

SYSTEMATICS AND EVOLUTIONARY HISTORY OF SUBTERRANEAN *GYRINOPHILUS* SALAMANDERS

MATTHEW L. NIEMILLER¹, BRIAN T. MILLER², BENJAMIN M. FITZPATRICK¹

¹University of Tennessee, Department of Ecology & Evolutionary Biology, Knoxville, TN 37996 USA

²Middle Tennessee State University, Department of Biology, Murfreesboro, TN 37132 USA

The Tennessee Cave Salamander (*Gyrinophilus palleucus*) complex comprises populations of stygobitic, neotenic salamanders endemic to subterranean waters of central and east Tennessee, north Alabama, and northwest Georgia. Two species are currently recognized based on morphology, *G. palleucus* and *G. gulolineatus*, with the former comprising two subspecies, *G. p. palleucus* and *G. p. necturoides*. However, many populations are difficult to assign to any of the described taxa. The other obligate cave-dwelling congener is the West Virginia Spring Salamander (*G. subterraneus*), a metamorphosing subterranean species endemic to just a single cave in West Virginia. Our study of cave-inhabiting *Gyrinophilus* shows that the four nominal forms (*G. p. palleucus*, *G. p. necturoides*, *G. gulolineatus*, and *G. subterraneus*) arose recently, perhaps during the late Pliocene and Pleistocene, and are genealogically nested within the epigeic species, *G. porphyriticus*. Precise phylogenetic relationships are obscured by short branch lengths and discordant gene trees. However, other evidence supports independent origins for *G. palleucus*, *G. gulolineatus*, and *G. subterraneus*. Coalescent-based analysis of the distribution of haplotypes among species indicates that the process of divergence occurred in the presence of continuous or recurrent gene flow between subterranean populations and their surface-dwelling progenitor. Subterranean founder populations may have become isolated owing to extirpation of surface source populations. However, we propose that epigeic and hypogean forms evolved their distinct morphologies and life histories while experiencing repeated bouts of secondary contact and gene flow, a scenario we term the “periodic isolation” hypothesis.

1. Introduction

Gyrinophilus consists of large, semi- to permanently aquatic members of the Plethodontidae with four recognized species. Three of these are troglobitic and endemic to the Interior Low Plateau and Appalachian Valley of eastern North America. The fourth is trogliphilic, thriving in both hypogean and epigeic habitats. The Tennessee Cave Salamander (*G. palleucus*) complex comprises populations of paedomorphic salamanders in subterranean waters of middle and eastern Tennessee, northern Alabama, and northwestern Georgia (Petranka 1998; Miller and Niemiller 2008). These are large-bodied, permanently aquatic, gilled salamanders with reduced eyes, a broad head with a distinctly spatulate snout, and a long laterally-flattened tail. Two species are formally recognized within the complex: the Tennessee Cave Salamander, *G. palleucus*, and the Berry Cave Salamander, *G. gulolineatus*. The third troglobitic species is the West Virginia Spring Salamander, *G. subterraneus*, a single site endemic (General Davis Cave, Greenbrier Co., West Virginia). Unlike the *G. palleucus* complex, *G. subterraneus* undergoes metamorphosis, albeit at an exceptionally large size.

Because of their subterranean existence and reclusive nature,

the life history and ecology of cave-dwelling *Gyrinophilus* are poorly understood. Eggs have never been discovered for any taxon and little is known about reproduction in general (Petranka 1998). Because of few known localities and potential threats to several populations, the *G. palleucus* complex is state listed in Tennessee, Alabama, and Georgia, and NatureServe (2009) ranks *G. palleucus* and *G. gulolineatus* as globally imperiled (G2G3) and critically imperiled (G1Q) respectively. Although the U.S. Fish and Wildlife Service listed *G. palleucus* as a Category 2 candidate for federal listing in 1994, the species has not been included on more recent federal lists. *G. subterraneus* is state listed in West Virginia and NatureServe ranks this species as G1 (critically imperiled).

Taxonomy of *Gyrinophilus* has remained largely unchanged since Brandon's (1966) morphological investigation, with the exception of the description of *G. subterraneus* (Besharse and Holsinger 1977). However, recent studies (Osborn 2005; Niemiller et al. 2008; Niemiller et al., unpublished data) have re-examined the morphology and phylogenetic relationships of cave-dwelling *Gyrinophilus*. Here we review the systematics and evolutionary history of cave-dwelling *Gyrinophilus* in light of these new data.

Current data support the classification of *G. gulolineatus*, *G. palleucus*, and *G. subterraneus* as distinct from the surface form, *G. porphyriticus*, and suggest that the cave forms arose independently via rapid adaptive evolution in the absence of complete geographic isolation.

2. Tennessee Cave Salamander (*Gyrinophilus palleucus*)

Gyrinophilus palleucus was discovered by E.C. McCrady at Sinking Cove Cave, located on the Eastern Escarpment of the Cumberland Plateau in southern Franklin County, Tennessee (McCrady 1954). The discovery was first presented at the Tennessee Academy of Science annual meeting in December 1944 where McCrady exhibited preserved specimens and images of gilled salamanders with reduced eyes that he presumed were neotenic (McCrady 1945, 1954). McCrady delayed formal description of the species until 1954 in hopes of substantiating his presumption. H.C. Yeatman later confirmed neoteny in August 1954 when an adult male was collected from the type locality with a spermatophore protruding from its cloaca (Lazell and Brandon 1962). Though neotenic, *G. palleucus* has been induced to metamorphose in the lab (Dent and Kirby-Smith 1963; Brandon 1971), and transformed individuals have been collected in nature (reviewed in Miller and Niemiller 2008). Neotenic *G. palleucus* are similar in body form to larvae of the troglomorphic *G. porphyriticus*. However, *G. palleucus* differs from larval *G. porphyriticus* by having smaller eyes, more premaxillary, prevomerine, and pterygoid teeth, a wider head, and a more spatulate snout (McCrady 1954; Brandon 1966). Relative eye size has been the main character used to distinguish *G. palleucus* from *G. porphyriticus* morphologically (Miller and Niemiller 2008).

Two subspecies of *G. palleucus* are currently recognized. The Pale Salamander (Fig. 1a), *G. p. palleucus*, differs from the other described taxa by having pale, immaculate body pigmentation. *Gyrinophilus p. palleucus* is found in caves along the Eastern Escarpment of the Cumberland Plateau in southern Franklin and Marion Cos., Tennessee, and Jackson Co., Alabama (Miller and Niemiller 2008). All subterranean streams associated with *G. p. palleucus* localities ultimately drain into the Tennessee River. The Big Mouth Cave Salamander (Fig. 1b), *G. p. necturoides*, has nineteen trunk vertebrae, and a dark purplish-brown dorsum with heavy spotting in adults. The type locality of Big Mouth Cave is located 35 km north-northeast of the range of *G. p. palleucus* at the base of the Western Escarpment of the Cumberland Plateau in southeast Grundy Co., Tennessee. McCrady (1954) believed this population warranted subspecies

designation, which was later formally described by Lazell and Brandon (1962). The Big Mouth Cave Salamander differs from populations of *G. p. palleucus* in the Crow Creek drainage of southern Franklin County by possessing an additional trunk vertebra and a dark, spotted dorsal color pattern. No other populations resembling *G. p. necturoides* were known from the Western Escarpment of the Cumberland Plateau for more than thirty years. Recently, Miller and Niemiller (2008) reported *G. p. necturoides* from several new caves from the Western Escarpment of Cumberland Plateau, Eastern Highland Rim, and Central Basin of Tennessee.

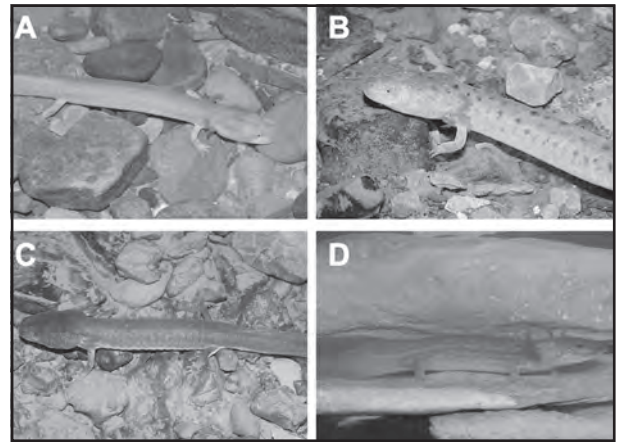


Figure 1: Obligate cave-dwelling *Gyrinophilus*: (A) Pale Salamander (*G. p. palleucus*), (B) Big Mouth Cave Salamander (*G. p. necturoides*), (C) Berry Cave Salamander (*G. gulolineatus*), and (D) West Virginia Spring Salamander (*G. subterraneus*). Photographs A, B, and C by M.L. Niemiller and D by D. Fenolio.

Outside of Tennessee, several populations of *G. palleucus* have not been assigned to subspecies in northwest Georgia (Cooper 1968; Buhlmann 2001) and northern Alabama (Brandon 1966; Cooper and Cooper 1968; Mount 1975). Previous authors have described some populations in northeast Alabama as intergrades between *G. p. necturoides* and *G. p. palleucus* (Lazell and Brandon 1962; Brandon 1966; Cooper and Cooper 1968), whereas Mount (1975) considered all populations of *G. palleucus* in Alabama as intergrades. Individuals of these populations have uniformly dark dorsal pigmentation with some individuals possessing spotting, intermediate trunk vertebrae number, and relative eye size nearer to *G. p. palleucus*. However, detailed morphological data are lacking for most populations.

3. Berry Cave Salamander (*G. gulolineatus*)

The Berry Cave Salamander (Fig. 1c) was described originally as a subspecies of *G. palleucus* by Brandon (1965), and is known from just eight caves in the Clinch

and Tennessee River watersheds of the Appalachian Valley and Ridge physiographic province in Knox, McMinn, and Roane counties of east Tennessee. *Gyrinophilus gulolineatus* differs from *G. p. palleucus* by having darker dorsal pigmentation and generally fewer trunk vertebrae (18 in 80% of *G. gulolineatus* versus 52% of *G. p. palleucus*), and from *G. p. necturoides* by possessing fewer trunk vertebrae (18 in *G. gulolineatus*, 19 in *G. p. necturoides*). Moreover, *G. gulolineatus* differs from both subspecies of *G. palleucus* by having a distinct dark stripe on the anterior half of the throat (in some populations), having a wider head with a more spatulate snout, and attaining a greater adult size (up to 136 mm SVL). Metamorphosed specimens of *G. p. palleucus* and *G. gulolineatus* differ in tooth counts, relative eye size, and division of the premaxillary bone (Simmons 1976; Brandon et al. 1986).

The taxonomic status of *G. gulolineatus* has been the subject of debate (Brandon et al. 1986; Collins 1991; Petranka 1998), although most authorities now treat the taxon as a species. Brandon et al. (1986) suggested *G. gulolineatus* be considered a separate species based on osteological evidence of transformed adults, morphological differentiation of larviform adults, and allopatry. Collins (1991, 1997) later advocated the elevation to species status. However, Redmond and Scott (1996) and Petranka (1998) treated *G. gulolineatus* as a subspecies of *G. palleucus*, a classification also currently employed by the state agencies of Tennessee.

4. West Virginia Spring Salamander (*G. subterraneus*)

The West Virginia Spring Salamander (Fig. 1d) was described by Besharse and Holsinger (1977) as a troglobitic species endemic to a single locality-General Davis Cave, in Greenbrier Co., West Virginia. Unlike *G. palleucus*, *G. subterraneus* regularly undergoes metamorphosis in nature, but at an extremely large size (>95 mm SVL, Besharse and Holsinger 1977). At the type locality, *G. subterraneus* occurs syntopically with *G. porphyriticus*. However, these species can be distinguished morphologically in several ways (Besharse and Holsinger 1977; Osbourn 2005). Larval *G. subterraneus* are larger and more robust relative to *G. porphyriticus*. In addition, larvae possess pale pinkish skin with darker reticulations and typically have two or three irregular lateral rows of pale yellow spots that are absent in larvae of *G. porphyriticus*. Moreover, *G. subterraneus* larvae have reduced eyes, wider heads, and more premaxillary and prevomerine teeth (Besharse and Holsinger 1977), but not to the extent exhibited in *G. palleucus* or *G. gulolineatus*. Metamorphosed *G. subterraneus* typically are gaunt in appearance and retain the pale reticulate coloration and

reduced eyes of larvae (Besharse and Holsinger 1977; Osbourn 2005). In addition, the premaxilla is undivided in *G. subterraneus* (also in metamorphosed *G. palleucus* and *G. gulolineatus*), but is divided in *G. porphyriticus*.

Several authors have questioned the validity of *G. subterraneus* as a species in spite of distinct morphological differences. It has been argued that *G. porphyriticus* is highly polymorphic with regards to coloration, eye size, and neoteny (Blaney and Blaney 1978) or is phenotypically plastic (Howard et al. 1984). Therefore, *G. subterraneus* represents just one of several possible phenotypes. Blaney and Blaney argued that speciation between *G. subterraneus* and *G. porphyriticus* has yet to occur and *G. subterraneus* represents a (conspecific) transitional cave form. The key argument for specific status is the co-occurrence of two distinct forms in General Davis Cave; transformed adults recognizable as *G. porphyriticus* and transformed adults with small eyes, undivided premaxilla, and distinct coloration.

5. Species Relationships

Few studies have examined the systematic relationships of taxa within *Gyrinophilus* in a phylogenetic context. Recognition of the current subspecies of *G. porphyriticus* and the obligate cave taxa is based primarily on the morphological analyses of Brandon (1966). Allozyme data (Addison Wynn, unpublished data) supports the recognition of two species within the *G. palleucus* complex: *G. palleucus* and *G. gulolineatus*. *Gyrinophilus gulolineatus* populations sampled have three unique alleles not shared with *G. palleucus*. Likewise, allozyme electrophoresis conducted by Howard et al. (1984) revealed six unique alleles in *G. subterraneus* not shared with *G. porphyriticus*. Although sample sizes were small, the authors felt *G. subterraneus* likely was a valid species and isolated from *G. porphyriticus*. Baldwin (2002) found that *G. p. palleucus* and *G. p. necturoides* form a monophyletic group sister to *G. porphyriticus* based on sequences of the ND4 and cyt *b* mtDNA genes and the RAG-1 nuclear gene.

Molecular analyses of mtDNA 12S and cyt *b* and nuclear RAG-1 genes (Niemiller et al. 2008; Niemiller et al., unpublished data) are consistent with current taxonomy describing four genetic clusters corresponding to *G. palleucus* (*palleucus* + *necturoides*), *G. gulolineatus*, *G. subterraneus*, and *G. porphyriticus*. The arguments below might not satisfy strict adherents of species definitions based on reproductive isolation or genealogical exclusivity, however, the nominal taxa are morphologically and genetically distinct (but see below for discussion on *G. subterraneus*) and are certainly valuable subjects for

studying speciation as an evolutionary process of divergence regardless of their Linnean ranking (Mallet 2008).

The data are consistent with continued recognition of the *G. gulolineatus* as a species. It maintains its distinctiveness despite geographic overlap and interbreeding with *G. porphyriticus*. Both DNA sequence data sets show clusters corresponding closely to *G. gulolineatus*. The subspecies of *G. palleucus* are morphologically distinct, but have been considered conspecific because of reported intergrades or populations that appear to include both spotted and unspotted forms. MtDNA divergence between *G. p. palleucus* and *G. p. necturoides* is low and haplotypes from the two taxa do not fall into distinct genealogical clusters (Fig. 2). RAG-1 haplotypes are more consistent with recognition of two groups (Figs. 3 and 4 in Niemiller et al. 2008). Many taxonomists have a love-hate relationship with subspecies as a Linnean rank. As evolutionary biologists, we find the designation useful as shorthand for a pattern of geographic variation suggesting incipient speciation, local adaptation, or other significant divergence. Based on the present data, we see no reason to alter the current taxonomy.

Brandon (1966) speculated that populations of *G. palleucus* inhabiting the Central Basin of Tennessee might represent an undescribed form (populations 17-18 in Niemiller et al. 2008). However, little DNA differentiation was found between these sites and other *G. palleucus* (Niemiller et al. 2008). Adjacent *G. p. necturoides* show substantial variation in dorsal coloration and degree of spotting (populations 9–13 in Niemiller et al. 2008), and populations in the Central Basin fall within that range of variation. Newly discovered populations along the Collins River in Warren County (populations 14–16 in Niemiller et al. 2008) and Duck River in Marshall and Maury counties (populations 19-20 in Niemiller et al. 2008) also show little genetic differentiation and possess coloration similar to *G. p. necturoides*. We propose extension of the range of *G. p. necturoides* to include populations in the Central Basin, and along the Duck River and Collins River.

6. Evolution of Cave Dwelling *Gyrinophilus*

A bifurcating species phylogeny was not resolved by analysis of mtDNA and *RAG-1* gene trees (Fig. 2; Fig. 3 in Niemiller et al. 2008). Genealogical discordance was manifested as shared haplotypes and more recent common ancestry of some heterospecific versus conspecific alleles. Haplotypes of *G. palleucus*, *G. gulolineatus*, and *G. subterraneus* are nested within the gene trees of *G. porphyriticus*, consistent with a scenario where each cave-adapted lineage contains a sample of ancestral Spring Salamander lineages. Our

interpretation is that the species tree is a true polytomy, representing simultaneous and independent evolution of three subterranean specialists. That is, *G. palleucus*, *G. gulolineatus*, and *G. subterraneus* each arose from a single widespread epigeal ancestor (Phase 1 speciation in Holsinger 2000). This proposition is also supported by distributional and geologic evidence. *Gyrinophilus gulolineatus* is known from caves within the East Tennessee aquifer system, which is isolated from aquifer systems to the west by a zone of faulting along the eastern escarpment of the Cumberland Plateau. This fault acts as a significant barrier for dispersal of subterranean fauna. The distributions of other stygobitic fauna, such as the Southern Cavefish (*Typhlichthys subterraneus*) and stygobitic crayfishes (*Orconectes* and *Cambarus*), exemplify this barrier; these species are abundant west of the fault but absent from the Valley and Ridge to the east. The existence of *G. gulolineatus* to the east of this subterranean barrier and *G. palleucus* to the west are consistent with the hypothesis of at least two independent invasions and adaptation to caves by ancestral *G. porphyriticus*.

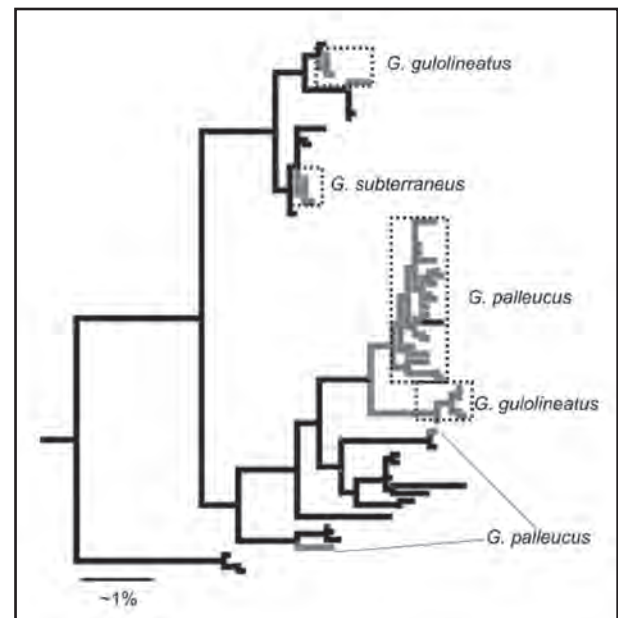


Figure 2: MtDNA sequences from obligate cave-dwelling *Gyrinophilus* (in red) are nested within the gene tree of the troglophile *G. porphyriticus* (in black) showing evidence of recent origin and multiple independent invasions. Data from nuclear *RAG-1* are similar (Niemiller et al. 2008).

Two alternative hypotheses describe the origin of subterranean species. The “climate-relict” model proposes allopatric speciation after populations of cold-adapted species become stranded in caves due to climate change (Vandel 1964; Holsinger 2000). The “adaptive-shift” model proposes parapatric speciation driven by divergent selection

between subterranean and surface habitats (Howarth 1973). Brandon (1971) postulated that the *G. palleucus* complex evolved from an epigeal, metamorphosing ancestor similar to present-day *G. porphyriticus* during the Pleistocene as fluctuating climatic conditions forced surface populations at the periphery of the species' range underground, and consequently isolating and facilitating speciation and evolution of troglomorphic characters as predicted by the climate-relict model. MtDNA and RAG-1 genealogies support the hypothesis that all three subterranean forms are recently derived from a *G. porphyriticus*-like ancestor with divergence estimates ranging from 61000 to 2.6 million years ago (Pleistocene to mid-Pliocene) (Niemiller et al. 2008). However, the data do not support the allopatric speciation scenario of the climate-relict model.

Evidence of gene flow (Niemiller et al. 2008) and the present-day ranges of the subterranean and surface forms suggest that cave-adapted *Gyrinophilus* did not diverge from their surface ancestor in strict allopatry. Under the adaptive-shift hypothesis, speciation by divergent selection results in parapatric or sympatric distributions of the incipient sister taxa (Rivera et al. 2002). Current distributions of *G. porphyriticus* and *G. palleucus* are parapatric, while *G. porphyriticus* overlaps the ranges of *G. gulolineatus* and *G. subterraneus*. However, inferring an adaptive-shift scenario based exclusively on geographic distributions is unreliable because post-speciation range shifts also may result in present-day parapatric or sympatric distributions. Alternatively, subterranean founder populations may have become temporarily isolated, but repeated bouts of recolonization by epigeal populations, possibly associated with climate change, allowed for secondary contact and gene flow, a scenario we term the "periodic isolation" hypothesis. Coalescent-based analyses (Niemiller et al. 2008) support divergence-with-gene-flow over strictly allopatric speciation with the distribution of migration events occurring over several thousand years. However, the genetic data cannot distinguish between continuous contact and alternating periods of contact and isolation. During the Pleistocene, *Gyrinophilus* are proposed to have been separated during warm periods and broadly overlapping during glacial periods when favorable cool, moist conditions would have been widespread at lower elevations (Brandon 1971). Given that interglacial periods have generally been shorter than glacial periods, and given that subterranean and surface-dwelling *Gyrinophilus* are not geographically isolated at present, periods of true isolation were likely brief relative to periods favorable to geographic overlap. Given evidence of historical and contemporary hybridization, it is clear that complete reproductive isolation did not evolve during any putative

period of geographic isolation.

Divergence in the face of continuous or episodic gene flow could be facilitated by a number of factors including assortative mating, selection against hybrids, or habitat isolation as subterranean population became more specialized and spread deeper underground (Rivera et al. 2002; Niemiller et al. 2008). In *Gyrinophilus*, access to breeding habitat may be a primary ecological advantage of subterranean colonization. However, permanent residence in caves requires special sensory, metabolic, and life history adaptations for efficient foraging and resource use (Romero and Green 2005) and these adaptations likely involve life history trade-offs. Reduction in eye size might be a pleiotropic response to selection favoring hypertrophy of other sensory systems (Jeffery 2005). Neoteny and an obligate aquatic life cycle in subterranean salamanders is probably adaptive in taking advantage of aquatic resources (Bruce 1979), while metamorphosis is favored in the small surface streams inhabited by epigeal salamanders.

Temperate cave fauna have often been viewed as isolated and relictual, originating in response to changing climatic conditions. Evidence from cave-dwelling *Gyrinophilus* and other temperate cave fauna illustrate rapid and adaptive divergence with ongoing or period bouts of gene flow; we term this scenario the "periodic isolation" hypothesis where subterranean populations are established and isolated temporarily from epigeal populations, but repeated bouts of recolonization, gene flow, and re-isolation occur, perhaps in response to climate change, during the speciation process. With increasing volumes of molecular data for cave-dwelling taxa and development of sophisticated statistical models to analyze such data, we expect discovery of more examples of divergence-with-gene-flow in cave-associated organisms.

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