricted organisms are endemic to a small range. Because of their conservation status and the physical difficulty of studying cave-restricted species, research on the evolutionary history and population dynamics of cave species is challenging. We have developed and characterized novel microsatellite markers using transcriptomic methods for the cave salamander, *Eurycea lucifuga*. Since this species is commonly found in caves across a wide expanse of the United States, these markers will facilitate studies of the cave habitat in a system that is relevant and tractable.

**Methods and Results**

The cave salamander, *Eurycea lucifuga*, is cave-dwelling Plethodontid species inhabiting a large range which extends across the Central and Eastern United States (Petranka 1998). In contrast with many vertebrate species that inhabit caves, which are often endemics of conservation concern, *E. lucifuga* is commonly found across its range in far greater numbers. The species therefore is well suited to studies of the population dynamics and evolutionary history of a unique and conservationally important habitat. The development of molecular resources, e.g. microsatellite markers, in *E. lucifuga* will allow more detailed study of ecological and evolutionary processes such as gene flow within and among cave systems, evolutionary dynamics of morphological change, and phylogenetic exploration of the relationship between *E. lucifuga* and closely related species.

Tail clips were taken from two individuals collected from different localities in Southwestern Virginia in Spring 2014 (Smokehole Cave 37.31402N 80.50894W, and Blankenship Blowhole 37.3135N 80.84987W; VADGIF permit #042984), and immediately preserved in RNAlater (Life Technologies). Whole sample RNA was extracted using a Qiagen RNEasy Mini Kit (Qiagen, Valencia, CA) following manufacturer suggested protocols and sent to the Genomic Core Facility at the University of Virginia's Department of Biology for the construction of individual paired-end, non-normalized cDNA libraries using a NEBNextmRNA library Prep Master Mix Set (New England Biolab Inc., Ipswich, MA). Individual libraries were given barcodes and pooled, and sent to Genewiz (GENEWIZ Inc., South Plainfield, NJ) and sequenced on an Illumina HiSeq 2500 using a PE 2x100bp format. Sequences for each sample were

assembled using the program Trinity v.11-10-2013 (Grabherr *et al.* 2011), and a summary of the sequencing results for each library can be found in Table 1. A custom BLAST search was used to locate regions of the mitochondrial genes cytb and nd2 sequenced previously from *E. lucifuga* in order to confirm the identity of the samples. Putative microsatellite loci were identified in each group of transcripts using the program msatcommander v. 1.0.8 (Faircloth 2008), specifying that the program search for repeats of trinucleotides or greater with at least five repeats. We used a BLAST searches to confirm the presence of primer sequences in both samples, and discarded primers that were not present in both group of transcripts.

Following the method of Schuelke (2000), we screened each primer set for amplification and polymorphism using m13 fluorescently labeled tags. Each test was performed using eight samples collected from across the range, and confirmatory sequencing was done using fragment analysis on an ABI 3130 Sequencer (Life Technologies). Primer pairs that amplified in all samples and were polymorphic were ordered with fluorescent tags incorporated into each forward primer, and multiplexed. Thermocycler conditions used throughout the screening process are: 94° for 15:00, 40 cycles of 94° for 0:30, 60° for 1:30, and 72° for 1:30, and finally 72° for 10:00. After identifying putative microsatellite loci and using BLAST to validate them, we were left with 220 primer pairs to screen. Twenty-four of these amplified in all individuals and were polymorphic, (>2 alleles) and these were multiplexed into six multiplexes, each containing four loci (Table 2).

The variability of these multiplexes was tested using 261 samples of *Eurycea lucifuga* collected from 49 populations across its range (Table 3). Fragment analysis for this test

was performed at the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT). Allele number at each locus (*k*) ranged from 2-7 (Table 2). Observed and expected heterozygosity ($H_o$ and $H_s$) were estimated using GenoDive v.2.0b27 (Meirmans & Van Tienderen 2004), and are shown in Table 2. When analyzed in GenoDive v. 2.0b27, no loci significantly deviated from HWE within populations (Table 4). All analyses were run using 999 permutations. These markers will enable further research on population and evolutionary dynamics in *Eurycea lucifuga* and potentially close relatives.

**References**

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Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature biotechnology* **18**, 233-234.

## Tables

Table 1. Summary of the sequencing results and assembly of the two paired-end libraries.

| Library | Number of reads | Q>30 | Total transcripts | Median contig length | Total assembled bases |
|---|---|---|---|---|---|
| BLB | 128 mill | 89.96% | 112,449 | 355bp | 73,398,489 |
| Smoke | 300 mill | 90.26% | 92,594 | 362bp | 59,509,037 |

Table 2. Microsatellite markers developed for *Eurycea lucifuga*, reported with primer sequence information as well as multiplex configuration and motif. We characterized the number of alleles at each locus (*k*), as well as observed ($H_o$) and expected ($H_s$) heterozygosity.

| Marker name | Forward primer | Reverse Primer | Plex | Motif | *k* | Ho | Hs |
|---|---|---|---|---|---|---|---|
| E_luc_238 | ATGGCTGCGCTTTCTTGTAC | CTCTGTACAGGAGACGGGTG | 1 | AAG | 3 | 0.009 | 0.009 |
| E_luc_915 | TGCCGAAAGTTGCAGTGAAG | CGCATCGTCATCTGCAGAAG | 1 | ATC | 4 | 0.04 | 0.048 |
| E_luc_1405 | ACTGAGCAAACTTCGCATGG | TGTCCAGATGCCTCTACAGC | 1 | ACCT | 5 | 0.092 | 0.099 |
| E_luc_1259 | ACAGCTTGCTTACTTGGTGC | AAGGGAACAAGGCTCAGAGG | 2 | AAG | 4 | 0.473 | 0.519 |
| E_luc_1375 | ACAAGCTCCATTTGCACGAG | GTGGTAGCCCTGGTTCTAGG | 2 | AACC | 5 | 0.13 | 0.175 |
| E_luc_1284 | GGTCTTTGTCAGCAGTGCAG | CCGAGGGCCTAAGTCTAACC | 2 | AAGC | 5 | 0.194 | 0.273 |
| E_luc_423 | GGATGAAGAAGGGTACTGCG | GCTGACTCTTGCAGACTGTG | 3 | ACC | 3 | 0.198 | 0.204 |
| E_luc_1055 | TGTGGTTGTATGCTTATCAGGC | TTCTGTGTGCTCAAGGAGATG | 3 | AAT | 3 | 0.094 | 0.205 |
| E_luc_433 | TGGAAAGGAAGCCAAAGTCAC | GTGCCAAATCCCTCTGCATC | 4 | AAT | 4 | 0.107 | 0.267 |
| E_luc_971 | CAGCCACAATCCAAGAACCC | AAGCCGGAATAGTAGAGCCG | 4 | ATC | 7 | 0.431 | 0.543 |
| E_luc_240 | TGCTATGACCTCTGGCATCC | AAGTTCTCCAGAGGCCTTGG | 4 | AAAT | 2 | 0.174 | 0.25 |
| E_luc_2440 | GCAGCAGAAACAAGGACTGG | CCAGTCTGACAGTGCGGG | 4 | AGGG | 7 | 0.211 | 0.286 |
| E_luc_371 | GTATGTGTGCACTGCGAGAG | TCAGTGGCTTGGATCTGGTG | 5 | ACC | 5 | 0.033 | 0.04 |
| E_luc_2319 | ATCAACGTTCTGAATGCGCC | TGCACTGAACTAGGAGGGAC | 5 | AAT | 5 | 0.361 | 0.381 |
| E_luc_961 | TGTTGCAAAGTTCTGGTCGG | CGTGCTTTACTTCCTTGGCC | 5 | ACC | 4 | 0.206 | 0.215 |
| E_luc_808 | CCCAGAACATGCACAACCAG | TAGCGGCTGGAAGAAGGATC | 6 | AGC | 5 | 0.655 | 0.499 |
| E_luc_2336 | TTTCATGGCTGCTTGTACCC | ACATACTACAACTCGAGGTGC | 6 | AAAT | 3 | 0.04 | 0.046 |
| E_luc_566 | AGGGTTTAACTGCTGAAGGG | GCAAATCTCAGCCGTGTCTC | 6 | AAAT | 4 | 0.04 | 0.198 |
| E_luc_2121 | CCCTCCCTGTGCTTACTCTG | ACGATCTGACCTGATGACCG | 6 | AATT | 2 | 0.014 | 0.013 |

Table 3. Location and collection information of each cave locality from which samples were taken. N indicates the number of sampled individuals at each locality. General regions only are provided, due to cave conservation and safety concerns, but geographic coordinates are available upon request.

| Cave Name | State | N | | Cave Name | State | N |
|---|---|---|---|---|---|---|
| Bankley/Fairground | IN | 9 | | YMCA | KY | 8 |
| Donnehue | IN | 5 | | Bull Creek | MO | 9 |
| Roberts | IN | 4 | | Crighton Spring | MO | 5 |
| Robinson Ladder | IN | 4 | | Blue Moon | OK | 2 |
| Sullivan | IN | 6 | | Iron Gate | OK | 3 |
| The Lost River | IN | 4 | | Jail | OK | 9 |
| Adwell | KY | 1 | | January-Stansbury | OK | 7 |
| Big Hollow Cave | KY | 7 | | Survivalist | OK | 2 |
| C2 | KY | 7 | | Third | OK | 4 |
| Cadaverous Cave | KY | 5 | | Crews | TN | 4 |
| Crystal Cave | KY | 2 | | Eoff | TN | 6 |
| Falling Tree Cave | KY | 6 | | Gillespie | TN | 4 |
| Great Onyx Cave | KY | 11 | | Lost Puddle | TN | 1 |
| Hickory Cabin Cave | KY | 6 | | Mull Prowell | TN | 6 |
| Left Eye Cave | KY | 1 | | Pompie | TN | 2 |
| Little | KY | 2 | | Blankenship Blowhole | VA | 6 |
| Mammoth Cave | KY | 2 | | Byrd's Water | VA | 10 |
| Natural Bridge Cave | KY | 1 | | Smokehole | VA | 6 |
| New Discovery Entrance | KY | 11 | | Tawney's | VA | 3 |
| Phil Cave | KY | 4 | | Borehole | WV | 4 |
| Stan | KY | 2 | | Buckeye Creek | WV | 1 |
| Sturgeon Cave | KY | 1 | | Higganbotham | WV | 4 |
| Unknown Cave (Austin Ent.) | KY | 2 | | Mann | WV | 3 |
| Violet City Entrance | KY | 6 | | Spring | WV | 1 |
| White Cave | KY | 2 | | | | |

Table 4. Hardy-Weinberg estimations for each locus within each population. Significant departures from Hardy-Weinberg are indicated in bold.

| Population | 238 | 915 | 1405 | 1259 | 1375 | 1284 | 423 | 1055 | 433 | 971 | 240 | 2440 | 371 | 2319 | 961 | 808 | 2336 | 566 | 2121 | Multi-locus |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Adwell** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Austen** | --- | 0 | 0 | 0 | 0 | 0 | --- | 1 | 0.5 | 0 | 0 | 0 | --- | 0 | 0 | 0 | --- | --- | --- | 0.25 |
| **Buckeye_Creek** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Bankley** | --- | --- | -0.091 | 0.216 | --- | -0.231 | -0.212 | 0 | --- | -0.116 | 0.158 | -0.067 | --- | 0 | -0.164 | 0.048 | --- | **0.536** | --- | 0.034 |
| **Big** | --- | --- | 0 | 0.321 | 0 | -0.125 | 0 | 1 | 0.647 | 0.419 | 0.368 | -0.358 | --- | 0.213 | -0.125 | -0.2 | --- | 1 | --- | 0.214 |
| **Blue_Moon** | --- | --- | 0 | --- | 0 | --- | --- | --- | --- | -1 | --- | 0 | --- | --- | --- | -1 | --- | --- | --- | -0.5 |
| **Black** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Blankenship** | --- | --- | --- | 0.062 | --- | --- | --- | --- | 0.5 | 0.524 | --- | --- | --- | --- | --- | -0.538 | 0.333 | --- | --- | 0.141 |
| **Borehole** | --- | --- | --- | 0 | --- | --- | --- | --- | --- | 0.5 | --- | --- | --- | --- | --- | -0.5 | --- | 1 | 0 | 0.25 |
| **Bull_Creek** | --- | --- | --- | -0.333 | -0.44 | --- | --- | --- | **0.59** | -0.514 | --- | --- | 0 | -0.067 | --- | -0.778 | --- | 0.636 | --- | -0.173 |
| **Byrd** | --- | 0.64 | 1 | 0.237 | 0.64 | 1 | --- | 1 | **0.673** | 0.308 | 0 | 0.64 | 0.654 | 0.64 | 0.5 | -0.191 | 0.386 | 1 | 0 | 0.463 |
| **C2** | --- | --- | 0 | -0.429 | 1 | -0.111 | 0 | -0.091 | 0.5 | 0 | 0.727 | 0.41 | --- | 0.368 | 0.122 | 0.294 | --- | --- | --- | 0.252 |
| **Cadaverous** | --- | --- | --- | -0.5 | 1 | 0.636 | -0.143 | 1 | 0.5 | -0.333 | -0.6 | 1 | --- | --- | -0.2 | -0.067 | --- | --- | --- | 0.197 |
| **Crews** | --- | --- | --- | --- | 0 | -1 | 0 | --- | --- | -1 | --- | --- | 0 | 0 | --- | 0 | --- | --- | --- | -0.286 |
| **Crystal** | --- | --- | --- | 1 | --- | 0 | 0 | --- | 0 | --- | 0 | --- | --- | -1 | 0 | --- | --- | --- | --- | 0.125 |
| **Crighton** | --- | --- | 0 | -0.143 | 1 | --- | --- | --- | 1 | 0.273 | --- | 0.6 | --- | --- | --- | -1 | 0 | --- | --- | 0.226 |
| **Culver** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Donnehue** | --- | --- | --- | -0.333 | --- | 0.6 | -0.143 | 0 | 1 | **0.75** | 0.6 | --- | --- | --- | --- | 0.04 | --- | 1 | --- | 0.432 |
| **Lost** | --- | --- | --- | -0.282 | 0 | 0.487 | -0.154 | --- | --- | -0.25 | -0.25 | --- | --- | --- | -0.111 | 0.211 | --- | 0.643 | --- | 0.046 |
| **Fall** | --- | --- | 0 | 0.318 | 0 | -0.053 | --- | 0.615 | 0 | 0.211 | 0.615 | 0.211 | --- | 0.024 | -0.111 | 0.118 | --- | --- | --- | 0.204 |
| **Gillespie** | --- | 0 | -0.091 | --- | -0.091 | --- | 1 | --- | --- | 0.182 | --- | 0.571 | 0 | 0 | 0 | 0.143 | --- | --- | --- | 0.2 |
| **Great_Onyx** | --- | --- | 0 | -0.033 | 0 | 0.304 | 0.268 | 0.574 | 0.223 | 0.459 | 0.259 | 0.231 | --- | 0.32 | -0.026 | -0.19 | 0 | **1** | --- | 0.237 |
| **Higganbotham** | --- | --- | --- | -1 | 0 | --- | --- | --- | --- | 0.6 | --- | 0 | --- | --- | --- | 0 | --- | --- | --- | 0 |
| **Higganbotham2** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Hickory** | --- | --- | 0 | -0.163 | --- | -0.143 | 1 | -0.111 | --- | 1 | -0.25 | -0.053 | --- | 0.259 | 0 | 0.25 | --- | --- | --- | 0.128 |
| **Historical** | --- | --- | 0 | 0 | 0 | 0 | 0 | --- | 0 | 0.5 | 1 | -1 | --- | -0.333 | 0.5 | -0.333 | --- | --- | --- | 0.154 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Iron_Gate** | --- | --- | --- | --- | 0 | --- | --- | --- | --- | 0 | --- | 1 | --- | --- | 0 | -1 | --- | 1 | --- | 0.333 |
| **Jail** | --- | -0.067 | --- | -0.032 | -0.2 | --- | --- | --- | **1** | -0.366 | --- | 0 | --- | 0 | 1 | **-0.8** | --- | **0.644** | --- | 0.028 |
| **January** | --- | -0.091 | --- | 0 | 1 | --- | --- | --- | 0.5 | -0.714 | --- | --- | --- | -0.2 | --- | **-1** | --- | 1 | --- | 0.016 |
| **Left** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Little** | --- | --- | --- | 0 | --- | 0 | 0 | --- | 0.5 | 1 | 1 | 0 | --- | 0.5 | --- | 0 | --- | --- | --- | 0.545 |
| **Puddle** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Mann** | --- | --- | --- | 1 | --- | --- | 0 | --- | --- | 0.2 | --- | --- | --- | --- | --- | -1 | --- | 0.6 | --- | 0.263 |
| **Natural** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **New** | --- | --- | 0 | 0.151 | --- | 0.64 | 0 | 0.31 | 0.5 | 0.474 | 0.259 | -0.163 | --- | 0.124 | 0.268 | -0.149 | --- | --- | --- | 0.209 |
| **Eoff** | --- | 0 | --- | 0.706 | -0.053 | -0.053 | -0.111 | -0.429 | --- | 0.091 | --- | 0 | 0 | 0.362 | 0.062 | -0.395 | --- | 0 | --- | 0.043 |
| **Pagoda** | --- | 0 | --- | -0.333 | --- | 0 | --- | 1 | 1 | --- | 0 | --- | --- | -0.333 | -0.143 | 0 | --- | --- | --- | 0.216 |
| **Phil** | --- | --- | 0 | 0.294 | --- | 0 | -0.2 | 1 | 1 | 0 | 0 | -0.091 | --- | -0.412 | 0 | 0 | --- | --- | --- | 0.165 |
| **Pompie** | --- | --- | 0 | 1 | --- | 0 | 0 | --- | --- | 0.5 | --- | 0 | 0 | -1 | --- | 0 | --- | 1 | --- | 0.308 |
| **Prowell** | -0.154 | --- | -0.111 | -0.667 | -0.25 | 0.615 | -0.429 | 0.394 | 0.286 | 0.286 | --- | -0.081 | --- | -0.19 | --- | -0.25 | --- | 1 | 0 | 0.024 |
| **Roberts** | --- | --- | --- | 0.571 | --- | 1 | -0.2 | 1 | --- | 0.667 | 0 | --- | --- | --- | --- | -0.5 | --- | 0 | --- | 0.362 |
| **Robinson** | --- | --- | --- | -0.059 | --- | 0.294 | -0.2 | --- | 1 | 0.625 | 0 | --- | --- | --- | -0.2 | -0.412 | --- | --- | --- | 0.174 |
| **Survivalist** | --- | --- | --- | -1 | 0 | --- | --- | --- | --- | -1 | --- | 1 | --- | 0 | --- | -1 | --- | --- | --- | -0.167 |
| **Silver** | --- | --- | --- | -0.391 | 0.615 | 1 | -0.25 | 0.333 | 0.5 | 0 | 0.615 | -0.053 | --- | 0.302 | -0.154 | 0 | --- | --- | --- | 0.21 |
| **Smoke** | --- | --- | --- | 0.706 | --- | --- | 0 | --- | --- | 0.211 | --- | --- | --- | --- | --- | -0.25 | --- | --- | --- | 0.18 |
| **Spring** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Stan** | --- | --- | 0 | -1 | --- | 1 | --- | 0 | --- | --- | 0 | 0 | --- | -0.333 | --- | 0 | --- | --- | --- | 0.053 |
| **Sturgeon** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | --- |
| **Sullivan** | --- | --- | --- | -0.081 | --- | -0.429 | -0.111 | --- | 1 | 0.211 | 0.062 | --- | --- | --- | -0.111 | 0 | --- | **0.706** | --- | 0.149 |
| **Tawney** | --- | --- | --- | 1 | --- | --- | 0 | --- | --- | 0.2 | --- | --- | --- | --- | --- | 0.111 | 0 | --- | --- | 0.314 |
| **Third** | --- | --- | --- | --- | 0 | --- | --- | --- | --- | -0.2 | --- | --- | --- | -0.2 | --- | -1 | --- | 1 | --- | -0.071 |
| **Violet** | --- | --- | --- | 0.524 | 0 | -0.111 | --- | 0.333 | 0.5 | --- | -0.25 | 0.062 | 0 | -0.081 | -0.111 | --- | --- | 0.062 | --- | 0.121 |
| **White** | --- | --- | --- | 0 | --- | 0 | --- | --- | --- | 0 | -1 | 0.5 | --- | 1 | 0 | --- | --- | --- | --- | 0.222 |
| **YMCA** | --- | --- | --- | -0.111 | --- | -0.077 | -0.167 | -0.077 | 0.65 | 0.192 | 0.741 | -0.273 | --- | 0.079 | -0.077 | -0.105 | --- | --- | --- | 0.089 |

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Overall** | 0.011 | 0.165 | 0.077 | 0.087 | 0.257 | 0.29 | 0.045 | 0.541 | 0.597 | 0.197 | 0.303 | 0.261 | 0.164 | 0.051 | 0.042 | -0.31 | 0.094 | 0.8 | 0.114 | 0.18 |