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Mitochondrial sequence data indicate "Vicariance by Erosion" as a mechanism of species diversification in North American *Ptomaphagus* (Coleoptera, Leiodidae, Cholevinae) cave beetles

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Abstract

Small carrion beetles (Coleoptera: Leiodidae: Cholevinae) are members of cave communities around the world and important models for understanding the colonization of caves, adaptation to cave life, and the diversification of cave-adapted lineages. We developed a molecular phylogeny to examine the diversification of the *hirtus*-group of the small carrion beetle genus *Ptomaphagus*. The *hirtus*-group has no surface-dwelling members; it consists of 19 short-range endemic cave- and soil-dwelling species in the central and southeastern United States of America. Taxonomic, phylogenetic and biogeographic data were previously interpreted to suggest the *hirtus*-group diversified within the past 350,000 years through a series of cave colonization and speciation events related to Pleistocene climate fluctuations. However, our time-calibrated molecular phylogeny resulting from the analysis of 2,300 nucleotides from five genes across three mitochondrial regions (*cox1*, *cytb*, *rrnL-trnL-nad1*) for all members of the clade paints a different picture. We identify three stages of diversification in the *hirtus*-group: (1) ~10 million years ago (mya), the lineage that develops into *P. shapardi*, a soil-dwelling species from the Ozarks, diverged from the lineage that gives rise to the 18 cave-obligate members of the group; (2) between 8.5 mya and 6 mya, seven geographically distinct lineages diverged across Kentucky, Tennessee, Alabama and Georgia; six of these lineages represent a single species today, whereas (3) the 'South Cumberlands' lineage in Tennessee and Alabama diversified

into 12 species over the past ~6 my. While the events triggering diversification during the first two stages remain to be determined, the distributions, phylogenetic relationships and divergence times in the South Cumberlands lineage are consistent with populations being isolated by vicariant events as the southern Cumberland Plateau eroded and fragmented over millions of years.

Keywords
Cumberland Plateau, speciation, microphthalmy, troglobiont, biodiversity hotspot

Introduction

Lacking light and typically low in energy resources, caves represent a challenging environment to adapt to. In spite of these challenges, subterranean habitats harbor communities of taxonomically diverse species assemblages. The small carrion beetles (Coleoptera: Leiodidae: Cholevinae) are a significant component of cave biodiversity in temperate regions, having colonized caves on numerous occasions worldwide (Peck 1973, Ribera et al. 2010, Fresneda et al. 2011). Cave-adapted species that have reduced eyes (microphthalmy) or are eyeless (anophthalmy) have evolved many times in the family (Fig. 1) (e.g. Peck 1973, Fresneda et al. 2011, Peck and Wynne 2013).

As common and speciose cave inhabitants, leiodid beetles can provide insights into the colonization of caves, adaptation to cave life, and the diversification of cave-adapted lineages. Recent molecular work on a Palearctic radiation of subterranean leiodids (Leiodidae: Cholevinae: Leptodirini) provided insight into the timing of cave colonization, life history evolution, and diversification in the group (Ribera et al. 2010, Fresneda et al. 2011, Rizzo et al. 2013, Cieslak et al. 2014, Njunjić et al. 2018). Using a molecular clock calibrated by the tectonic separation of the Corso-Sardinian plate, Ribera et al. (2010) showed this was an ancient invasion of cave habitats, with the earliest subterranean lineages diverging in the Oligocene, around 30 million years ago (mya). They also traced subsequent evolution within the group, uncovering continued life-history evolution and diversification post-cave invasion (Cieslak et al. 2014).

Studies of leiodid beetles have also provided insight into the molecular changes associated with cave adaptation. The first transcriptome study on a cave species, the Nearctic leiodid *Ptomaphagus hirtus* (Leiodidae: Cholevinae: Ptomaphagini), revealed the conservation and expression of all genes known to be specifically required for phototransduction despite an extreme reduction of the visual system (Fig. 1). This observation was complemented by light-dark choice tests, which uncovered a strong negative photoresponse in *P. hirtus* (Friedrich et al. 2011). In addition, the conservation of circadian clock gene expression and the loss of expression of several genes in the ommochrome eye pigmentation pathway were observed (Friedrich et al. 2011).

The genus *Ptomaphagus* (subgenus *Adelops*) has been described as the most ecologically versatile group of New World Leiodidae (Peck 1973). Peck (1973) divided

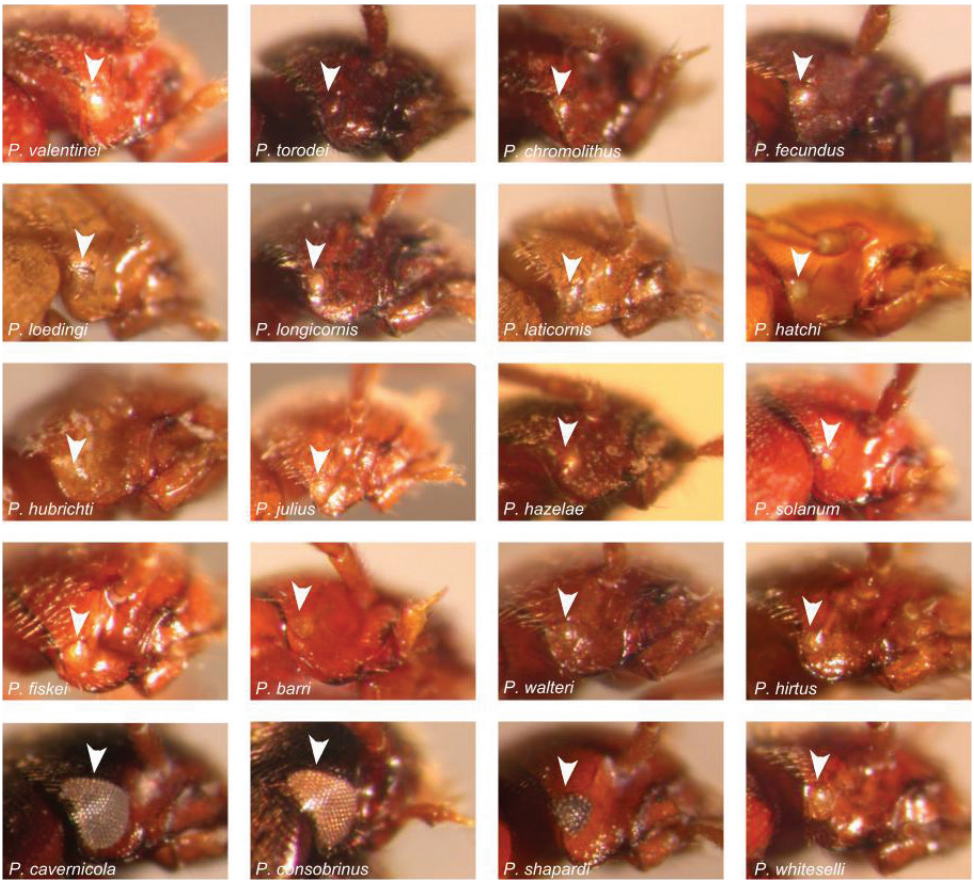

Figure 1. Eye morphologies in *Ptomaphagus*. Lateral view of head capsule and compound eye or eyelets (arrowheads) of *Ptomaphagus* species discussed in this paper. *Ptomaphagus cavernicola* and *P. consobrinus* are macrophthalmic and were used as outgroups in this study. *Ptomaphagus shapardi*, the only soil-dwelling species in the *hirtus*-group, has reduced eyes and is considered microphthalmic. The other 17 members of the *hirtus*-group are extremely microphthalmic.

Adelops into the *hirtus*-, *consobrinus*- and *cavernicola*-groups. Most members of the *consobrinus*- and *cavernicola*-groups are fully eyed (macrophthalmic), winged, and have large distribution ranges. Various lineages within all three groups are either facultative cave dwellers (eutroglophiles) or obligatory cave dwellers (troglobionts) (Sket 2008, Peck and Newton 2017). The trend toward cave-dwelling is most pronounced in the 19 species of the *hirtus*-group. Distributed across the central and southeastern United States of America, the *hirtus*-group is the largest Nearctic clade of cave-adapted Leiodidae (Peck 1973, 1984, 1986). With the exception of one soil-dwelling eutroglophile, all *hirtus*-group species are troglobionts, exhibiting extreme reduction and reorganization of the ancestral compound eye state to miniaturized camera-type eyelets (microphthalmy) and loss of wings (Fig. 1) (Peck 1973).

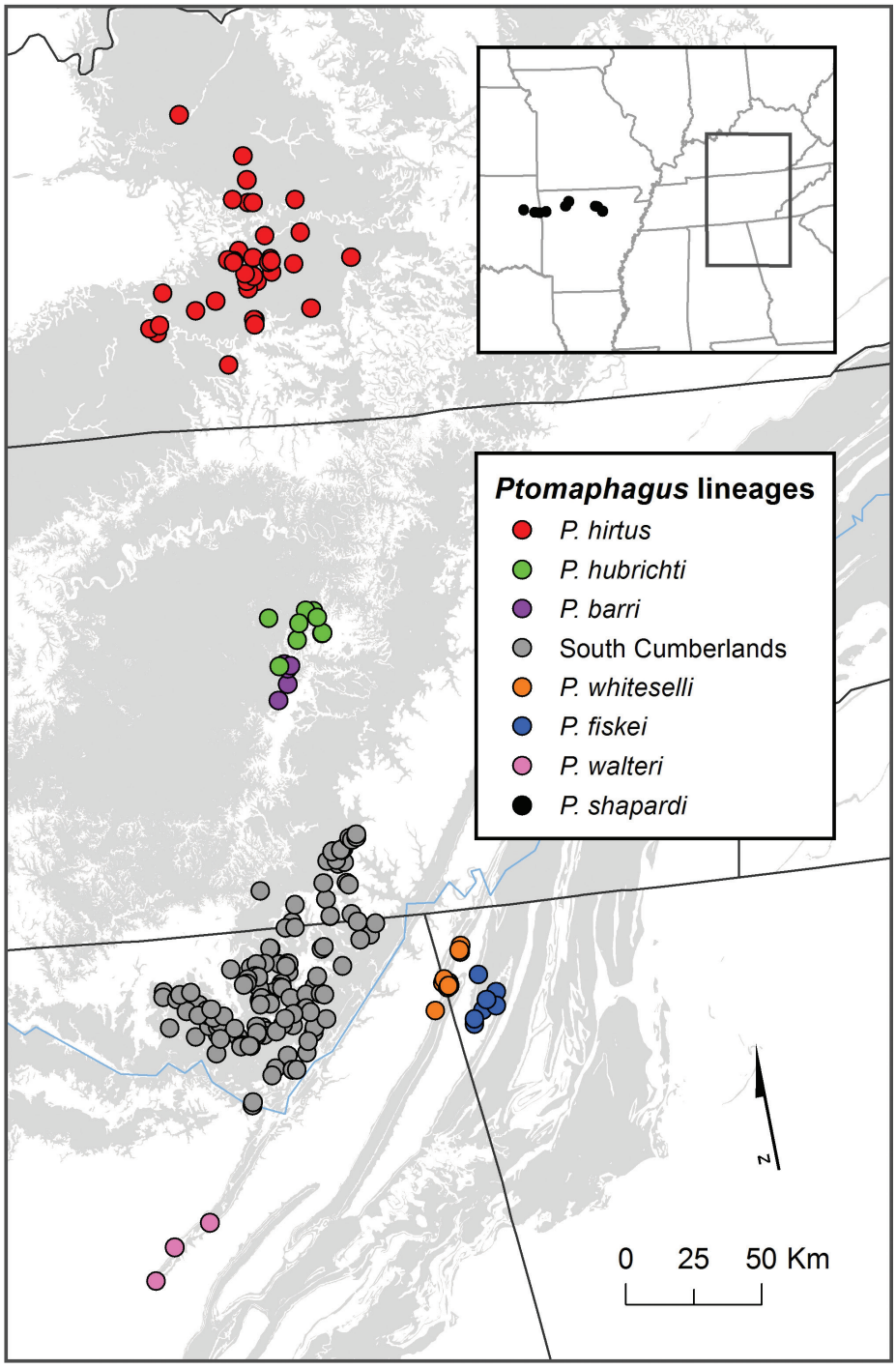

Figure 2A. Distribution of *hirtus*-group species. All known sites for *hirtus*-group species in Kentucky, Tennessee, Alabama, and Georgia. *P. shapardi* sites in Oklahoma and Arkansas are indicated in upper right inset map. A dozen species from the southern Cumberland Plateau in Tennessee and Alabama are combined.

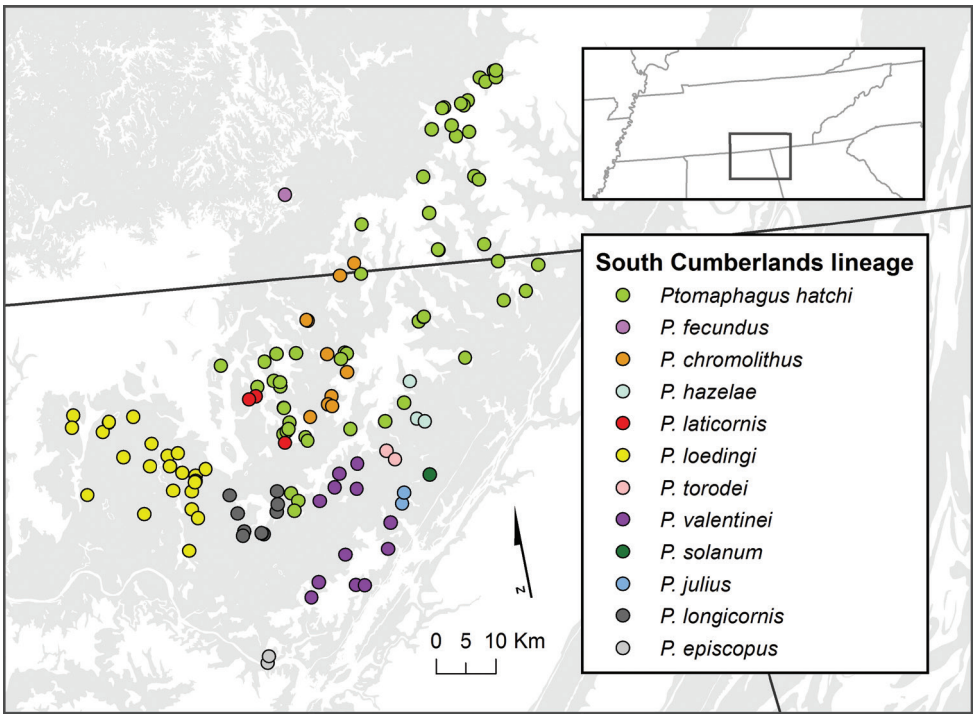

Figure 2B. Distribution of *hirtus*-group species. All known sites for members of the South Cumberlands lineage in Tennessee and Alabama. Exposed karst is shown in gray.

Members of the *hirtus*-group are found in four ecoregions (Omernik 1987): the Ridge and Valley, the Southwestern Appalachians, the Interior Plateau, and the Ozarks. *Ptomaphagus shapardi*, the soil-dwelling eutroglophile, is found in Arkansas and Oklahoma. The other species are troglobionts. *Ptomaphagus hirtus* is found in the Mammoth Cave region of central Kentucky, two species (*P. barri* and *P. hubrichti*) are found in central Tennessee, two species (*P. fiskei* and *P. whiteselli*) are found in northwest Georgia and one species (*P. walteri*) is known from the southern end of the Sequatchie Valley in Alabama. The other twelve *hirtus*-group species are clustered in the southern Cumberland Plateau of south-central Tennessee and northeast Alabama (Fig. 2A, B; Peck 1973).

As is typical for cave species, all *hirtus*-group species have small ranges. With distribution ranges <10,000 km^2, all 18 cave-obligate *hirtus*-group species are short-range endemics (Harvey 2002). Indeed, many are extreme short range endemics (Niemiller et al. 2017) with species ranges <100 km^2. Half of the *hirtus*-group species are known from five or fewer caves (Peck 1973).

Using morphological characters, Peck (1973, 1984) developed a phylogenetic framework of *Adelops* and *hirtus*-group diversification but left many relationships unresolved. Based on a lack of sympatry between *hirtus*-group species and the presence of microphthalmic eyes (as opposed to anophthalmy), Peck hypothesized that the *hirtus*-group invaded caves and diversified recently. In a version of the Climatic

Relict Hypothesis (reviewed in Culver and Pipan 2009), he suggested that glacial-interglacial cycles of the Pleistocene led to the isolation and diversification of most *hirtus*-group species over the last 350,000 years (Peck 1973, Peck 1984). Peck (1984) further hypothesized that *Ptomaphagus* populations retreated to cave- or cave-like habitats during warm and dry interglacial periods, eventually becoming cave-limited and, as a consequence, ultimately reproductively isolated. Following Peck (1984), the *hirtus*-group has been noted as an example of the Climatic Relict Hypothesis in the literature (e.g. Culver and Pipan 2009). The timing of Peck's (1984) scenario for the diversification of the *hirtus*-group (over the past ~350,000 years during the middle and late Pleistocene) differs markedly from divergence times subsequently estimated by molecular clock dating approaches for the European cave-dwelling Leiodidae, where most congeners diverged in the Pliocene or Miocene, as long as 15 million of years ago (Ribera et al. 2010).

Confronted with these divergent models for the timing of diversification in palearctic vs. nearctic cave-dwelling Leiodidae we investigated the diversification of the *hirtus*-group with molecular data. We aimed to (1) develop a molecular phylogeny for the group; (2) estimate the timing and pattern of diversification in the *hirtus*-group, and (3) gain insight into how these cave beetles diversified across distinct ecoregions and in the southern Cumberland Plateau.

Materials and methods

Specimens

Representatives of all 19 species of the *hirtus*-group were collected from 2012 to 2014. The Tennessee Wildlife Resources Agency permitted work in Tennessee (permit #1605). The Georgia Department of Natural Resources permitted work in Georgia (permit #8934). The National Park Service permitted collection of *P. hirtus* from Mammoth Cave National Park (permit #MACA-2013-SCI-0008). *P. shapardi* specimens from Oklahoma were collected by Matthew Niemiller. Two outgroup species representing the other main lineages in the subgenus *Adelops* (*P. cavernicola* and *P. consobrinus*) were collected in Florida. All beetles were collected by hand, typically with an aspirator or moist brush, and stored in 95% ethanol at -20°C. Seven species were collected from their type locality and several other species were collected from sites <1 km from their type locality. Sampling localities and species names are detailed in Table 1.

Molecular methods

We amplified and sequenced three regions of the mitochondrial genome (*cox1*, *cytb*, *rrnL-trnL-nad1*) totaling on average 2300 bp. These regions were previously used in

Table 1. *Ptomaphagus* specimens, sampling locations and Genbank accession numbers.

Species	Specimen	Locality	*cox1*	*cytb*	*rrnL-nad1*
P. cavernicola Schwarz, 1898	KSZ13-127	USA: Warrens Cave, Alachua County, Florida	KT167442	KT167490	KT167394
P. consobrinus (LeConte, 1853)	KSZ13-128	USA: Tallahassee, Florida	KT167443	KT167491	KT167395
P. barri Peck, 1973	TCN35_1	USA: Gunters Cave, Cannon County, Tennessee	KT167444	KT167492	KT167396
	TCN37_1	USA: Pleasant Ridge Cave, Cannon County, Tennessee	KT167445	KT167493	KT167397
	TCN37_2	USA: Pleasant Ridge Cave, Cannon County, Tennessee	KT167446	KT167494	KT167398
	TCN78_1	USA: Frog Hole Cave, Cannon County, Tennessee	KT167447	KT167495	KT167399
P. chromolithus Peck, 1984	AJK601_1	USA: Dub Green Cave, Jackson County, Alabama	KT167448	KT167496	KT167400
	AJK601_2	USA: Dub Green Cave, Jackson County, Alabama	KT167449	KT167497	KT167401
P. episcopus Peck, 1973	AMS3278_1	USA: Bloody Head Cave, Marshall County, Alabama	KT167450	KT167498	KT167402
P. fecundus Barr, 1963	TFR2_2	USA: Caney Hollow Cave, Franklin County, Tennessee	KT167451	KT167499	KT167403
P. fiskei Peck, 1973	GWK57_1	USA: Pigeon Cave, Walker County, Georgia	KT167452	KT167500	KT167404
	GWK57_2	USA: Pigeon Cave, Walker County, Georgia	KT167453	KT167501	KT167405
P. hatchi Jeannel, 1933	AJK289_1	USA: Kyles Cave, Jackson County, Alabama	KT167454	KT167502	KT167406
	AJK289_2	USA: Kyles Cave, Jackson County, Alabama	KT167455	KT167503	KT167407
	AJK826_1	USA: Roadside Cave, Jackson County, Alabama	KT167456	KT167504	KT167408
	AJK826_2	USA: Roadside Cave, Jackson County, Alabama	KT167457	KT167505	KT167409
	TFR423_6	USA: Grapevine Cave, Franklin County, Tennessee	KT167458	KT167506	KT167410
	TFR423_7	USA: Grapevine Cave, Franklin County, Tennessee	KT167459	KT167507	KT167411
	TGD10_1	USA: Crystal Cave, Grundy County, Tennessee	KT167460	KT167508	KT167412
	TGD10_2	USA: Crystal Cave, Grundy County, Tennessee	KT167461	KT167509	KT167413
P. hazelae Peck, 1973	AJK459_1	USA: Geiger Cave, Jackson County, Alabama	KT167462	KT167510	KT167414
P. hirtus (Tellkampf, 1844)	KWH_1	USA: White Cave, Edmonson County, Kentucky	KT167463	KT167511	KT167415
	KWH_2	USA: White Cave, Edmonson County, Kentucky	KT167464	KT167512	KT167416
P. hubrichti Barr, 1958	TCN26_1	USA: Tenpenny Cave, Cannon County, Tennessee	KT167465	KT167513	KT167417
	TCN26_2	USA: Tenpenny Cave, Cannon County, Tennessee	KT167466	KT167514	KT167418
	TDK8_1	USA: Cripps Mill Cave, DeKalb County, Tennessee	KT167467	KT167515	KT167419
	TDK8_2	USA: Cripps Mill Cave, DeKalb County, Tennessee	KT167468	KT167516	KT167420
P. julius Peck, 1973	AJK974_1	USA: House of Happiness Cave, Jackson County, Alabama	KT167469	KT167517	KT167421
	AJK974_2	USA: House of Happiness Cave, Jackson County, Alabama	KT167470	KT167518	KT167422
P. laticornis Jeannel, 1949	AJK290_1	USA: Rousseau Entrance to Gary Self Pit, Jackson County, Alabama	KT167471	KT167519	KT167423
	AJK290_2	USA: Rousseau Entrance to Gary Self Pit, Jackson County, Alabama	KT167472	KT167520	KT167424
P. loedingi (Hatch, 1933)	AMD120_3	USA: Cold Spring Cave, Madison County, Alabama	KT167473	KT167521	KT167425
	AMD120_4	USA: Cold Spring Cave, Madison County, Alabama	KT167474	KT167522	KT167426
	AMD60_2	USA: Cave Spring Cave, Madison County, Alabama	KT167475	KT167523	KT167427
	AMD60_3	USA: Cave Spring Cave, Madison County, Alabama	KT167476	KT167524	KT167428
P. longicornis Jeannel, 1949	AJK310_1	USA: Crossings Cave, Jackson County, Alabama	KT167477	KT167525	KT167429
	AJK310_2	USA: Crossings Cave, Jackson County, Alabama	KT167478	KT167526	KT167430
	AMD6_1	USA: Hering Cave, Madison County, Alabama	KT167479	KT167527	KT167431

Species	Specimen	Locality	*cox1*	*cytb*	*rrnL-nad1*
P. shapardi Sanderson, 1939	KSZ13-137	USA: Wady Cave #86, Adair County, Oklahoma	KT167480	KT167528	KT167432
P. solanum Peck, 1973	AJK166_1	USA: Sheldon's Cave, Jackson County, Alabama	KT167481	KT167529	KT167433
P. torodei Peck, 1984	AJK1068_1	USA: Two Way Cave, Jackson County, Alabama	KT167482	KT167530	KT167434
	AJK1068_2	USA: Two Way Cave, Jackson County, Alabama	KT167483	KT167531	KT167435
P. valentinei Jeannel, 1949	AJK174_1	USA: Schiffman Cave, Jackson County, Alabama	KT167484	KT167532	KT167436
	AJK174_2	USA: Schiffman Cave, Jackson County, Alabama	KT167485	KT167533	KT167437
P. walteri Peck, 1973	ABA355_1	USA: Bryant Cave, Blount County, Alabama	KT167486	KT167534	KT167438
	ABA355_2	USA: Bryant Cave, Blount County, Alabama	KT167487	KT167535	KT167439
P. whiteselli Barr, 1963	GDD66_1	USA: Byers Cave, Dade County, Georgia	KT167488	KT167536	KT167440
	GDD66_2	USA: Byers Cave, Dade County, Georgia	KT167489	KT167537	KT167441

studies of palearctic Leiodidae (Ribera et al. 2010, Fresneda et al. 2011, Rizzo et al. 2013, Cieslak et al. 2014). DNA extractions and PCR amplifications followed standard protocols (Dixon and Zigler 2011). The primers used were based on Ribera et al. (2010) but modified based on transcriptome sequence information available from *Ptomaphagus hirtus* (Friedrich et al. 2011) (Table 2). Both strands of successful PCRs were sequenced on an ABI3730. Sequences were aligned and edited in Sequencher (v. 4.9, GeneCodes Corp).

We sequenced the three gene regions from 48 individuals from 29 populations, including all 19 species of the *hirtus*-group and two outgroups (*P. cavernicola* and *P. consobrinus*) representing the other *Adelops* species-groups (Peck 1973). In 19 cases, we sequenced two individuals from the same cave. For five species, we sequenced individuals from more than one cave. All sequences have been submitted to Genbank (KT167394-KT167537; Table 1).

Table 2. Primers used, in 5' to 3' orientation. Primers were based on Ribera et al. (2010) but modified based on sequences available from *Ptomaphagus hirtus* (Friedrich et al. 2011).

Gene Region	Primer	Orientation	Sequence
cox1	hatchi.COIfor	Forward	CTGGTGGTGGGGATCCAATTC
	hirtus.COIfor	Forward	CAGGAGGTGGAGATCCTATTC
	hatchi.COIrev	Reverse	GCTTAAATTCATTGCACTAATCTGC
	hatchi.COIrev2	Reverse	TAAATTCATTGCACTAATCTGCCAT
cytB	CB3	Forward	GAGGAGCTACAGTTATTACAAA
	CB4	Reverse	AATAAAAAATATCATTCTGGTTGAAT
rrnL-trnL-nad1	16SaR	Forward	CGCCTGTTTAWCAAAAACAT
	16SaNew	Forward	CTTAAGTCTAATCTGCCCAATG
	16Sc	Forward	GATTGCGACCTCGATGTTGGA
	nad1	Reverse	ATTAGAATTTGAAGATCAACCTG
	16Sb	Reverse	CCGATTTAAACTCAGATCATGT
	nadnew	Reverse	ATTTCATAAGAAATAGTTTGAGC

Molecular tree estimation

The lack of indels or stop codons in the protein-coding *cox1*, *cytb*, and *nad1* regions allowed for unambiguous multiple sequence alignment across all sites. The ribosomal and transfer RNA coding *rrnL-trnL* sequences were aligned using MAFFT (Katoh et al. 2005). Similar alignments were obtained using MUSCLE (Edgar 2004) and CLUSTAL (Larkin et al. 2007). Bayesian analysis was conducted with MrBayes v.3.2.2 (Ronquist and Huelsenbeck 2003), using four partitions (*cox1*, *cytb*, *rrnL-trnL*, *nad1*). Evolutionary models were estimated prior to the analysis with jModelTest v.2.1 (Posada 2008). GTR+I+Γ was the preferred model for all partitions except *nad1* (TIM1+I+Γ) by AIC. MrBayes ran for 60 million generations using default values and the GTR+I+Γ model for each partition, saving every 6,000th tree. 10% of values were discarded as burnin. Similar results were obtained using codon-position partitions for the three protein coding regions and from a two partition approach (protein coding and non-protein coding sequences); both yielded similar or identical topologies with the only differences occurring at poorly-supported nodes.

Maximum likelihood searches were conducted using RAxML (Stamatakis 2014) with a GTR+Γ model used for each of the four partitions. Branch support was evaluated by rapid bootstrapping over 1,000 replicates (Suppl. material 3). Maximum likelihood analyses yielded similar topologies and node support to the Bayesian analyses.

Divergence time estimation

Divergence time estimates were generated with BEAST v1.8.1 (Drummond et al. 2012) using a total chain length of 60,000,000 generations with sampling every 1,000 generations. A Yule type speciation model with a wide normal distribution of tree height (treeModel.rootHeight = normal, initial: 15, Mean 15, Stdev 6, 95% of distribution between 5.495 mya and 24.89 mya) was used to model the tree. (We obtained similar results for tree height when using a uniform prior distribution of tree height between 5-25 mya.) The four data partitions shared a lognormal relaxed clock model with a substitution rate of 0.01 substitutions/site/my (stddev 0.002) (Rizzo et al. 2013). Each partition was assigned its own GTR+I+Γ substitution model. Tree topology was constrained to require monophyletic clades recovered with greater than 0.90 posterior probability in both MrBayes trees to appear in the final tree. We confirmed the convergence of parameter estimates by examination of three simultaneous runs using Tracer v1.5. Resulting tree files were summarized using TreeAnnotator v1.8.1, discarding 25% of samples as burnin and visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Phylogenetic relationships and molecular clock estimates of diversification in the *hirtus*-group based on mitochondrial DNA sequences

Adopting the approach of Ribera et al. (2010), we produced a molecular phylogeny for the *hirtus*-group based on sequences from five genes across three mitochondrial regions, applying the molecular clock developed for the same genes in this subfamily of beetles (Leiodidae: Cholevinae) to establish divergence times for the phylogeny. Using that approach, the *hirtus*-group is estimated to have diverged from the *consobrinus*- and *cavernicola*-groups around 15 mya (Fig. 3). Within the *hirtus*-group we observed three stages of diversification. First, we found strong support for the soil-dwelling and geographically separated *P. shapardi* as sister species to the 18 cave-obligate species of the *hirtus*-group, with the two lineages diverging ~10 mya (Fig. 3). This divides the sole member of the *hirtus*-group from the Ozarks from the rest of the species in the southeastern United States (Fig. 2A). In the second stage of diversification, seven geographically distinct lineages originated between 8.5 and 6 mya (Fig. 3) across Kentucky, Tennessee, Alabama and Georgia, spreading *hirtus*-group members across several ecoregions (Fig. 2A). We were unable to resolve the branching order of these lineages with high confidence (Fig. 3). Six of these lineages are currently represented by a single species - *P. barri*, *P. fiskei*, *P. hirtus*, *P. hubrichti*, *P. walteri* and *P. whiteselli* (Figs 2A, 3). The seventh lineage contains all the species from the southern Cumberland Plateau (Fig. 2B), which form a well-supported clade (Fig. 3). In the third stage of diversification, the South Cumberlands lineage expanded from one to 12 species beginning around 6 mya. The branching order in the South Cumberlands clade is well-resolved (Fig. 3).

Ptomaphagus hatchi is polyphyletic

Most specimens sampled for a single presumed species formed monophyletic groups in our molecular phylogeny. This was not the case, however, for the *P. hatchi* specimens sampled from four different sites. Our molecular phylogenetic analysis reveals that *P. hatchi* constitutes a polyphyletic clade with respect to six species (*P. chromolithus*, *P. episcopus*, *P. fecundus*, *P. laticornis*, *P. torodei* and *P. valentinei*) with which it has overlapping or adjacent distribution ranges on the southern Cumberland Plateau (Figs 2, 3). One well supported clade joins several populations of *P. hatchi* with *P. episcopus*, *P. fecundus* and *P. chromolithus* (Fig. 3). A second well supported clade joins *P. hatchi* with *P. laticornis* (Fig. 3). We further noted that the intraspecific (between cave) divergence between *P. hatchi* populations was high, ranging from 1.2 to 5.1% P-distance for the *cox1* gene (Suppl. material 2). Intraspecific (between cave) distances for populations of other *Ptomaphagus* species are all < 2.0% (Suppl. material 2). In several cases, the intraspecific *P. hatchi* divergence is greater than the interspecific divergence observed between other *Ptomaphagus* species.

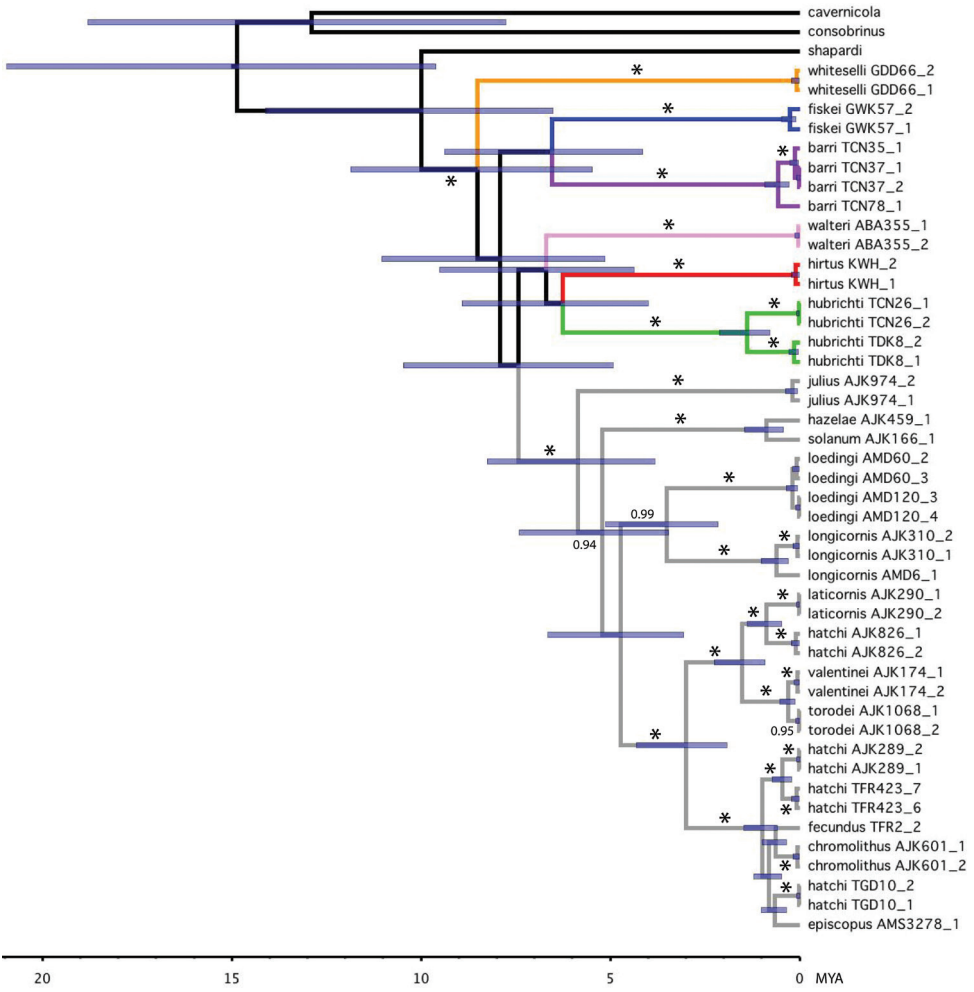

Figure 3. Ultrametric tree for the *hirtus*-group. Bayesian tree estimated from combined partial mitochondrial sequence data. Branches supported by posterior probability >0.90 are labeled with values or, for branches with posterior probability of 1.0, an asterisk. Blue bars indicate 95% confidence intervals of estimated ages for the nodes. Taxa are labeled with species name and specimen identifier (Table 1). Scale at bottom indicates divergence times in millions of years as estimated by BEAST (Drummond et al. 2012). Branch colors correspond to those in Figure 2A.

Intraspecific molecular variation

To assess genetic diversification within and between lineages, we also surveyed intraspecific variation at population (within cave) and species (between cave) levels. In 19 cases (representing 14 species), we sequenced two individuals from the same cave. Variation between individuals from the same cave was low, with a mean *cox1* p-distance of 0.15% (N = 19, range = 0.00 - 0.58%) (Suppl. material 1). In all 19 cases individuals from the same cave were each other's closest relatives (Fig. 3).

For five species, we sampled animals from multiple caves. Specifically, we sampled *P. hubrichti*, *P. loedingi* and *P. longicornis* from two caves, *P. barri* from three caves, and *P. hatchi* from four caves. Intraspecific variation between caves had a mean *cox1* p-distance of 2.1% for all pairwise comparisons (N = 12, range = 0.2 - 5.1%) (Suppl. material 2). Intraspecific variation between caves across the 2300 bp of all five sampled loci was slightly lower (mean p-distance = 1.7%), consistent with the previous observation that the *cox1* region evolves faster than the other mitochondrial regions in this group (Ribera et al. 2010) (Suppl. material 2).

Discussion

The *hirtus*-group diversified in three stages

Our molecular phylogeny provides a time-calibrated picture of the diversification of the *hirtus*-group, shedding new light on the origins of an important component of cave biodiversity in North America. The integration of molecular and biogeographic data now suggests that the *hirtus*-group diversified in three stages. The first stage, occurring mid-Miocene ~10 mya, separated the sole extant eutroglophile in the *hirtus*-group, *P. shapardi*, located in the central United States from the 18 exclusively troglobiotic species located in the southeastern United States (Figs 2A, 3). The second stage of diversification spawned seven lineages across the southeastern United States during the late Miocene, 8.5-6 mya (Figs 2A, 3). In the third stage, further fine scale diversification occurred in the southern Cumberland Plateau region during the Pliocene and Pleistocene over the past six million years (Figs 2B, 3).

How many times have *Ptomaphagus* invaded cave habitats?

During the second stage of *hirtus*-group diversification, the lineages that diversified into the 18 cave-obligate members of the *hirtus*-group were established in five distinct geographic regions (Fig. 2A): the Mammoth Cave region of Kentucky, central Tennessee, northwest Georgia, the southern end of the Sequatchie Valley in Alabama, and the southern Cumberland Plateau in Tennessee and Alabama. Although further investigation of the deeper nodes of the *hirtus*-group phylogeny is still warranted, it appears that the two species in central Tennessee (*P. barri* and *P. hubrichti*) do not form a monophyletic group, nor do the two species in northwest Georgia (*P. fiskei* and *P. whiteselli*). This suggests that distinct *hirtus*-group lineages invaded underground habitats as many as seven times. Any alternative explanation requires one or more episodes of up to 75 km long-distance dispersal across significant non-karst terrain separating current species distributions. We do not favor long-distance dispersal as an explanation for current species distributions as the dispersal ability of *hirtus*-group

members appears to be quite limited. All species are wingless and small, and none have been collected in surface habitats. Even short-distance migrations appear unlikely, as two species have never been collected in the same cave, which is particularly notable in the case of the cave- and species-rich southern Cumberland Plateau. In many cases, uninhabited cave habitats are present within just one or a few kilometers of known *hirtus*-group populations (Peck 1973), indicating extremely low vagility across the *hirtus*-group.

Further consistent with minimal dispersal ability is the fact that the southern Cumberland Plateau lineage diversified into twelve species over the past 6 million years and no members of this lineage appear to have migrated from the southern Cumberland Plateau. Notwithstanding the strong circumstantial evidence, long-distance migration cannot be completely discounted as an explanation for some aspects of the species distributions we see today. For instance, long-distance dispersal has been proposed for one group of troglobiotic leiodids in the palearctic realm (*Troglocharinus* of Spain; Rizzo et al. 2013). Rizzo et al. (2013) proposed these beetles expanded their range in the early Pliocene from the central Pyrenees across a significant non-karst region to coastal karst habitats 60–70 km away, via stepping-stone migration across the surface during a permissive climate period, followed by subsequent isolation of the two lineages (Rizzo et al. 2013).

The limited dispersal capacity of troglomorphic (microphthalmic and wingless) *Ptomaphagus* species suggests that troglomorphy developed multiple times through convergent evolution in this group. This scenario and its implications could be further scrutinized at the molecular level. Previous transcriptome analyses on *P. hirtus*, for example, identified several genes in the ommochrome eye pigmentation pathway that are no longer expressed in *P. hirtus*, consistent with the lack of pigment granules in the highly reduced eyelets of this species (Friedrich et al. 2011). Similarly reduced and non-pigmented eyelets are found in all other cave-adapted species of the *hirtus*-group. A single cave colonization event during early *hirtus*-group evolution would predict shared lack-of-function mutations underlying the regression of eyes and eye pigmentation across the *hirtus*-group. Multiple independent cave colonization events, in contrast, would be reflected by a lack of shared lack-of-function mutations in eye pigmentation genes. This approach, which can be extended to other cave adaptive traits, will ultimately require genomic analyses to determine whether identical mutations are present in geographically distinct populations (consistent with a single cave invasion and the evolution of troglomorphy followed by long-distance dispersal) or whether different mutations are present in different lineages (consistent with multiple cave invasions followed by the convergent evolution of troglomorphy). Consistent with independent cave invasions and evidence for the feasibility of such an approach, distinct mutations have been observed in the pigmentation gene *cinnabar* and in the opsin genes of subterranean diving beetles (Leys et al. 2005, Tierney et al. 2015) and in *rhodopsin* genes of cavefish (Niemiller et al. 2013) in geographically distinct populations of those groups.

A new model of *hirtus*-group diversification: Vicariance by erosion

The Climatic Relict Hypothesis suggests that a species' initial colonization of caves occurred when it sought refuge from environmental stressors in the surface environment (reviewed in Culver and Pipan 2009). The Climatic Relict Hypothesis is often proposed for troglobiotic taxa lacking close relatives on the surface, with those relatives presumably extinct as a result of the changing surface conditions that forced the proto-troglobiotic taxa underground. The Climatic Relict Hypothesis can be supported by evidence that the cave species originated or diversified during a period of climate change. A convincing case for the Climatic Relict Hypothesis was developed by Leys et al. (2003) for Australian diving beetles where numerous independent invasions of isolated calcrete aquifers occurred. Leys et al. (2003) used a molecular clock to show that the timing of these invasions correlated with increasing aridity moving across the region from north to south during the Pliocene. In North America, the Climatic Relict Hypothesis has often been suggested as a driver of cave colonization related to climate change during late Pleistocene glaciation events (Culver and Pipan 2009). Peck (1973, 1984) offered the *hirtus*-group as a possible example of cave colonization via the Climatic Relict Hypothesis. To explain the species diversity of the *hirtus*-group, Peck (1973, 1984) suggested that the first *Ptomaphagus* to invade cave habitats subsequently dispersed to nearby caves during recent Pleistocene interglacial periods via cool and moist habitats such as leaf litter, talus or moss mats, after which the species became more completely cave adapted and isolated.

Our time-calibrated molecular phylogeny rejects the hypothesis of middle to late Pleistocene diversification in the *hirtus*-group. We found the timing of most speciation events in the *hirtus*-group to be an order of magnitude greater than Peck hypothesized (Peck 1973, 1984). Our divergence time estimates are similar to those derived from molecular data in other troglobiotic groups (e.g., Leys et al. 2003, Faille et al. 2010, Ribera et al. 2010, Derkarabetian et al. 2010, Niemiller et al. 2012). The divergence times estimated by these studies indicate that the species in these groups are much older than the recent Pleistocene. With most diversification of the *hirtus*-group occurring in the Miocene and Pliocene, we can rule out the Climatic Relict Hypothesis related to Pleistocene glaciations as a general rule for the *hirtus*-group.

Now recognizing that the third stage of *hirtus*-group diversification, the radiation in the southern Cumberland Plateau, began ~6 mya, we hypothesize that cave-adapted *Ptomaphagus* populations distributed throughout the region were isolated by vicariant events as the southern Cumberland Plateau eroded and fragmented over millions of years. Barr and Holsinger (1985) suggest gene flow between cave systems can be reduced via "simple erosion that divides a karst area into isolated segments by cutting down into underlying noncavernous strata." This mechanism is particularly applicable to terrestrial troglobionts, whose populations are unlikely to maintain connectivity via deep groundwater connections, as might be the case in aquatic troglobionts (e.g., Fenolio et al. 2017). The southern Cumberland Plateau is greatly dissected, with numerous isolated and peripheral mountains and ridges, and *hirtus*-group species are fre-

quently limited to a single isolated ridge. For example, *P. longicornis* is limited to caves on Keel Mountain (in Madison and Jackson Counties, Alabama), *P. julius* is limited to caves on July Mountain (Jackson County, Alabama), and *P. solanum* is limited to Tater Knob (Jackson County, Alabama). Each of these ridges is an isolated remnant of the Cumberland Plateau (Fig. 4). Further consistent with the vicariance by erosion model (Fig. 5), the earliest lineages to diverge in the South Cumberlands clade (*P. julius*, *P. hazelae* + *P. solanum*, *P. loedingi* and *P. longicornis*) are found in peripheral and isolated ridges at the edge of the southern Cumberland Plateau, whereas later-diverging lineages are concentrated in the more intact central region of the southern Cumberland Plateau (Figs 3, 4).

Although we lack a clear picture of the timing and pattern of erosion and fragmentation of the southern Cumberland Plateau, it is clear that the ~6 my over which the South Cumberlands lineage diversified is sufficient for significant erosion and cave development to have occurred. In support of this, on the western edge of the Cumberland Plateau in middle Tennessee, extensive stream incision, erosion and cave development occurred over a similar period of time. In this region, the oldest caves (now located 60–90 m above current river level) were estimated to be 3.5–5.7 my old based on the dating of radioactive cave sediments (Sasowsky et al. 1995, Anthony and Granger 2004, 2007). Assuming a similar degree of erosion in the southern Cumberland Plateau, it is reasonable to assume that the *hirtus*-group has been heavily impacted by fragmentation, resulting in cave population isolation events over the past 6 my.

Questions arising from the present study

Understanding the lack of *hirtus*-group species diversity in the Mammoth Cave region

The *hirtus*-group is widespread in the Mammoth Cave region of Kentucky and in the southern Cumberland Plateau of Tennessee and Alabama (Fig. 2). Measured as extent of occupancy (Bachman et al. 2011), the range extent of *hirtus*-group species in the two regions is similar: 3,952 km^2 in the Mammoth Cave region and 4,151 km^2 in the southern Cumberland Plateau. It is striking that, across these similar ranges, only one species (*P. hirtus*) is present in the Mammoth Cave region, whereas 12 species are present in the southern Cumberland Plateau. As discussed above, the diversification in the southern Cumberland Plateau may be related to more frequent occurrence of vicariance by erosion, in contrast to the more continuous karst of the Interior Plateaus in the Mammoth Cave region. Indeed, such patterns have been proposed in the past (e.g., Barr and Holsinger 1985 and references therein).

One prediction from this scenario is that *P. hirtus* evolved less genetic diversity across its range than we observed across the South Cumberlands lineage. Alternatively, *P. hirtus* may represent a collection of cryptic species, as has been observed in numerous cave lineages (e.g., Trontelj et al. 2009, Derkarabetian et al. 2010, García-Machado et al. 2011, Hedin 2015, Zhang and Li 2013). Our analysis presented here sampled only a single *P. hirtus*

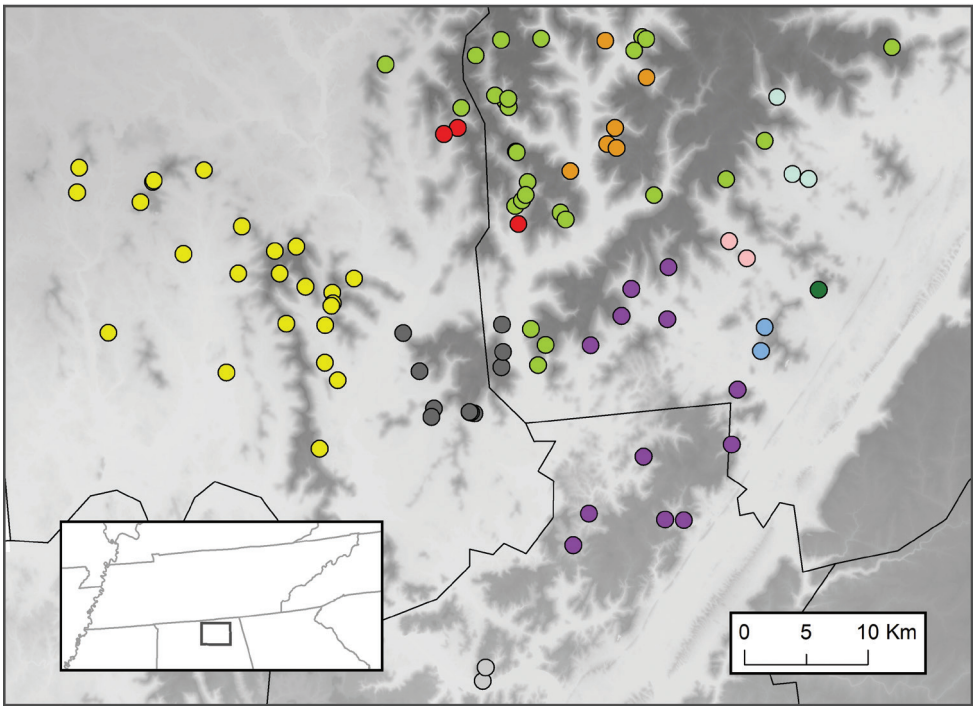

Figure 4. Distribution of *Ptomaphagus* species on the southern Cumberland Plateau, overlaid on a digital elevation model. Higher elevations (to 500 m) are indicated by darker shades, lower elevations (to 180 m) by lighter shades. *Ptomaphagus* species diverging early in the South Cumberlands lineage are limited to isolated ridges and mountains on the fringes of the plateau. These species are *P. loedingi* (yellow), *P. longicornis* (dark gray), *P. julius* (blue), *P. solanum* (dark green) and *P. hazelae* (light blue). The colors used here correspond to those in Figure 2B.

population (Table 1). Thus, further phylogeographic and population genetic studies of *P. hirtus* are warranted to clarify whether current species definitions are consistent with the genetic diversity present in the region. Such studies are further recommended given the evidence of cryptic species in what has previously been considered a single taxon, *P. hatchi*.

Molecular evidence that *Ptomaphagus hatchi* is polyphyletic

With the exception of *P. hirtus*, *P. hatchi* has the largest range extent of any *hirtus*-group species. *P. hatchi* is also the only member of the *hirtus*-group with a range that overlaps those of other species in the group. We found that the current species definition of *P. hatchi* is polyphyletic with respect to six species (*P. chromolithus*, *P. episcopus*, *P. fecundus*, *P. laticornis*, *P. torodei* and *P. valentinei*) from the southern Cumberland Plateau lineage (Figs 2, 3). Peck's (1983, 1984) study of species boundaries within *P. hatchi* relied on distinctions in the female spermatheca (Peck 1984) and evidence of

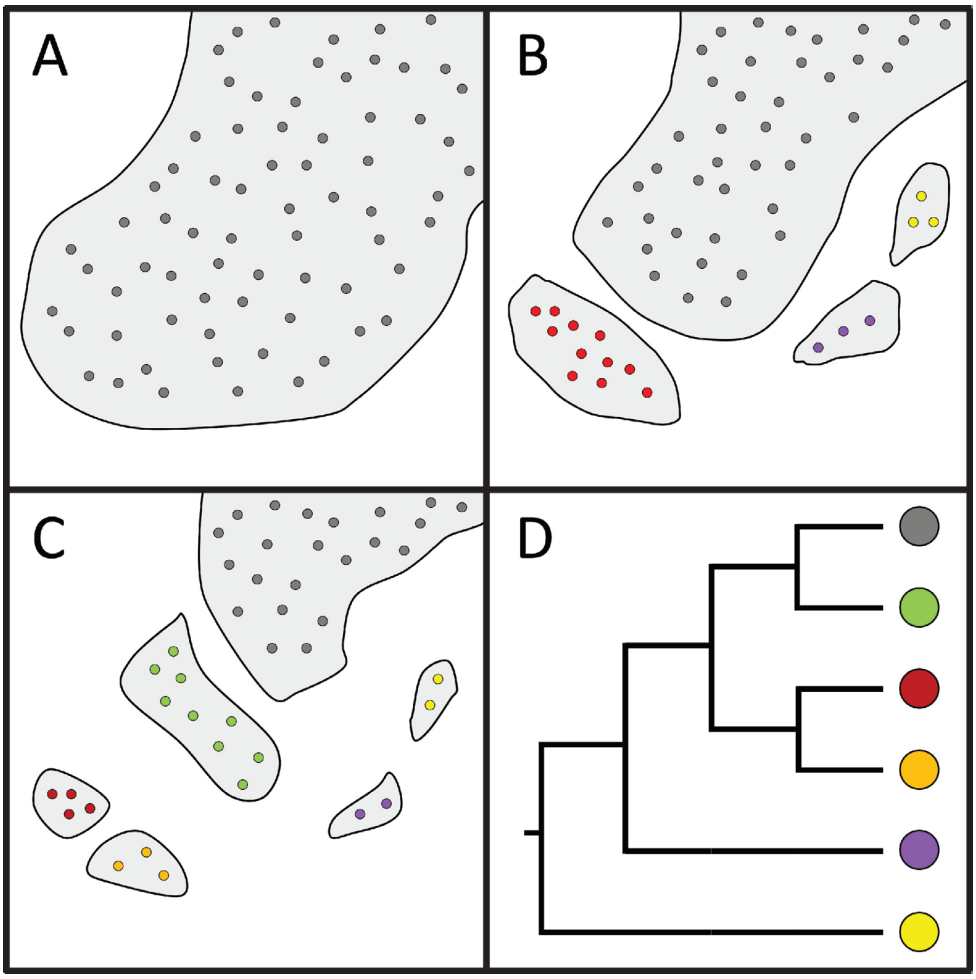

Figure 5. Biogeographic and phylogenetic expectations for a 'vicariance by erosion' scenario as hypothesized for the southern Cumberland Plateau. **A–C** Karst (gray) erodes and fragments over time, leading to the isolation and divergence of cave populations (colored circles) in the remaining patches of karst **D** A phylogeny consistent with the vicariance by erosion process, with taxa that diverge early distributed at the periphery of the eroding region.

reproductive isolation in various interpopulational crosses (Peck 1983). In the most recent taxonomic revision, Peck (1984) restricted *P. hatchi* to populations with 'form I' spermathecae and described *P. laticornis* (with 'form II' spermathecae) and *P. chromolithus* (with 'form III' spermathecae). He also described *P. torodei* as a close relative of *P. valentinei* and raised *P. fecundus* to full species status (Peck 1984).

Significantly, our mitochondrial sequence divergence data do not align with these distinctions. This could be due to a more dynamic variability of spermatheca form than previously envisioned. Alternatively, 'form I' spermathecae may represent an ancestral

state that has been retained in some but not all descendant lineages (now called *P. hatchi*), leading to the erroneous support for a polyphyletic taxon based on the shared similarity of a plesiomorphic character state, i.e. symplesiomorphy. It is also possible that hybridization and introgression have confused the molecular phylogenetic picture of these lineages as we only analysed mitochondrial DNA. This, however, seems unlikely as two species of *Ptomaphagus* (or two spermathecal forms) have never been reported from the same cave (Peck 1984). At present, the available molecular data are too limited to recommend taxonomic revision, but further molecular analysis of these taxa across their distributions has now become essential to gain a reliable understanding of the *hirtus*-group diversification.

"Vicariance by erosion" and the development of a cave biodiversity hotspot on the southern Cumberland Plateau

Exhibiting high levels of taxonomic diversity and endemism, the southern Cumberland Plateau is a hotspot for cave biodiversity. This region has been compared to other centers of cave biodiversity such as the Dinaric karst of Slovenia, the French Pyrenees and the Mammoth Cave region (Culver et al. 2006). This biodiversity peaks in a six-county region in northeast Alabama and south-central Tennessee. Totaling ~10,000 sq km, this region has more than 4400 known caves that support more than 150 troglobionts (Culver et al. 2000, Niemiller and Zigler 2013). Members of the *hirtus*-group compose nearly 10% of the troglobionts present in the southern Cumberland Plateau.

With a few exceptions, we lack time-calibrated molecular phylogenies for troglobionts from the southern Cumberland Plateau. This has limited our understanding of the development of biodiversity in this cave biodiversity hotspot. Important exceptions exist for several aquatic taxa including crustaceans, fish and salamanders (Buhay and Crandall 2005, and comment in Trontelj 2007, Niemiller et al. 2008, Niemiller et al. 2012). These studies indicate diversification during the Pleistocene for all of these taxonomically diverse groups. Here, we provide the first time-calibrated study of a diverse terrestrial troglobiont group from the region. We found that the diversification of the South Cumberlands lineage of the *hirtus*-group extended across ~6 my, beginning near the end of the Miocene and continuing through the Pliocene and Pleistocene (Fig. 3). Thus, it appears troglobionts have accumulated in the southern Cumberland Plateau since at least the late Miocene.

We therefore suggest that vicariance by erosion played a generally significant role in the accumulation of troglobiotic species in this biodiversity hotspot. Better models of the fragmentation of the southern Cumberland Plateau and the development of time-calibrated phylogenies for other species-rich terrestrial troglobiotic taxa from the region (such as pseudoscorpions and millipedes) would allow further evaluation of "vicariance by erosion" model (Fig. 5) for the southern Cumberland Plateau cave biodiversity hotspot.

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Supplementary material 1

Table S1. Mean *cox1* P-distances between specimens from the same cave

Authors: Vincent L. Leray, Jason Caravas, Markus Friedrich, Kirk S. Zigler

Data type: statistical data

Link: https://doi.org/10.3897/subtbiol.29.31377.suppl1

Supplementary material 2

Table S2. Mean *cox1* and five gene (*cox1*, *cytb*, *rrnL-trnL-nad1*) P-distances between conspecific individuals from different caves

Authors: Vincent L. Leray, Jason Caravas, Markus Friedrich, Kirk S. Zigler

Data type: statistical data

Link: https://doi.org/10.3897/subtbiol.29.31377.suppl2

Supplementary material 3

Figure S1. Maximum likelihood majority rule consensus bootstrap tree

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Data type: phylogenetic data

Explanation note: Maximum likelihood tree estimated from combined partial mitochondrial sequence data. Bootstrap values (from 1000 replicates) are indicated above branches. Taxa are labeled with species name and specimen identifier (Table 1).

Link: https://doi.org/10.3897/subtbiol.29.31377.suppl3