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Evan H. Campbell Grant

Kevin P. Mulder

Adrianne B. Brand

Douglas B. Chambers

Addison H. Wynn

See next page for additional authors

Authors

Evan H. Campbell Grant, Kevin P. Mulder, Adrianne B. Brand, Douglas B. Chambers, Addison H. Wynn, Grace Capshaw, Matthew L. Niemiller, John G. Phillips, Jeremy F. Jacobs, Shawn R. Kuchta, and Rayna C. Bell

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RESEARCH ARTICLE

Speciation with gene flow in a narrow endemic West Virginia cave salamander (*Gyrinophilus subterraneus*)

Evan H. Campbell Grant[1] · Kevin P. Mulder[2,3,4] · Adrianne B. Brand[1] · Douglas B. Chambers[5] · Addison H. Wynn[2] · Grace Capshaw[2,6] · Matthew L. Niemiller[7] · John G. Phillips[8] · Jeremy F. Jacobs[2] · Shawn R. Kuchta[9] · Rayna C. Bell[2,10]

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Abstract

Due to their limited geographic distributions and specialized ecologies, cave species are often highly endemic and can be especially vulnerable to habitat degradation within and surrounding the cave systems they inhabit. We investigated the evolutionary history of the West Virginia Spring Salamander (*Gyrinophilus subterraneus)*, estimated the population trend from historic and current survey data, and assessed the current potential for water quality threats to the cave habitat. Our genomic data (mtDNA sequence and ddRADseq-derived SNPs) reveal two, distinct evolutionary lineages within General Davis Cave corresponding to *G. subterraneus* and its widely distributed sister species, *Gyrinophilus porphyriticus*, that are also differentiable based on morphological traits. Genomic models of evolutionary history strongly support asymmetric and continuous gene flow between the two lineages, and hybrid classification analyses identify only parental and first generation cross (F1) progeny. Collectively, these results point to a rare case of sympatric speciation occurring within the cave, leading to strong support for continuing to recognize *G. subterraneus* as a distinct and unique species. Due to its specialized habitat requirements, the complete distribution of *G. subterraneus* is unresolved, but using survey data in its type locality (and currently the only known occupied site), we find that the population within General Davis Cave has possibly declined over the last 45 years. Finally, our measures of cave and surface stream water quality did not reveal evidence of water quality impairment and provide important baselines for future monitoring. In addition, our unexpected finding of a hybrid zone and partial reproductive isolation between *G. subterraneus* and *G. porphyriticus* warrants further attention to better understand the evolutionary and conservation implications of occasional hybridization between the species.

Keywords Endangered species · Hybrid zone · Sympatric speciation · Amphibian decline · Troglobitic

Evan H. Campbell Grant and Kevin P. Mulder have contributed equally.

✉ Evan H. Campbell Grant
ehgrant@usgs.gov

[1] U.S. Geological Survey, Patuxent Wildlife Research Center, S.O. Conte Anadromous Fish Research Laboratory, 1 Migratory Way, Turners Falls, MA 10376, USA

[2] Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, 1000 Constitution Ave, Washington, DC 20560, USA

[3] CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Universidade Do Porto, Campus de Vairão, 4485-661 Vairão, Portugal

[4] Center for Conservation Genomics, Smithsonian Conservation Biology Institute, National Zoological Park, 3001 Connecticut Avenue NW, Washington, DC 20008, USA

[5] U.S. Geological Survey, Virginia-West Virginia Water Science Center, 11 Dunbar Street, Charleston, WV 25301, USA

[6] Department of Biology, University of Maryland, College Park, MD 20742, USA

[7] Department of Biological Sciences, The University of Alabama in Huntsville, Huntsville, AL 35899, USA

[8] Department of Biological Sciences, University of Idaho, Moscow, ID 83843, USA

[9] Department of Biological Sciences, Ohio Center for Ecological and Evolutionary Studies, Ohio University, Athens, OH 45701, USA

[10] Herpetology Department, California Academy of Sciences, 55 Music Concourse Dr, San Francisco, CA 94118, USA

Introduction

Studies of the origins and adaptations of cave-dwelling species have made major contributions to our understanding of evolutionary biology. Repeated transitions to subterranean lifestyles within and across species provide the ideal context for investigating the roles of geographic isolation and local adaptation in lineage divergence (Coyne and Orr 2004). Likewise, the recurring evolution of shared suites of characters in cave-dwelling lineages enable comparative studies of regressive evolution and selection for cave-adapted traits such as eye reduction or loss, lower metabolic rates, and increased longevity (Culver 1982; Jeffery 2005; Protas et al. 2007; Riddle et al. 2021). Due to their limited geographic distributions and specialized ecologies, as well as the disconnected nature of subterranean environments, cave faunas are often highly endemic and can be especially vulnerable to habitat degradation within and surrounding the cave systems they inhabit (Culver 1986; Devitt et al. 2019; Cardoso et al. 2021). As such, these habitats have high conservation value and disproportionally contribute to regional biodiversity, but because cave and karst systems are strongly tied to the surface watershed, the conservation of these subterranean ecosystems is especially challenging. Here we investigate the evolutionary history, current population status, and potential threats to a cave-dwelling salamander in West Virginia.

The West Virginia Spring Salamander (*Gyrinophilus subterraneus* Besharse and Holsinger 1977) is a cave-obligate (troglobitic) salamander only known from General Davis Cave (Greenbrier County, West Virginia, USA), a stream-passage cave within the Greenbrier Limestone formation. The species has an aquatic larval (gilled) stage and although larvae reach large body sizes, sexual maturity has only been observed in transformed individuals (Besharse and Holsinger 1977). Phylogenetic analyses of the *Gyrinophilus* genus indicate that *G. subterraneus* forms a monophyletic group within the Spring Salamanders [*Gyrinophilus porphyriticus* (Green 1827)], a species complex that occurs throughout much of the eastern United States and that co-occurs with *G. subterraneus* within General Davis Cave (Fig. 1; Besharse and Holsinger 1977; Osbourn 2005; Niemiller et al. 2010). This phylogenetic placement is consistent with a relatively recent origin of the cave-dwelling *G. subterraneus* and divergence time estimates (based on substitution rate and fossil-calibration approaches) suggest that they may have become isolated from the more widespread, surface-dwelling *G. porphyriticus* in the Pleistocene (Kuchta et al. 2016). Some authors have proposed that *G. subterraneus* is not a valid species and merely represents local adaptation in the widely variable (Blaney and Blaney 1978) and phenotypically plastic *G. porphyriticus* (Howard et al. 1984). Morphological differences between the species include smaller eyes and wider heads in larval *G. subterraneus*, and smaller eyes and paler skin in transformed adult *G. subterraneus*, all of which are features that are associated with cave-dwelling in many organisms (Barr 1968), including other troglobitic species in the genus *Gyrinophilus* (Cooper and Cooper 1968; Brandon 1971). More extensive

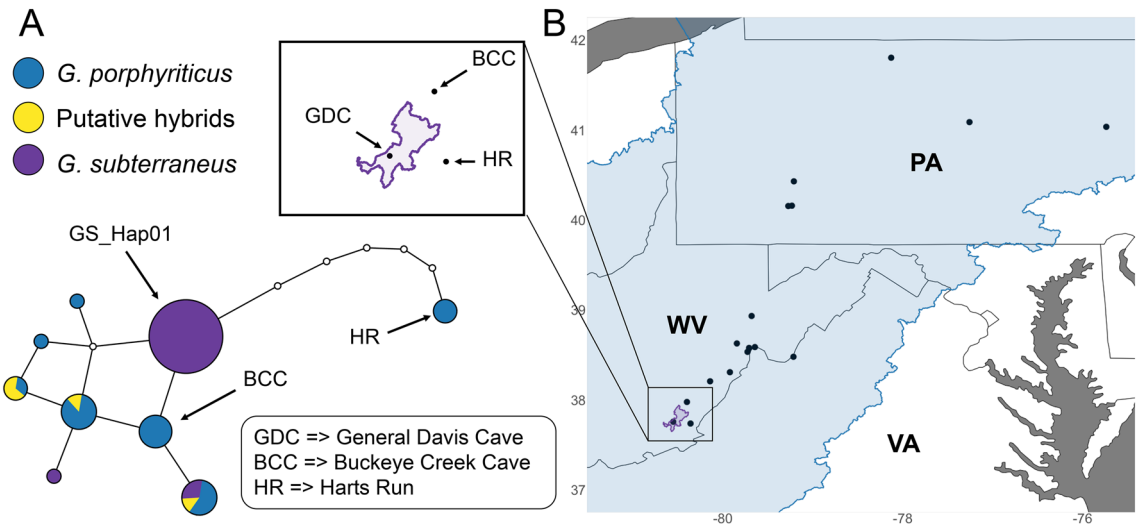

Fig. 1 **A** Mitochondrial haplotype network of the cytochrome B gene for all 61 *Gyrinophilus* salamanders found within General Davis Cave (both *G. porphyriticus* and *G. subterraneus*) and two nearby populations of *G. porphyriticus* (Harts Run and Buckeye Creek Cave, ~ 17 and 28 km away). Colors are based on the genetic identification from the nuclear data. **B** Map of sampling localities included in the study. Range of *G. porphyriticus* indicated in blue, and range of *G. subterraneus* indicated in purple based on shapefiles downloaded from Sciencebase

genetic data and analyses are needed to clarify the evolutionary history of the species and the basis of this morphological differentiation (Niemiller et al. 2009a).

Although *G. subterraneus* and *G. porphyriticus* are currently sympatric in General Davis Cave, it is unclear whether *G. subterraneus* arose via allopatric speciation or in the presence of gene flow with surface-dwelling *G. porphyriticus*. In addition, the extent of any ongoing gene flow between the species is unknown. For instance, demographic models of divergence in Tennessee cave-dwelling salamanders (*Gyrinophilus palleucus* (McCrady 1954) and *Gyrinophilus gulolineatus* (Brandon 1965)) with respect to surface-dwelling *G. porphyriticus* support a history of divergence with gene flow (Niemiller et al. 2008), suggesting there is strong divergent selection between subterranean and surface habitats. Yet, hybridization occurs between *G. gulolineatus* and *G. porphyriticus* in at least one cave system in which they both occur (Kuchta et al. 2016). Consequently, fine-scale genetic sampling with demographic modeling approaches are needed to understand whether phenotypic differences between *G. subterraneus* and *G. porphyriticus* reflect local adaptation in the face of historical and/or ongoing gene flow. In addition, genetic sampling of salamanders throughout the accessible regions of the cave may clarify whether the species occur in sympatry throughout the cave or whether the surface-dwelling *G. porphyriticus* are primarily concentrated near the entrance with the cave-adapted *G. subterraneus* occupying deeper portions of the cave.

Within General Davis Cave, *G. subterraneus* appears to be more abundant than *G. porphyriticus* (Besharse and Holsinger 1977), and both species likely prey upon the rich invertebrate fauna that inhabits the cave (Culver et al. 1973). Only a handful of scientific surveys have reported count data of salamander abundance within the cave, however, and consequently the population demography of *G. subterraneus* has not yet been formally assessed. The stream that flows through General Davis Cave is fed by a surface stream that first flows through Sinks-of-the-Run Cave. The first ~ 4000 m of the stream passage have been surveyed, along which the stream depth varies from 15 to 30 cm, and salamanders (species unidentified) have been reported as far as 1800 m into the cave (Besharse and Holsinger 1977). Though cave ecosystems are characterized by more stable environmental conditions, allochthonous inputs (organic matter and nutrients derived from outside) form the basis of most subterranean food webs (Culver 1982), and these sensitive ecosystems thus face threats from terrestrial surface activities, including land use changes (Urich 2002; Niemiller and Taylor 2019). For caves with subterranean streams, like General Davis Cave, aquatic cave organisms such as salamanders may also be sensitive to changes to streamflow and environmental contamination (Eamus et al. 2016; Hutchins 2018; Burri et al. 2019). Here we (1) ascertain the evolutionary history of *G. subterraneus* with respect to *G. porphyriticus* within and around General Davis Cave using mtDNA sequence and ddRADseq-derived SNPs, (2) model the current population status and trend of *Gyrinophilus* salamanders within General Davis Cave based on historic and newly collected survey data, and (3) evaluate the ecological setting and potential threats for the General Davis Cave habitat.

Methods

Genetic sample collection

We sampled DNA from tissue sourced from four expeditions into the cave and its immediate surroundings: 10 samples from 1988 [USNM 525271-525280; described in Niemiller et al. 2010)], 41 samples from 2007/2008 (collected by MLN; described in Niemiller et al. 2010), 10 samples from 2015 (collected by JGP; described below), and 11 samples from 2018 (collected by EHCG & ABB, described below). To obtain sufficient sample sizes and fine-scale geographic coverage for genetic analyses, we supplemented our field sampling of *G. porphyriticus* with voucher specimens collected by previous researchers between 1981 and 1991 that were archived at the Smithsonian Institution's National Museum of Natural History (USNM). The source of voucher specimen DNA was frozen tissue or blood cells that were originally removed from the vouchers when producing serum for protein electrophoresis. The other tissues were non-lethally sampled tail tips. Because our sampling for genetic analyses spans multiple visits to the same small population, and cave salamanders are long-lived, we aimed to account for the possibility of sampling tail tips from the same individual multiple times. For one specimen (USNM 525291), we also included a separate DNA extraction for both blood and tissue to test if our genomic analyses (see below) would identify the re-sampled individual. The total dataset consisted of 93 samples of which 41 were field identified as *G. subterraneus* (based on overall appearance including eye size and coloration), and 61 samples were from inside General Davis Cave (see Table S1 for a full summary of all data).

Mitochondrial sequence data collection and haplotype network estimation

The mitochondrial genome is a maternally inherited genetic marker that can provide insights into sex-biased dispersal and asymmetric gene-flow. Previous studies of the genus *Gyrinophilus* sequenced cytochrome B (Niemiller et al. 2008; Kuchta et al. 2016), and thus to ensure compatibility of our datasets we developed new primers to sit slightly inside the previously sequenced region for a target sequence

of 645 base pairs (bps): GYR_for50 (5′ CCCCATCAAAYY TATCATACTTATGAA 3′) and GYR_rev730 (5′ TGGGTC TCCAAGGAGGTTYG 3′). Genomic DNA was amplified using Bioline Taq following manufacturer guidelines in 25 µl reactions and adding 0.4 µM of each primer and 2 mM of $MgCl_2$. PCRs were run using a touchdown of 10 cycles (95 °C for 30 s, 60 °C to 55 °C for 30 s, and 45 s of 72 °C) followed by an additional 34 cycles at an annealing temperature of 55 °C and adding an initial 5 min of 95 °C and a final extension of 10 min at 72 °C. We then cleaned the amplified PCR products using ExoSAP-IT (United States Biochemical) and sequenced the cleaned amplicons using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc). The sequenced products were filtered using Sephadex and then analyzed on an Applied Biosystems 3730 DNA Analyzer. We generated 645 base pairs (bps) of cytochrome-b sequence data for 22 samples [GenBank (records ONS524106-ONS524112, ONS524150-524164 provided upon manuscript acceptance)], and combined them with 37 sequences of 783 bps that were previously generated following Niemiller et al. (2008) [GenBank (record ON524113-ONS524149 provided upon manuscript acceptance)], and two sequences from a previous study (Kuchta et al. 2016; GenBank KT794463 and KT794465) for a total dataset of 61 sequences. All mitochondrial sequences were trimmed to 612 bps and aligned using MUSCLE 3.8.425 (Edgar 2004) as implemented in Geneious prime 2019 (Kearse et al. 2012) for a maximum of eight iterations. We estimated a haplotype network using TCS 1.21 (Clement et al. 2000) for all the samples found in General Davis Cave and the nearby Harts Run and Buckeye Creek Cave localities (~ 17 km E and 28 km NE respectively), and created the network figure with tcsBU (Múrias Dos Santos et al. 2015). Haplotype diversity was calculated using the hap.div function of the R-package Pegas (Paradis 2010) implemented in R 3.6.3, and mean uncorrected sequence divergence between *G. porphyriticus* and *G. subterraneus* found within General Davis Cave was estimated using MEGA X (Kumar et al. 2018).

Genome-wide SNP dataset collection

We applied double-digest RAD sequencing (ddRAD; Peterson et al. 2012) to generate 10,000 s of independent markers across the nuclear genome. This technique is inexpensive, does not require a priori knowledge of the genome, and can detect recent evolutionary divergence between closely related species (Andrews et al. 2016). Double-digest RAD-seq (ddRAD) libraries were prepared following the general protocol by Peterson et al. (2012) but with some adjustments. The restriction enzymes SphI and EcoRI (NEB, Ipswich, MA, USA) and size selection window of 450–550 bps were chosen as they have been shown to reduce the number of loci to a feasible number for the large genomes of salamanders (Weisrock et al. 2018). P1 adapters were designed to include both an 8 bp Unique Molecular Identifier (UMI) and staggered inline barcode lengths to increase sequence diversity at the start of the read and enable removal of PCR duplicates during bioinformatic processing. The universal P2 adapter was also split into three different adapters that included staggered length NNNs that were not used as barcodes but to introduce sequence diversity at the start of the reverse read.

Genomic DNA was extracted from all 93 samples using either Qiagen Blood and Tissue kits or protein precipitation (Qiagen, Valencia, CA, USA) and quantified by Qubit 2.0. Approximately 3000 ng of DNA was digested using both restriction enzymes and incubated for 3 h at 37 °C. Following a bead clean-up using Kapa pure beads (Roche, Pleasanton, CA, USA), samples were quantified again and prepared for post-ligation pooling at equimolar amounts by adjusting the amount of input DNA to the individual ligation reactions. Samples were ligated to a universal P2 adapter and 24 unique P1 adapters. Following ligation, samples were combined into pools of 8 for further processing and we combined three pools at different equimolar concentrations to maximize input DNA per pool. After a bead clean-up we performed a size selection on the pools using the Blue Pippin (Sage Science, Beverly, MA, USA) 2% gel cassettes and the internal V1 marker, using the tight setting centered at 500 bps. The resulting size-selected pools were amplified in 7 unique reactions of 25ul using modified primers. The primers were slightly shorter following Peterson et al. (2012), but included the new TruSeq 8 bp Unique Dual Index (UDI) barcodes on both primers to catch and remove potential index hopping (Costello et al. 2018). Samples were sequenced on part of a NovaSeq S4 run using 150 bps paired-end reads at the DNA technologies core at UC Davis with a 5% PhiX spike in.

We identified and removed PCR duplicate reads by their unique molecular identifier (UMI) with the *clonefilter* script included in Stacks 2.41 (Rochette et al. 2019) and subsequently demultiplexed the remaining reads using ipyrad 0.9 (Eaton and Overcast 2020). We ran all samples (N = 93) through the Stacks 2.41 denovo_map.pl pipeline (Rochette et al. 2019) using both forward and reverse reads. A prior sensitivity analysis on 12 representative samples was used to identify the optimum clustering threshold (Paris et al. 2017; Rochette and Catchen 2017). In short, we ran the full pipeline with different iterations of allowed mismatches within stacks (M ≥ 1–6) and chose the setting that maximizes the number of polymorphic loci in 80% of individuals (M = 4). Using this optimum number, we varied the number mismatches between stacks (n ≥ 1–6) and chose the number in which the number of polymorphic loci in 80% of individuals asymptotes and the distribution of Single Nucleotide Polymorphisms (SNPs) across loci was close to constant (n = 4).

Following the de novo stacks pipeline, we removed loci found in less than 70% of samples (R70) and removed samples with more than 35% missing data as an initial filtering.

The remaining samples (N = 87) were used for relatedness analyses to identify potential re-captures and close kin using the KING method (Manichaikul et al. 2010), as implemented in VCFtools (Danecek et al. 2011) as retaining these samples could bias subsequent analyses (O'Connell et al. 2019). Following the KING method manual, we identified pairs of duplicates (Phi > 0.34) or 1st degree kin (0.34 < Phi > 0.17) and out of those identified pairs, we removed the sample with more missing data to obtain our final set of samples for subsequent population genetic analyses (N = 81). We further filtered the 81-sample dataset to only retain high-quality SNPs. Specifically, we removed any SNPs with an average coverage higher than twice the standard deviation of the mean (≥ 124) and with an allele balance of under 0.3, or above 0.7, to remove potential paralogs and repetitive elements. We did not filter for Hardy–Weinberg equilibrium (HWE) as our sampling was geographically structured and included putative hybrids between species that could provide biologically relevant departures of HWE (Pearman et al. 2021), and instead opted to only remove SNPs that had an F_{IS} lower than zero. We subsequently removed all loci with any missing data and selected one SNP per locus to minimize the effects of linkage disequilibrium (LD), resulting in a final dataset of 9681 high quality, unlinked SNPs. Given the short sequence length and the lack of a reference genome, we did not attempt to conduct outlier analyses to detect and/or remove SNPs that may be under selection (Lowry et al. 2017).

Population clustering and ancestry

To assess genetic differentiation between *G. subterraneus* and *G. porphyriticus*, we used the program Admixture (Alexander et al. 2009) with clustering values K = 1 to K = 14 for our full set of samples (N = 81) and a second set of analyses with only the subset of samples from General Davis Cave (N = 52). Prior to population clustering analyses we removed singleton and doubleton SNPs from our dataset, as they can negatively influence population structure analyses (Linck and Battey 2019). We conducted a principal component analysis for a complementary visual representation of the SNP dataset. Our Admixture results indicated that some individuals within the cave may have mixed *G. subterraneus* and *G. porphyriticus* ancestry (see "Results" section. To quantitatively assess whether these individuals were hybrids, we applied the R package HIest (Fitzpatrick 2012) to all individuals found within the cave (N = 52) to score both the ancestry coefficient and the hybrid index. This method uses a maximum likelihood method to jointly infer the ancestry index (S; proportion of the genome descending from each parental lineage) and the interclass heterozygosity (H; heterozygosity of diagnostic loci). H values close to one indicate recent hybridization (e.g., a first generation hybrid; F1) and values closer to 0 indicate hybridization occurring in the distant past. By considering both values we can quantify the timing and direction of hybridization. We first identified diagnostic SNPs between individuals that were confidently assigned to either *G. porphyriticus* or *G. subterraneus* (Q > 0.90 in Admixture analysis, 34 *G. subterraneus* and 13 *G. porphyriticus*). Based on these two parental groups, we estimated allele frequencies for each locus using VCFtools and only retained loci fixed between lineages (parental allele frequencies > 0.9 or < 0.1, final dataset of 478 fixed SNPs). We subsequently estimated S and H for all 52 cave samples using the 'SANN' method, with 1000 MCMC iterations, a starting grid = 99, and surf = TRUE and plotted all individuals along their estimated H and S scores. Finally, to better understand the spatial overlap of *G. porphyriticus* and *G. subterraneus* within the cave environment, we plotted genetic assignments of individuals with precise capture locations within the cave (N = 35) along a fine-scale transect from the entrance of General Davis Cave to approximately 450 m along the main cave passage.

Evolutionary history of Gyrinophilus salamanders in General Davis Cave

To test if *G. porphyriticus* and *G. subterraneus* evolved in sympatry with continuous gene-flow, or alternatively, that they diverged in allopatry and have since come into secondary contact, we applied δaδi (Gutenkunst et al. 2009) to our genomic data. δaδi uses a diffusion approximation method to model different demographic scenarios and compare them to the folded two-dimensional site frequency spectrum (2D-SFS) to determine which model best fits the empirical dataset. We calculated the 2D-SFS from our filtered SNPs, using all samples from General Davis Cave with the exception of the hybrids (see "Results" section), and we used the easySFS script (https://github.com/isaacovercast/easySFS), to downproject our data and optimize the number of unlinked SNPs and individuals to maximize the number of segregating sites (24 individuals of *G. subterraneus* with 25,303 SNPs, and 7 individuals of *G. porphyriticus* with 24,959 SNPs). We additionally used an intermediate downprojection with even sampling for both clades to ensure uneven sampling did not impact our results (10 individuals each, and 22,438 SNPs for *G. subterraneus* and 19,894 SNPs for *G. porphyriticus*). Following the δaδi optimization scripts from Portik et al. (2017), we performed consecutive rounds of optimizations on five potential demographic models (divergence in allopatry without migration, two populations with continuous symmetric migration, two populations with continuous asymmetric migration, divergence in allopatry and subsequent

symmetric migration, or divergence in allopatry and subsequent asymmetric migration). For each round, we ran multiple replicates and used parameter estimates from the best scoring replicate to seed the subsequent searches. We used the default settings in dadi_pipeline (replicates = 10, 20, 30, 40; maxiter = 3, 5, 10, 15; fold = 3, 2, 2, 1), and optimized parameters using the Nelder-Mead method (optimize_log_fmin). Every model was run five times for both SFS projections to ensure results were not impacted by a local optimum. We identified the best-supported model by comparing AIC scores and visually inspected modelfit by comparing the residuals between the model and the data. Additional goodness of fit tests were performed following Barratt et al. (2018) by simulating 200 different SFSs for our best-ranked model and optimizing modelfit using the same optimization pipeline (replicates = 20, 30, 50; maxiter = 5, 10, 20; fold = 3, 2, 1) and comparing the score obtained from our empirical data to the distribution of scores of the Pearson's chi-squared statistics, to determine if our data fell within the distribution of possibilities.

Morphological divergence between *G. porphyriticus* and *G. subterraneus*

To characterize differences in relative eye size, head width, and body size among larval and transformed *G. porphyriticus* and *G. subterraneus* we examined 32 ethanol-preserved specimens from the USNM (see Table S3). This sampling included all USNM specimens of *G. subterraneus* and *G. porphyriticus* from General Davis Cave including the vouchers for the three *G. subterraneus* and seven *G. porphyriticus* from 1988 used in the genetic analysis, the holotype and eight paratypes of *G. subterraneus,* as well as comparative samples of 8 larvae and 3 transformed *G. porphyriticus* from the stream outside Overholt Blowing Cave (Pocahontas County, West Virginia), a cave in the Greenbrier River drainage ca. 62 km NE of General Davis Cave. Measurements were: (1) snout to anterior vent length (SVL), (2) trunk length, measured between the anterior and posterior limb insertions, (3) cranial length, measured ventrally from tip of snout to midpoint of gular fold, (4) cranial width, measured at articulation of jaw, and (5) eye diameter, measured from the anterior to posterior corners of the left eyelid in transformed animals, and the width of the exposed eye visible between the anterior and posterior edges of the surrounding skin in branchiated individuals. Eye diameter was measured with an optical micrometer to the nearest 0.1 mm. All other measurements were made with a ruler to the nearest millimeter, with the exception of small larvae, where cranial length and cranial width were also made with an optical micrometer to the nearest 0.1 mm. Measurements were corrected for size by dividing them by SVL and we plotted the size-corrected measurements by species and by life-stage using violin-plots with ggplot2 as implemented in R 3.6.3 (R Core Team 2020), and applied a two-sample Welch t test to determine if the means were significantly different. Although individuals captured during our 2018 field surveys (see below) were identified to species based on this same set of morphological traits, it was not possible to measure live animals in the cave to the same level of precision as preserved specimens in the laboratory, and thus we only present the preserved specimen measurements.

To characterize differences in cranial traits, we examined a subset of ethanol-preserved specimens of *G. porphyriticus* (7 transformed individuals) and *G. subterraneus* (2 larval and 1 transformed individual) collected from General Davis Cave during the 1988 expedition. We used X-ray microcomputed tomographic (μCT) imaging, which facilitates the collection and analysis of high-resolution osteological data in a non-destructive manner. We visualized and compared the condition of the anterior rami of the premaxillary bones among *Gyrinophilus* specimens. The formation of a suture between the paired anterior rami to form a bipartite (unfused) premaxilla upon metamorphosis is an ancestral pattern of development in plethodontid salamanders (Wake 1966). All larval *Gyrinophilus* have an undivided premaxilla that divides upon metamorphosis in *G. porphyriticus*, but the post-metamorphic retention of a fused premaxilla in adult *G. subterraneus* individuals is considered a diagnostic trait of the species (Besharse and Holsinger 1977). All specimens were scanned using a GE Phoenix v|tomelx m imaging system at the USNM. Imaging parameters were optimized for each scan, ranging from 90 to 100 kV, 170–180 μA, and 131–250 ms exposure time, with isometric voxel sizes of 12.78–23.08 μm. We digitally reconstructed the μCT data to create 3D surface models of the cranial bones using Mimics image processing software (Materialise NV, USA).

Salamander population status in General Davis Cave

To assess the current population status and trends of *G. subterraneus* and co-occurring *G. porphyriticus,* we conducted new surveys, searched the literature for published survey data, and communicated with researchers who had previously surveyed the cave to assemble all known count data for *Gyrinophilus* species in General Davis Cave. Salamanders were identified to species in the field based on eye size, head width, and coloration (Besharse and Holsinger 1977). One author (JP) conducted one survey on 01 June 2015, entering via the upper entrance passage, which intersects with the stream after ~ 150 m, and beginning surveys where the cave entrance intersects the stream, ending a short distance past the 'breakdown matrix.' Two authors (EHCG and ABB) led two surveys with 6 observers on 27–28 August 2018, beginning surveys at the same point and surveying continuously for 450 m up to where the

cave passage became inaccessible (i.e., until the 'breakdown matrix'). Surveyors in 2018 used a meter tape so that salamander encounters could be indexed to exact position within the cave. During the 2018 survey, all observers maintained ~ 25 m space between observers and searched the stream and banks for salamanders, turning cover and using dipnets in the water to capture salamanders. During 2018 surveys, individuals were marked with Visual Implant Elastomer (VIE; Northwestern Marine Technology, Inc., Shaw Island, Washington, USA). VIE is a two-part silicone-based material which cures to a pliable consistency, which can be seen using UV or blue light with amber filtering glasses. Individuals were temporarily restrained in individual plastic zip-top baggies and given a unique mark by combining 4 colors and up to 6 marking locations (Grant 2008), ventral and adjacent to each limb, injected using a 29 gauge insulin needle disinfected between individuals using 70% ethanol pads. Following marking, individuals were photographed in a scaled plexiglass box (dorsal, ventral, and lateral views) and tail clip samples were taken for genetic analyses. During the 2018 surveys we practiced strict decontamination to minimize the possible risk of spread of amphibian pathogens as well as white-nose syndrome in bats. All equipment that was used in the cave was disinfected using 10% bleach or Lysol followed by a hot water wash and heated dry.

Previous published and unpublished survey data include 17 visits to the cave between 1975 and 2008 (See Supplemental Methods and reported in Niemiller et al. 2010). Information on survey methods and protocols is not available in detail for each of the historic (pre-2007) surveys, but is presumed to be similar to the approach reported in a 1985 internal survey report to WV Department of Natural Resources from T. Pauley, B. McDonald, and R. Bartgis: "The survey was done with the same method used by heritage staff in the 1979 and 1980 surveys, so numbers would be comparable. The survey consists of counting all *Gyrinophilus* encountered, noting species and age class (adult vs. larvae). The area surveyed is the stream passage to 600 ft (183 m) above and 100 ft (30.5 m) below the entrance passage. The salamanders are encountered in the stream and on the adjacent stream banks. They have not been found in adjacent passages, where there is little or no suitable habitat." Additional surveys (reported in Niemiller et al. 2010) were reported to have been conducted between May and October within the first 290 m of cave stream habitat and used visual encounter surveys of the stream and banks, combined with active cover searches of rocks, logs, cobble and leaf litter.

Because the length of the cave surveyed differed among sampling occasions, we calculated an observed density of salamanders for each survey occasion (count/m) based on field notes and reports so that data could be compared among years.

Acknowledging that the counts likely include some observation error, we fit a state-process model that includes both process noise and observation error. This model estimates the true population component and a component for the partially observed counts. The true population state at time t is the exponential population growth model on the log scale:

$$\log\left(N_{t+1}\right) = \log\left(N_t\right) + r_t$$

where N_{t+1} is the population density in year $t+1$, N_t is the population density in year t, and r_t is the stochastic population growth rate. The stochastic population growth rates are realizations of a random normal process with mean $\bar{r}$ and variance σ_r^2:

$$r_t \sim Normal\left(\bar{r}, \sigma_r^2\right)$$

where $\bar{r} = 1$ is the prior expected average population growth rate, and the variance represents environmental stochasticity (i.e., process error; prior = 0.0001).

We next specify an observation process model that links the true population state and the observed data:

$$y_t = \log\left(N_t\right) + \varepsilon_t$$

where ε_t is the observation error with prior mean $\mu = 0$ and observation variance $\sigma_y^2 = 1$:

$$\varepsilon_t \sim Normal\left(\mu, \sigma_y^2\right)$$

Models were fit using a Bayesian approach with Markov chain Monte Carlo in the programs R v.3.6.3 (R Core Team 2020) using JAGS (Plummer 2003) via the R package *jagsUI* (Kellner 2016). We initiated model runs with three chains, a burnin period of 1,000, and thinning by 600, and ran the MCMC chains for 800,000 iterations. We assessed convergence using the $\widehat{R}$ statistic ($\widehat{R} < 1.1$; Brooks and Gelman 1998) and visual inspection of traceplots.

Cave and surface stream water sampling

To characterize the water quality conditions in General Davis Cave, we collected samples from the General Davis Cave stream on 27 August 2018. The cave stream was sampled at the zero-point of the survey transect, and at the 300 and 450 m transect locations. Field measurements were recorded using standard USGS protocols (Wilde 2008) at each sampling point and at two additional points where obvious seeps were entering the main cave stream, at the 82 and 230 m transect locations. As the cave stream was quite small, typically less than 1.1 m wide and less than 0.3 m deep, grab samples were collected with an open-mouth bottle and composited in a 3 L HDPE bottle and stored at ambient stream temperature until the sampling team left the cave.

Upon leaving the cave, samples were processed in a mobile laboratory van according to USGS protocols (U.S. Geological Survey 2002).

Laboratory analyses were performed by two USGS labs, the USGS National Water Quality Laboratory for nutrients and common ions and the USGS California Water Science Center's Pesticide Fate Research Laboratory for pesticides. All results are stored in the USGS National Water Information System (NWIS) and are available online (https://waterdata.usgs.gov/nwis/inventory?agency_code=USGS&site_no=374520080331601).

Results

Mitochondrial haplotype diversity and structure

Consistent with previous studies (Niemiller et al. 2009a), our mitochondrial dataset indicated that some mitochondrial haplotypes are shared between *G. subterraneus* and *G. porphyriticus* (Fig. 1). The vast majority of *G. subterraneus* samples (32 of 35) share the same haplotype (GS_Hap01); several closely related haplotypes (differing in 1–3 SNPs) included both *G. subterraneus* and *G. porphyriticus* individuals found within the cave (Fig. 1). The four individuals identified as hybrids based on their nuclear genotypes (see below), did not exhibit the GS_Hap01 haplotype and instead had haplotypes that are more typical of the *G. porphyriticus* found within the cave. Haplotype diversity was low for *G. subterraneus* (0.16, N = 35) relative to *G. porphyriticus* found within the cave (0.73, N = 13). Mean uncorrected sequence divergence between *G. subterraneus* and *G. porphyriticus* within General Davis Cave was low (0.336%).

Genome-wide SNP dataset assembly and relatedness analyses

Out of 93 genetic samples, 87 individuals had less than 35% missing data after our initial broad filtering and were included for the relatedness analyses. Average relatedness scores were low (− 0.61), indicating that we generally sampled unrelated individuals, but three pairs of samples had scores indicative of duplicate samples (0.34–0.5). One pair was our control specimen (USNM 525291) with two separate tissue extractions (score of 0.47), and the other two pairs (0.45 and 0.47) were individuals from General Davis Cave that were both initially caught and tail clipped in August 2007. One of these individuals was a hybrid (see below for hybrid identification) that was recaptured in January 2015, and the other was a *G. subterraneus* recaptured in November 2008. Three additional pairs of samples had scores (0.24, 0.25 and 0.31) indicative of 1st degree kin (either siblings or parent-offspring). All recaptures and 1st degree kin pairs were reduced to one sample, removing the sample with the most missing data, resulting in a final dataset of 81 samples.

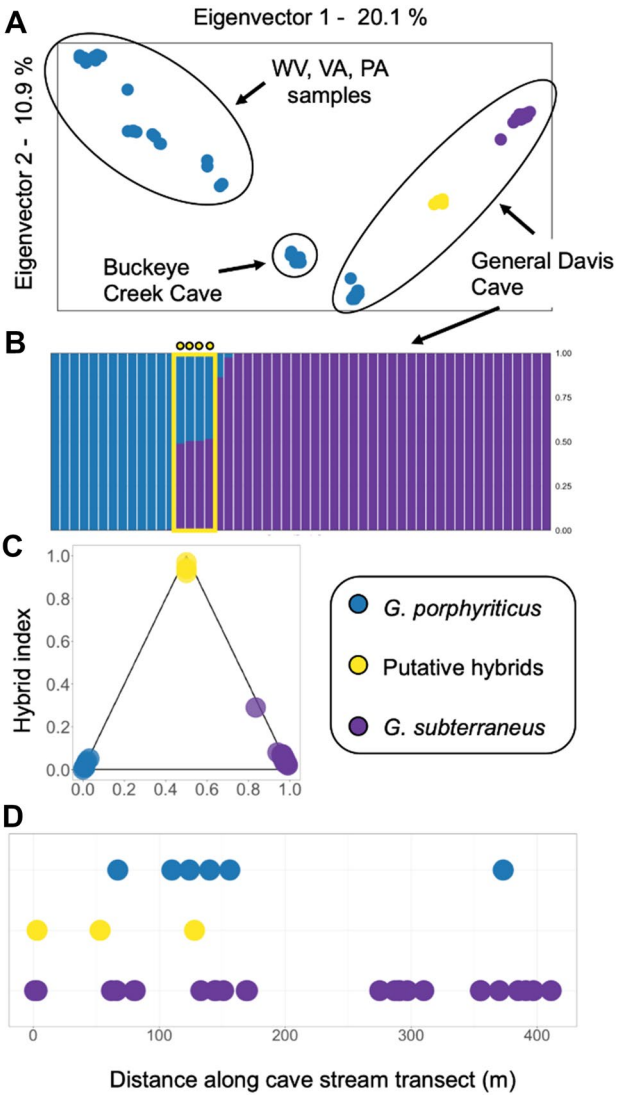

Fig. 2 Results of the nuclear genetic analyses of *Gyrinophilus subterraneus* and *G. porphyriticus* samples. **A** Principal component analysis of all 81 samples. Axis length is proportional to variation explained. **B** Admixture analyses at K = 2 for the 52 individuals found inside General Davis Cave. Every bar represents the assignment probability for a given individual to each species and samples are ordered by population. **C** Hybrid index analyses of all 52 samples collected within General Davis Cave. The x-axis is a representation of the proportion of the genome corresponding to each of the parental species. The y-axis represents the hybrid index (derived from estimates of heterozygosity) and ranges from 0 (no signal or very ancient hybridization) to 1 (a first generation hybrid between parental species). **D** Genetic identification of all samples collected within General Davis Cave with known distance along the cave stream transect (N = 35). The three F1 hybrids with known sampling locations are centered at the start of the cave stream transect. Samples without exact collecting location information were not included

Population clustering and *G. porphyriticus* ancestry within and around General Davis Cave

Principal component analyses of genomic variation suggested a deeper split between *G. porphyriticus* and *G. subterraneus*, with several genetically intermediate individuals, and substructure within the *G. porphyriticus* clade (Fig. 2A). Admixture analyses of the samples found within General Davis Cave showed K = 2 (Fig. 2B) as the best fit with a Cross Validation (CV) score of 0.42 (Fig. S2A). This indicates that the samples in the cave do not form a panmictic population and that dividing the samples into two groups is a better fit of the data to the model. At K = 2 the pure genetic assignments (above 90%) corresponded exactly with morphologically identified *G. porphyriticus* or *G. subterraneus* (Fig. 2B). Four samples from General Davis Cave identified as *G. porphyriticus* based on morphological traits, exhibited intermediate ancestry assignments (Fig. 2B). Analyses of the full dataset of 81 samples also showed a big drop in CV score from K = 1 to K = 2 (0.60 to 0.44), but additional higher values of K had slightly lower cross validation scores (0.36–0.38) and further divided the larger, more widespread *G. porphyriticus* clade into smaller groups corresponding with geographic clusters (Figs. S1, S2B).

Our hybrid index analyses of all the cave samples provided finer resolution of the mixed assignment patterns we observed in the Admixture analyses. Within General Davis Cave we found both pure *G. porphyriticus* and pure *G. subterraneus* ancestry, as well as four individuals with a Hybrid Index score close to 1, which matches the expectation for a first-generation offspring of a *G. porphyriticus* and *G. subterraneus* cross (F1; Fig. 2C). These were the same individuals with intermediate assignments in the Admixture analyses and formed an individual cluster in the principal component analysis. These hybrid individuals were collected during several different expeditions to the cave (1988, 2007–2008, 2015) and were all transformed. *Gyrinophilus porphyriticus* were mostly encountered in the first 200 m of the cave though one individual was sampled near the furthest extent of the transect (~ 373 m). By contrast, *G. subterraneus* were encountered throughout the length of the transect (Fig. 2D). Correspondingly, hybrids were found at multiple sites along the transect and our data do not suggest that this hybrid zone follows a steep cline with increasing distance into the cave. The recaptured *G. subterraneus* was initially found 312 m along the cave stream in 2007 and found 370 m along the stream 15 months later. The recaptured hybrid was found 128 m along the cave stream in 2007; when it was recaptured in 2015, its location was not precisely recorded.

Evolutionary history of *Gyrinophilus* salamanders in General Davis Cave

Demographic modeling of the genomic data using δaδi strongly supported a history of sympatric divergence with asymmetric gene-flow (Fig. 3A and Table S2). In the overall best ranked model (AIC = 1695.12 for the 20 + 20 downprojection), the effective population size for *G. porphyriticus* was larger (0.053) compared to G. subterraneus (0.023), and gene flow was higher from *G. porphyriticus* into *G. subterraneus* (15.2) than from *G. subterraneus* to *G. porphyriticus* (1.7). The top ranked score within the second downprojection (40 + 14) was the same model, with similar estimated parameters (Fig. S3, Table S2). The best ranked model fit the data well with relatively low residuals (Fig. 3B), and simulations showed that the log likelihood from our empirical data sat on the higher end of the distribution of our simulated scores (Fig. 3C).

Morphological differentiation between *G. porphyriticus* and *G. subterraneus*

A total of 52 samples from inside General Davis Cave were included in the genetic analyses (Table S1). Of these, 35 were identified in the field as *G. subterraneus* and 17 as *G. porphyriticus* based on overall appearance, including differences in eye size and color pattern. Most salamanders (48 of 52) were assigned to the genetic clade that corresponds with their field identification, confirming that external morphological differences are largely adequate for species identification. The remaining four samples are putative F1 hybrids based on our genetic analyses and were all identified in the field as *G. porphyriticus*.

With respect to cranial morphology, we confirmed the presence of the fused premaxilla in the transformed *G. subterraneus* specimen versus unfused in all transformed specimens of *G. porphyriticus* (Fig. 4A). We CT scanned one individual classified as an F1 hybrid in our genetic analyses and its premaxilla is unfused as in *G. porphyriticus* (Fig. 4A). We also scanned two larval *G. subterraneus*, both of which exhibited the fused condition (data not shown).

Two individuals (USNM 198537) were removed from morphological analyses, USNM 198537 as the eye was damaged and USNM 252272 as it was an F1 hybrid. As expected, eye diameter was significantly different between the two species, with *G. subterraneus* having reduced eye size relative to *G. porphyriticus*, for both larval (Fig. 4B; *P* value = 0.00036) and transformed (Fig. 4C; *P* value = 0.00039) individuals, with no overlap in values. For cranial width there was overlap in values (Fig. S4), but the difference in the mean was still significant for larval individuals (*P* value = 0.00772), and significant for transformed individuals (*P* value = 0.0467). There was overlap in the

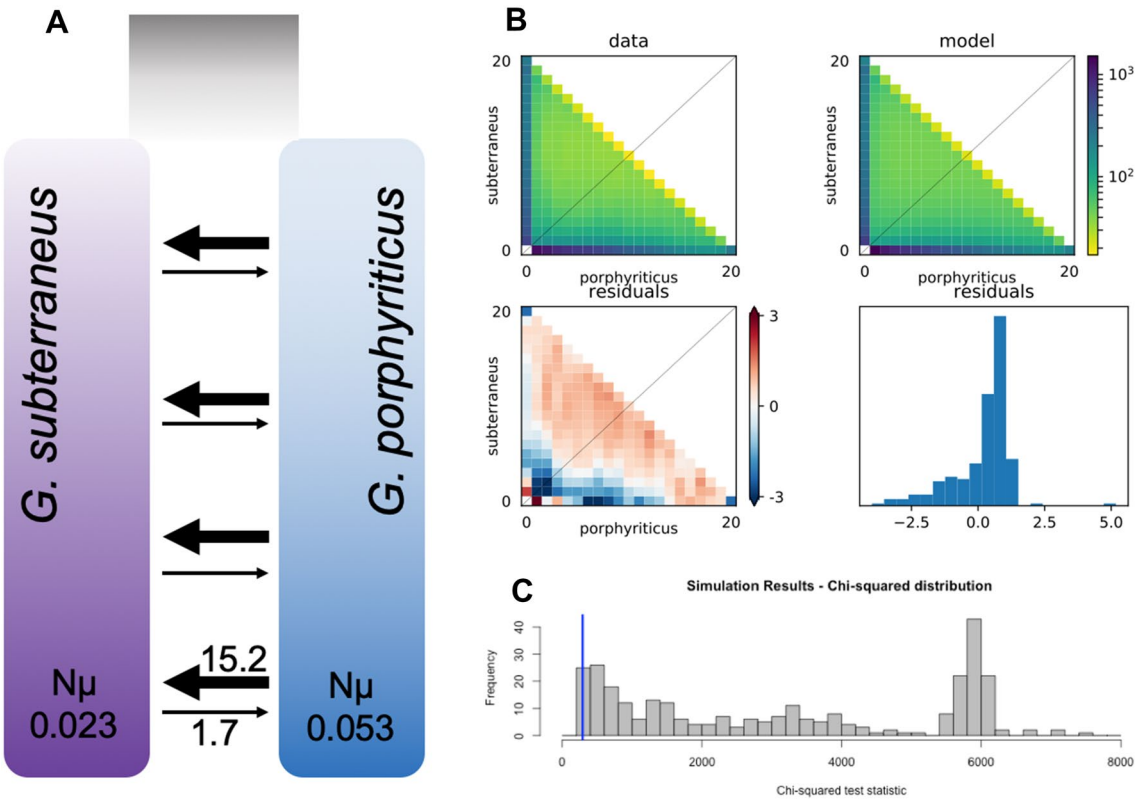

Fig. 3 Results of the demographic modelling based on the Site Frequency Spectrum of nuclear SNPs of the 20,20 downprojection. **A** Scheme depicting the best ranked demographic model (asym_mig) with the estimated effective population sizes (Nμ) and gene flow parameters indicated. **B** Graphic representation of the best ranked model and the empirical data of the site frequency spectrum with residuals between model and data. **C** Distribution of Pearsons chi-squared scores of the empirical data in the blue line, compared to 300 SFS simulations

overall size distributions between the species, though *G. subterraneus* tended to be larger as both larvae and transformed individuals (Fig. 4D), and to grow to larger sizes before transforming.

Population status within General Davis Cave

The 06 January 2015 survey found 3 *G. porphyriticus* (all transformed) and 7 *G. subterraneus* individuals (5 larvae and 2 transformed). Surveys in General Davis Cave on 27–28 August 2018 yielded only 6 individuals identified as *G. subterraneus* (5 larvae, 1 transformed) and we did not detect any *G. porphyriticus*, despite 6 observers searching 450 m of stream habitat over two consecutive days. By contrast, previous surveys for salamanders generally detected more individuals of both species within 290 m of surveyed habitat. Only 1 individual (the transformed *G. subterraneus*) was recaptured on both days in 2018.

Over the past 45 years, surveys have recorded high variation in the observed population density for *G. subterraneus* (Fig. 5A; estimated observation variance = 0.504, SD = 0.211). Despite this, we estimated that the population has undergone a decline, with a mean stochastic population growth rate, *r*, of -0.035 (SD = 0.057). The posterior probability distribution (Fig. 5A) includes zero. We used the posterior distribution to calculate the proportion of MCMC samples where the mean population growth rate (r) was less than 0, which can be interpreted as the probability of decline; for the full dataset we estimated this to be 81.4%. We also ran the analysis excluding the 2015 and 2018 survey data to determine if the most recent surveys (where few individuals were encountered) were influencing the overall mean estimate of the population trajectory. The results, excluding these survey data, results in a smaller population decline; the mean *r* for the 1973–2008 data is − 0.006 (SD = 0.070), and the probability of a decline was reduced to 57.6%. While the most recent surveys recorded fewer individuals than many of the past surveys, the mean density across the dataset is 0.049 individuals per m of stream; 13 of the previous 17 surveys (i.e., since 1990) had lower densities than this overall mean estimate. We also estimated a relatively high process variance (0.135 with SE = 0.172), which was unexpected in a

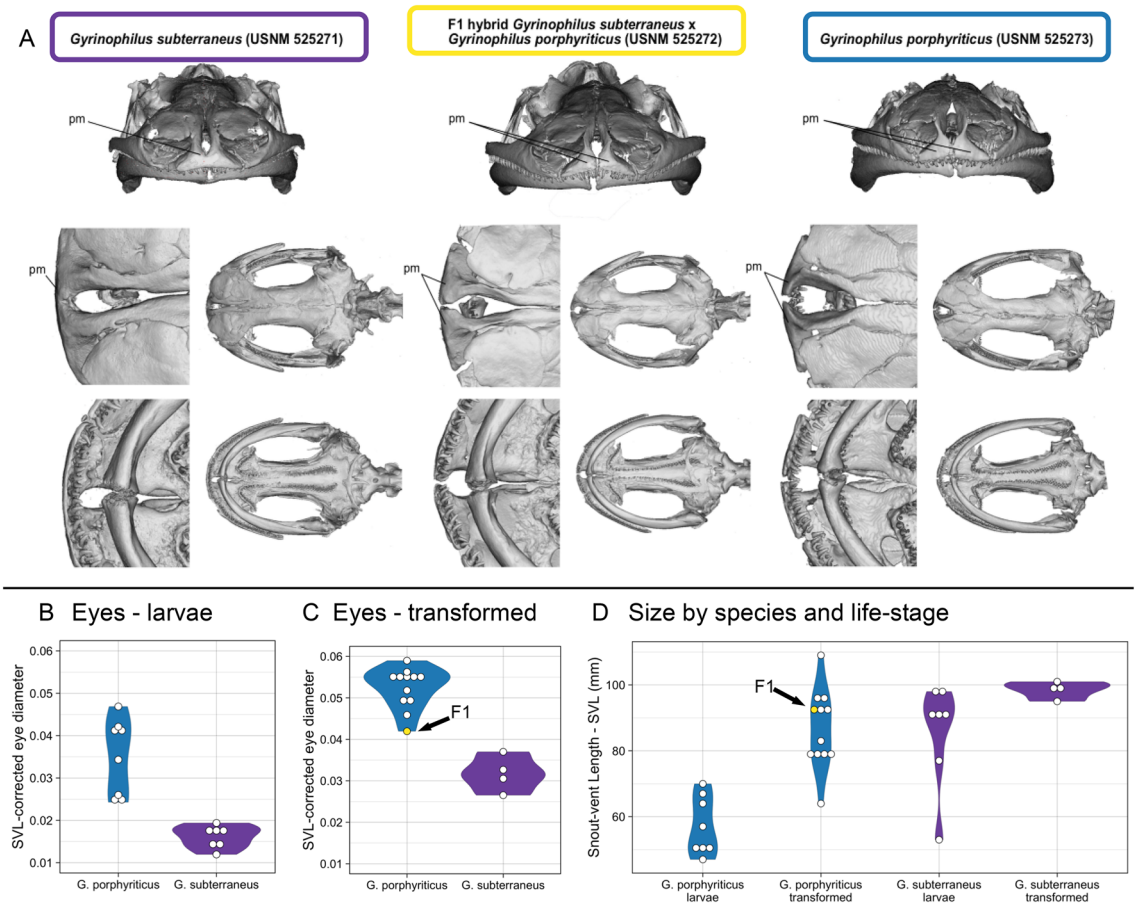

Fig. 4 **A** 3D surface models of the skulls of adult, metamorphosed *Gyrinophilus subterraneus* (USNM 525271), F1 hybrid (USNM 525272), and *G. porphyriticus* (USNM 525273) presented in rostral (top panel), dorsal (middle panel), and ventral (bottom panel) views. The premaxilla (pm) is indicated to highlight the variable condition between the species. The F1 hybrid illustrates the condition typical of *G. porphyriticus.* **B** Size-corrected eye diameter for larval specimens split by species. **C** Size-corrected eye diameter for transformed specimens split by species. The F1 hybrid (USNM 525272) was field identified as *G. porphyriticus,* but presented an intermediate eye diameter (see arrow). **D** Snout-vent length for specimens, split by both species and life-stage demonstrating that compared to *G. porphyriticus,* larval *G. subterraneus* grow to larger sizes before transforming

cave system assumed to have stable environmental conditions. *G. porphyriticus* had lower mean density (Fig. 5C; but with high observation variance = 0.947, SD = 0.049, likely due to the more variable initial survey records), and the population was relatively stable (Fig. 5D; $r = 0.149$, SD = 0.161) over the same time period. Given that the field identification matched the genetic assignment of species (except for the putative hybrids which all resembled *G. porphyriticus*), we assume that misidentification between species did not contribute to the variation in the observed counts.

Cave and surface stream water quality

The waters in all cave discharge samples were of calcium carbonate type, which was unsurprising considering the karst terrane of the area (Table S4). Of the 160 pesticides and pesticide degradation products analyzed for, none were present in detectable concentrations in the General Davis cave samples. Water-quality in the cave stream varied little along the survey transect. Field measurements were recorded at two seeps, both of which were flowing in from the left descending side of the cave stream. While field measurement values for the seep at the 82 m transect point differed little from cave stream measurements, the seep at the 230 m transect point differed, particularly in regard to specific conductance. Specific conductance in the 230 m transect point seep was 490 µS/cm, while cave stream specific conductance values ranged from 221 to 259 µS/cm.

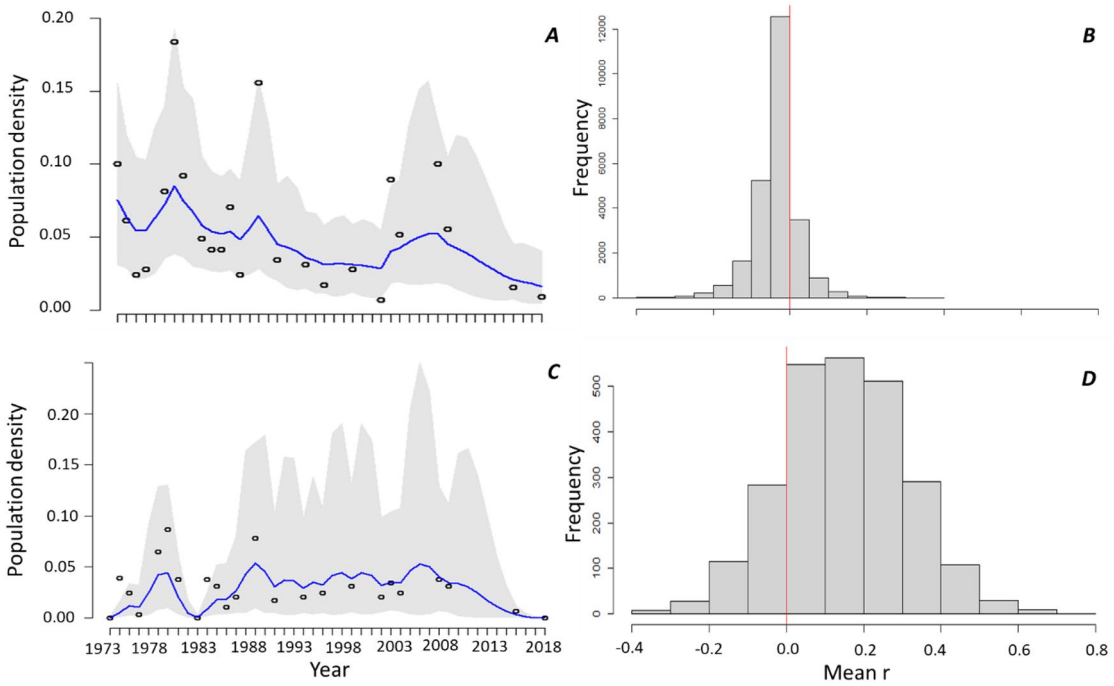

Fig. 5 Population trend in **A** *Gyrinophilus subterraneus* and **C** *G. porphyriticus* population density over time in General Davis Cave. The blue line is the fitted mean, the observed data are the open circles, and the 95% CI is in grey. Panels (**B**) and (**D**) are histograms of the mean stochastic population growth rate (*r*) for *G. subterraneus* (panel **B**) and *G. porphyriticus* (panel **D**) in General Davis Cave for surveys conducted between 1973 and 2018. Red line is the stochastic growth rate of a stable population

Discussion

Genomic and morphological data support evolutionary distinctiveness of *G. subterraneus*

When a new species originates, especially if it originates within another species' range, the ancestral taxon will almost always be rendered paraphyletic (Funk and Omland 2003; Kuchta and Wake 2016), and only given enough time will gene trees transition to reciprocal monophyly (Knowles and Carstens 2007). Previous phylogenetic inferences for the genus *Gyrinophilus* identified *G. subterraneus* as a monophyletic group (Mulder et al. in review); however, recognizing this evolutionary lineage as a distinct species renders the widespread *G. porphyriticus* paraphyletic (Kuchta et al. 2016; Mulder et al. in review). *Gyrinophilus porphyriticus* is considered a species complex with multiple deep evolutionary lineages that likely reflect undescribed species diversity and several of these lineages, like *G. subterraneus*, have been named as distinct species or subspecies by previous authors (Brandon 1966; Adams and Beachy 2001; Mulder et al. in review). These genetically distinct lineages typically occur in parapatry with a general pattern of isolation-by-distance across the large geographic range of *G. porphyriticus*. Within this broader context of phylogeographic structure of *G. porphyriticus*, the divergence between *G. subterraneus* and *G. porphyriticus* within and in the vicinity of General Davis Cave is generally unremarkable, except that individuals from both species occur in microsympatry within the cave. In particular, fine-scale nuclear genetic structure within General Davis Cave between *G. subterraneus* and sympatric *G. porphyriticus* is greater than between *G. porphyriticus* populations that are on average separated by over 200 km. Similarly, although the genetic distance between *G. subterraneus* mtDNA haplotypes and those of *G. porphyriticus* individuals collected within the cave was low (0.336% divergence), our results support mtDNA divergence between the two species on this very fine spatial scale. These patterns coupled with our model-based inferences of divergence with gene flow between *G. subterraneus* and *G. porphyriticus* within the cave indicate that genetic structuring is occurring in sympatry and thus there must be some intrinsic (e.g., genetic incompatibility) and/or extrinsic barriers (e.g., local adaptation) to genetic exchange between the species.

Our genetic analyses indicate the presence of several F1 hybrids (all transformed adults) in General Davis Cave and only a few individuals that may represent multi-generation backcrosses (Hybrid Index < 0.3). In addition, both *G. porphyriticus* and F1 hybrids were found across our cave stream transect, and thus it does not appear that hybridization follows a cline with increasing depth into the cave. The

absence of F2 and backcross individuals in our dataset indicates that although hybridization between *G. porphyriticus* and *G. subterraneus* occurs, these hybrids may have lower survival and/or reproductive fitness, which is consistent with an intermediate degree of reproductive isolation between the species (Servedio and Noor 2003). One of the recaptured individuals in our dataset, however, is an F1 hybrid that was sampled in 2007 and in 2015, suggesting that hybrid progeny have reasonably long lifespans. All four hybrids exhibited *G. porphyriticus* haplotypes, which may indicate that hybridization between the species is asymmetrical (i.e., female *G. porphyriticus* mate with male *G. subterraneus* but female *G. subterraneus* do not mate with male *G. porphyriticus*). Our model-based inferences of gene flow between the species also indicated strong asymmetry in gene flow (greater gene flow from *G. porphyriticus* to *G. subterraneus)* across the nuclear genome. These patterns may reflect sexual differences in dispersal, relative abundance, and/or mating behavior (Lamb and Avise 1986; Cahill et al. 2013). For instance, the original description of *G. subterraneus* reported that this species was four times more abundant within the cave than *G. porphyriticus* (Besharse and Holsinger 1977); consequently, female *G. subterraneus* may be less likely to mate with heterospecifics than are female *G. porphyriticus* simply by chance. Alternatively, this pattern may indicate strong selection against progeny from *G. porphyriticus* male and *G. subterraneus* female matings (Coyne 1998). A variety of asymmetric incompatibilities including nuclear-cytoplasmic, maternal-zygotic, or sex-chromosome/autosome interactions are known in the early evolutionary stages of reproductive isolation and speciation for both plants and animals (Turelli and Moyle 2007). Future studies investigating courtship behavior and hybrid fitness in *Gyrinophilus* will be an important step towards understanding the evolution of reproductive isolation in this genus.

Our measurements of preserved specimens confirmed that eye size and head width differ significantly in both larval and transformed life stages of *G. subterraneus* and *G. porphyriticus*. All individuals included in our genetic analyses were initially identified in the field based on external morphology and the vast majority (91%) of these field identifications matched genetic assignments, indicating strong concordance between genotype and external morphology. In addition, the individuals that were misidentified in the field were the F1 hybrids and were all identified as *G. porphyriticus*, suggesting that hybrid external morphology more closely resembles *G. porphyriticus* than *G. subterraneus*. This was also apparent in the measured hybrid specimen that grouped with *G. porphyriticus*, but exhibited the smallest relative eye size (see arrow Fig. 4C). Our more detailed investigation of internal cranial morphology clearly revealed the fused premaxilla of transformed *G. subterraneus*, one of the diagnostic traits for the species as indicated in the original description (Besharse and Holsinger 1977), whereas the transformed *G. porphyriticus* and one F1 we imaged, all exhibited a divided premaxilla. This suture forms at metamorphosis in *G. porphyriticus*, dividing the larval premaxilla into two elements; like *G. subterraneus*, this ontogenetic change also does not occur in the congeneric Tennessee Cave Salamander *G. palleucus* (Brandon et al. 1986). Collectively, the consistent differences in genotype and phenotype across our datasets are strong evidence of speciation (Wiens and Penkrot 2002; Johnson et al. 2018) and indicate that visual surveys with species identifications based on morphology are likely adequate for further field monitoring efforts.

Smaller eye size and broader head width in *G. subterraneus* likely reflect adaptations to living in the darkness of the cave environment. Many cave-adapted salamanders have reduced eyes or lose their eyes entirely (Mitchell and Reddell 1965; Brandon 1971; Besharse and Brandon 1973, 1974) presumably because they do not rely on vision to sense their environments and eye tissue is energetically costly during development. Some aquatic cave salamanders also exhibit relatively large heads with broad snouts, as well as increased numbers of teeth and sensory pores (Lazell and Brandon 1962; Brandon 1965, 1971; Brandon and Rutherford 1967). In dark cave environments where prey may be limited, this combination of traits likely provides an advantage in detecting prey movement and capture efficiency. Finally, our measurements of body size in larval and transformed *G. porphyriticus* and *G. subterraneus* are consistent with the observation that *G. subterraneus* attain larger sizes as larvae than do *G. porphyriticus* (Besharse and Holsinger 1977). Large *G. subterraneus* larvae are at or near sexual maturity with developed gonads and while it is unclear whether these individuals can reproduce prior to metamorphosis, these observations are consistent with an evolutionary transition to paedomorphosis (Besharse and Holsinger 1977), as documented in many cave-obligate salamander species (Brandon 1971; Bonett et al. 2014).

Current status and trend of the population within General Davis Cave suggests a declining population

Our analysis of the population survey data suggests that *G. subterraneus* may be in decline, despite high variance in counts over the 45-year record. Recent surveys detected relatively few individuals; excluding these surveys reduced the mean estimated decline but we still estimate that the population declined between 1973 and 2008, and surveys since 1990 have detected lower than average density of the species. By contrast, the co-occurring *G. porphyriticus* populations were relatively stable within General Davis Cave throughout the survey record. These differences in population stability within the accessible regions of the cave may

reflect the different ecologies of the species. Alternatively, these demographic differences may indicate environmental changes that are detrimental to the cave-adapted *G. subterraneus* but not to *G. porphyriticus*. Obligate cave species are expected to be more susceptible to environmental changes because of their specialized troglobitic adaptations (Mammola et al. 2019) and some cave-associated salamanders in urbanizing watersheds have suffered declines (Bendik et al. 2014). Deforestation, sedimentation, and water contamination are principal threats to karst systems (Harley et al. 2011; Mammola et al. 2019), but these threats are not apparent in the General Davis Cave watershed.

Assessing the population status of salamanders within General Davis Cave is complicated by several characteristics of our limited historical data. First, the extent of the cave surveyed by different authors was incompletely reported in the literature and field records, and thus one explanation for the high among-survey variation we estimated may be the uneven sampling effort across survey years. We inferred from field notes, personal communication with researchers, and the summary of survey effort reported in Niemiller et al. (2010) that historical surveys were not more than 290 m from where the stream channel intersects with the cave entrance passage. Our survey length in 2015 and 2018 was nearly twice this survey effort but still represents a small fraction of the cave; the cave has been mapped an additional ~ 2.5 km from the 'breakdown matrix,' beyond which we were unable to find access to the rest of the cave. We account for this source of potential bias in our demographic model by converting counts to density, and assuming that the density in the first 450 m is similar to those deeper in the cave. Second, our analysis does not adjust counts for heterogeneity in detection probability (the probability an individual available for detection is encountered during a survey event), which is known to be important in amphibian surveys in general, and stream salamander surveys in particular (e.g., Fields et al. 2017). We may reasonably assume, however, that detection probability is constant over time, as the stable environmental conditions in caves removes a major source of variation in detection rates (e.g., Connette et al. 2015). Third, survey counts are likely an undercount of the total population—which is typical in the study of free-ranging populations. For example, while Niemiller et al. (2010) do not report larvae smaller than 48 mm SVL during 2002, 2003, 2007, and 2008 surveys, our 2018 survey encountered almost entirely small (< 25 mm SVL) larvae and only a single transformed individual. It is unclear whether past survey methods could have biased detection toward larger individuals or whether our observations reflect a true change in the population demography. The most recent prior surveys (2007, 2008, and 2015) did not detect small larvae, despite sampling the streambed, but found large larvae and transformed individuals easily (Niemiller, Phillips, *personal observations*) and with less sampling effort than the 2018 surveys. Nevertheless, we can assume that any biases in the population counts (and availability of animals for detection during each survey) are constant over time, allowing us to draw conclusions from our analyses of the population data.

Interestingly, while larval *G. subterraneus* have been detected on most surveys, only a single larval *G. porphyriticus* was recorded across the 45-yr survey record (in 1980, when 13 larval *G. subterraneus* were recorded). While individual sizes and morphological features used to assign species identity were not recorded for most historic surveys, the strong concordance between morphology and genetics in our dataset suggests that misidentification was unlikely. Given that survey counts of *G. subterraneus* were typically higher than *G. porphyriticus*, and past surveyors report ease in finding metamorphosed salamanders and larger larvae (Niemiller et al. 2010), it is possible that larval *G. porphyriticus* were overlooked. However, *G. porphyriticus* (both metamorphosed and larval stages) are commonly observed in many other caves in the region during surveys (Brandon 1966; Osbourn 2005; Miller and Niemiller 2008; Niemiller et al. 2016; Zigler et al. 2020) including surveys conducted by the same researchers that have surveyed General Davis Cave. It seems unlikely that larger (> 40 mm SVL) larval *G. porphyriticus* would be overlooked for 45 years at General Davis Cave if they were present. Likewise, the common occurrence of larval *G. porphyriticus* in other caves across the species range indicates that *G. porphyriticus* can establish stable breeding populations within caves. This suggests that *G. porphyriticus* larvae should be capable of surviving the environmental conditions within General Davis Cave, which we find to be characteristic of karst systems.

Alternatively, the lower abundances of *G. porphyriticus* larvae in General Davis Cave may reflect differential survivorship in larval *Gyrinophilus* within the cave. Competition for food, shelter sites, breeding sites, and other resources may explain the lack of *G. porphyriticus* larvae if *G. subterraneus* larvae are better competitors. Spring salamanders are also known to be cannibalistic (Burton 1976; Gustafson 1993), and in the resource-poor cave environment, *G. subterraneus* may differentially predate *G. porphyriticus* larvae. These species interactions could also relegate larval *G. porphyriticus* to microhabitats within General Davis Cave that are inaccessible to humans. Finally, our observations may indicate that *G. porphyriticus* at General Davis Cave are facultative trogloxenes (i.e., they use the cave as a refuge but cannot complete their life cycle in the cave). Most *G. porphyriticus* were detected within the first 180 m of the cave, which is consistent with this hypothesis; however, *G. porphyriticus* is known to breed in other caves (Niemiller et al. 2009b), female *G. porphyriticus* with enlarged ova were observed during surveys in the 2000s, and the presence of F1 hybrids between both species all suggest

that *G. porphyriticus* reproduction may occur within General Davis Cave. More work is needed to understand the distribution and life history of *Gyrinophilus* within General Davis Cave, including competitive interactions between the species (and hybrids) across life stages.

Ecological setting and potential threats within General Davis Cave

Given the highly restricted range and limited genetic variation found in *G. subterraneus*, the species is likely vulnerable to habitat degradation in and above General Davis Cave. We assessed habitat conditions during the 2018 survey but found no evidence of water quality impairment in the cave stream. Water in the General Davis Cave comes primarily from the Davis Hollow Basin (Jones 1973), including surface drainage that enters the cave system via the Sinks-of-the-Run Cave sink. This relatively small basin is largely forested with little agricultural activity. No pesticides were detected in the cave stream samples, likely reflecting the forested land cover of the Davis Hollow Basin. We acknowledge that water-quality dynamics are impossible to ascertain based on a synoptic sample; however, our data provide important baselines for future monitoring. This cave's chemical hydrology is no doubt complex, including interactions of surface-water inputs via the Sinks-of-the-Run swallet and groundwater inputs both from transmission through the highly permeable carbonate rocks and through the conduits that are common in this karst terrane. The input of contaminants may vary over time and among inputs to the cave stream, but the forested watershed and the present sampling results indicate the potential current threat to water-quality impairment is low.

The direct connection to surface drainages was evident in the amount of leaf-litter and other allochthonous organic matter we encountered well inside the cave. With such a strong connection to surface hydrology, it is likely the cave stream is influenced by large rainfall events, including the June 2016 rainfall event that resulted in widespread severe flooding of the Greenbrier River in the area surrounding the cave. The 23 June 2016 rainfall events dropped between 203 and 238 mm of rain in a 24-h period throughout much of Greenbrier County (Austin et al. 2018), causing overland flow and increased streamflow in the region, some of which undoubtedly entered General Davis Cave via the Sinks-of-the-Run. The recurrence interval of floods of the magnitude occurring in June 2016 ranges from 41 years to over 2000 years (Austin et al. 2018). We found evidence that water levels in General Davis Cave had been much higher prior to the 2018 survey, as indicated by leaf fragments adhering to stalactites and in deposits above stream elevation. While it is not possible to confidently attribute these high-water marks to the June 2016 precipitation event, they are clear indications of a wide range of stage and mobilization as well as scouring and deposition of organic matter. Furthermore, and especially in steeper parts of the cave, the higher flows increase the stream's hydraulic radius and are likely to result in increased shear stress and mobilization of bottom material (Gordon et al. 2004), including substrate used as refuge by stream salamanders. Salamanders may avoid high flows by retreating to side channels and refugia in the cave. A more complete understanding of the cave stream's hydrology would require measurements of channel geometry and gradient, among other characteristics. While it is possible that increased flow and stream velocity represents a relatively rare disturbance of the cave habitat, the effects of these flow events on populations of stream salamanders are poorly understood.

Conclusions

Our genetic and morphological data point to a rare case of sympatric speciation occurring within General Davis Cave. Due to the temporal and geographic context of speciation of *G. subterraneus*, the paraphyletic nature of its placement within *G. porphyriticus* is expected and reflects the ongoing biological process of speciation (Niemiller et al. 2008; Kuchta et al. 2018; Mulder et al. in review). The combination of genetic and morphological differences between *G. subterraneus* and *G. porphyriticus* when they occur in direct sympatry provides strong support for continuing to recognize *G. subterraneus* as a distinct species. Due to its specialized habitat requirements, generally small number of encountered individuals during surveys, and ability to inhabit microhabitats in complex cave environments, the complete distribution of *G. subterraneus* is unresolved. First, much of General Davis Cave has not been systematically surveyed for the species, which limits our inferences of the estimated population size of *G. subterraneus* and the degree to which its range (in General Davis Cave) overlaps with that of *G. porphyriticus*. Second, *G. porphyriticus* has been documented in other hydrologically connected caves (e.g., Sinks-of-the-Run Cave) and the surface stream that feeds General Davis Cave as well as many other caves in the vicinity of General Davis Cave (Green and Brant 1966). It is possible that *G. subterraneus* occurs outside the known range within General Davis Cave and has been misidentified as *G. porphyriticus*, though no reports of the species from other caves are known. In terms of future threats, The Nature Conservancy owns an easement to the cave and owns title to the cave entrance, but the surface lands and the watershed above the cave entrance are privately owned and without any conservation protections. Effective conservation will require a better understanding of the *G. subterraneus* distribution within General Davis Cave,

surveys in the Sinks-of-the-Run Cave to determine whether *G. subterraneus* is present, and robust population survey methods and associated statistical models to provide better estimates of population size and demography. In addition, our analyses revealed hybridization between *G. subterraneus* and *G. porphyriticus* with evidence of partial reproductive isolation between the species, which provides an exciting avenue of future research to understand the process of morphological and genomic adaptation to cave environments.

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Author contributions EHCG, KPM, ABB and RCB designed the study; EHCG, KPM, ABB, DBC, AW, GC, MLN, JGP, JFJ, SRK, and RCB contributed data; EHCG, KPM, DBC and RCB analyzed data; EHCG, KPM and RCB wrote the first draft of the manuscript; all authors contributed to revisions. All authors whose names appear on the submission: (1) made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; (2) drafted the work or revised it critically for important intellectual content; (3) approved the version to be published; and (4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files. The cytochrome-b sequence data for 59 samples (SRA), under BioProject PRJNA824885 (samples: SRR19240940-SRR19241032) will be available from GenBank [ON524106-ON524164] and Illumina sequence data are available on the Sequence Read Archive (SRA).

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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