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Two new phreatic snails (Mollusca, Caenogastropoda, Cochliopidae) from the Edwards and Edwards-Trinity aquifers, Texas

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Abstract

The Edwards and Edwards-Trinity Aquifers of Texas have diverse stygofauna, including fifteen species of snails found in phreatic and hyporheic habitats. These species have the hallmarks of adaptation to subterranean environments including extremely small body size and the loss of pigmentation and eyes. Here we use an integrative taxonomic approach, using shell, radula, and anatomical features as well as mitochondrial and nuclear DNA data, to circumscribe a new genus and two new cavesnail species from Central Texas. *Vitropyrgus lillianae* **gen. et sp. nov.** is described from Comal Springs (Comal County) and Fessenden Springs (Kerr County) and distinguished by a glassy, highly sculptured shell and distinctively simple, unornamented penial morphology. We also describe *Phreatodrobia bulla* **sp. nov.** from Hidden Springs (Bell County), and several other springs in Bell & Williamson Counties, Texas. This species has a smooth, unsculptured teleoconch, a reflected and flared lip, and deeply concave operculum.

Keywords

Cave snails, Edwards Trinity Aquifer System, groundwater, hyporheic, phreatic, spring

Introduction

The Edwards, Trinity, and Edwards-Trinity aquifers, constitute a large, interconnected karst aquifer systems in Texas and Northern Mexico and provide habitat for a diverse stygofauna (Longley 1981) including snails, crustaceans, worms, beetles, fish, and salamanders. These aquifers are the primary drinking water source for several large metropolitan areas (Maclay 1995) and support ranching and agriculture across a large portion of the Edwards Plateau. The Edwards (Balcones Fault Zone) Aquifer, a long, narrow region of faults, is a particularly biodiverse and productive subterranean ecosystem (Hutchins et al. 2016) which harbors a diversity of subterranean snails commonly called cave snails.

Cave or phreatic snails inhabit groundwater systems such as subterranean streams and aquifers (Hershler and Longley 1986b). Recent surveys have also found some species in hyporheic habitats (Alvear et al. 2020a; Hutchins et al. 2020). They are typically small (<3 mm), depigmented, and eyeless, with a suite of additional morphological adaptations attributed to the effects of miniaturization (Rysiewska et al. 2016; Osikowski et al. 2017; Falniowski 2018). These include loss or reduction of gills, lengthy coiled intestines, simplification of gonadal organs (Hershler and Holsinger 1990), and convergent evolution of characteristic shell shapes (Falniowski 2018). The phreatic snails of the Edwards and Edwards-Trinity Aquifers include 14 nominal species, with nine species in the genus *Phreatodrobia* Hershler & Longley, 1986. There are also several monotypic genera including: *Phreatoceras* (Hershler & Longley, 1986), *Stygopyrgus* Hershler & Longley, 1986, and *Texapyrgus* Thompson & Hershler, 1991. *Balconorbis* Hershler & Longley, 1986 has a single species endemic to Texas with an additional species in contiguous limestone regions of northern Mexico. Finally, the species referred to as *Tryonia diaboli* (Pilsbry & Ferriss, 1906) is a subterranean species, but requires taxonomic study to determine its generic assignment.

Phreatic snails of the Edwards and Edwards-Trinity Aquifers are all members of the Cochliopidae Tryon, 1866, a group of small freshwater and estuarine snails, comprising 260+ species found in the Nearctic, Neotropical, and Afrotropical regions of the world (Clark 2019). The family was, until recently, part of the Hydrobiidae Stimpson, 1865 which included 400+ genera and is undergoing splitting and taxonomic revision (Wilke et al. 2001; Wilke et al. 2013; Wilke and Delicado 2019). The most recent revisionary work (Hershler and Thompson 1992), divided the Cochliopidae into three subfamilies, Cochliopinae Tryon, 1966, Littoridininae Thiele, 1928, and Semisalsinae Giusti & Pezzoli, 1980 largely diagnosed by glandular features of the male reproductive anatomy. But the relationships among Cochliopidae remain unsettled with nearly twenty genera not assigned to a subfamily, including *Phreatodrobia* and the other subterranean genera from the Edwards and Edwards-Trinity. Surveys of this region encountered phreatic snail populations that were provisionally identified from gross shell morphology as nominal species (Alvear et al. 2020a; Alvear et al. 2020b; Gibson et al. 2021a, 2021b; Hutchins et al. 2021), however additional sampling and more extensive study has allowed these to be distinguished. In this study, we use mitochondrial and nuclear genetic data along with anatomical characteristics to examine relationships among the cave snails of central Texas and describe two new species from this diverse region.

Materials and methods

Groundwater was sampled using different methods as appropriate for the substrate or habitat (Fig. 1). Flowing springs and wells with outflow pipes were sampled by placing a 100 μm drift net into the spring opening, securing it with available cobble or by wedging it between rocks (Fig. 2). The net remained in place for 2–7 days. Well samples where there was no outflow pipe were sampled using a bottle trap baited with pistachios left in place for two weeks. Mophead samples were taken by placing the cotton head of a mop into the spring for ~30 days. Hyporheic samples were taken by Bou-Rouch sampling (Bou and Rouch 1967), where a stainless steel spike was hammered 30–50 cm into the hyporheic zone of a stream or gravel bed, allowing water to be pumped through a 100 μm mesh net. Bou-Rouch samples were further processed using a modified elutriation method (Lackey and May 1971) to remove sediments and retain organic materials, including animals. The sample is washed and agitated using faucet water with a small hose to flush lower-density organic materials (including snails and snail shells) out of a sorting tray and into a 100 μm filter. Inorganic sediments remain in the bottom of the tray. Sediments are repeatedly washed until all organic materials are removed. All samples were immediately preserved in 95% ethanol (refreshed at least once and stored in a refrigerator) until sorting under a dissecting microscope.

Types and paratypes are deposited in the Philadelphia Academy of Natural Sciences at Drexel University (**ANSP**). Additional reference materials include lots from the Texas Memorial Museum (**TMM**) and paravouchers deposited in the Texas State University Aquifer Biodiversity Collection (ABC). For some of the methods, such as DNA extraction, the animals are destroyed, paravouchers are intact individuals from the same population.

Photovouchers were created prior to DNA extraction since the procedure resulted in the dissolution of fragile shells (Suppl. materials 2–4). DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit, incubated at 65 °C for 24 hours. The same digestion was used to retain radula and opercula as well as DNA. PCR was conducted using the Platinum SuperFi DNA Polymerase Master Mix Kit (Thermo-Fisher). We followed the manufacturer protocol for PCR, conducting a temperature gradient between 48–54 °C for each species and proceeding with the optimal temperature, typically 51.7 °C. Primers included COIH2198 and COIL1490 (Folmer et al. 1994) for COI and LSU 1 & 3 (Wade et al. 2001) for the nuclear Large Ribosomal Subunit (LSU). Amplicons were purified using the PCR DNA Fragment Extraction Kit (IBI Scientific, Peosta) and quantified with a Qubit 3.0 Fluorometer and Qubit dsDNA HS Assay Kit (Invitrogen). DNA sequencing was conducted at Eton Biosciences, Inc.

Figure 1. Spring sites in Texas. Type locality of *Vitropyrgus lillianae* gen. et sp. nov. Comal Springs Upwelling #7, New Braunfels, Comal County **A** at normal water flow conditions, photo by Marcus Gary **B** at low water flow conditions, photo by Randy Gibson **C** drift nets in water flow at Fessenden Springs, Kerr County, photo by K.E. Perez **D** type locality of *Phreatodrobia bulla* sp. nov. Hidden Springs, Bell County, photo by P. Diaz **E** Robertson spring run hyporheic sampling site, Bell County, photo by K.E. Perez.

Following sequencing, Geneious 10.2.6 was used to assemble contigs, trim and visually inspect sequences, and align them using the MUSCLE algorithm. Model selection (Kalyaanamoorthy et al. 2017), Maximum likelihood analyses (Nguyen et al. 2015), and 10,000 ultrafast bootstrap replicates (Hoang et al. 2018) were conducted in IQTREE 1.6.12. For COI a 74 terminal alignment was assembled including all new sequences generated and members of all genera in the Cochliopidae available on Genbank. *Pomatiopsis lapidaria* (Say, 1817) was included as an outgroup. The COI alignment was partitioned to allow evaluation of 1st, 2nd, and 3rd codon positions separately. PartitionFinder and ModelFinder (via IQTREE) were used to assess whether partitions should be analyzed separately or merged and to determine the best fit model for each partition.

For the nuclear LSU gene an alignment with all available sequences (23) was generated using MUSCLE as implemented at https://www.ebi.ac.uk/Tools/msa/muscle/ and analyzed in IQ-TREE with 10,000 ultra-fast bootstrap replicates. Other Cochliopidae were not available for this locus, so *Pyrgulopsis* Call & Pilsbry, 1886

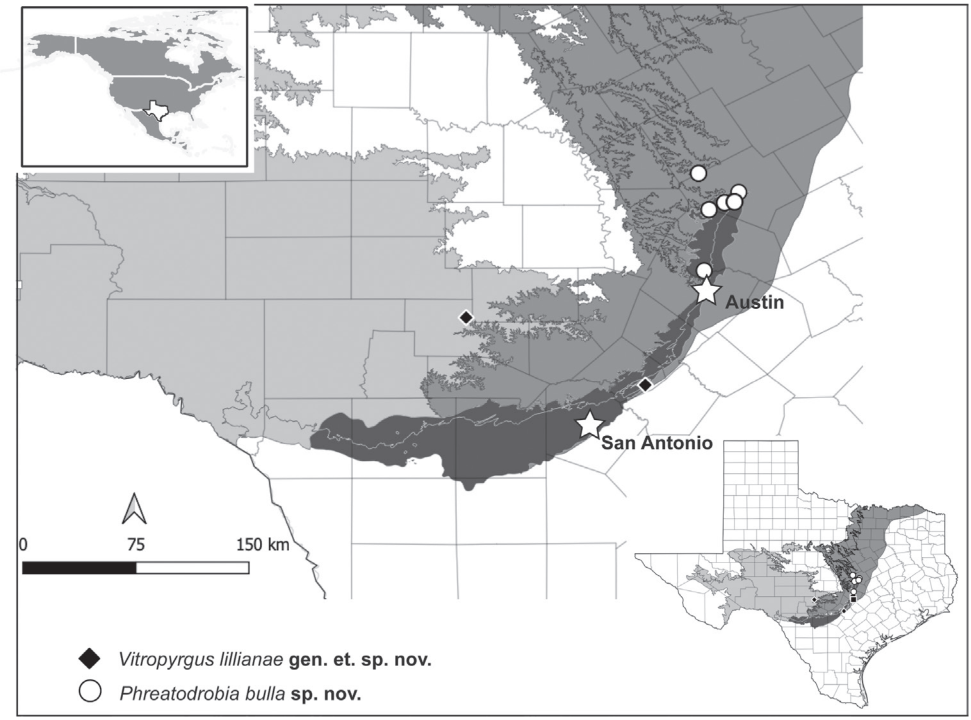

Figure 2. Map with known localities for *Vitropyrgus lillianae* gen et. sp. nov. and *Phreatodrobia bulla* sp. nov. The Edwards-Trinity Aquifer System is shown in gray shades with the lightest shade indicating the Edwards-Trinity, the medium gray indicating the Trinity, and the darkest portion indicating the Edwards portions. Major cities are indicated with a star and name label. Inset maps indicate the region of North America and state of Texas.

species were used as the outgroup. Model selection was conducted in IQ-TREE using the Bayesian Information Criterion (BIC). For the 74-terminal alignment for COI, the following models were used for 1st positions: TNe+I+G4, 2nd positions: TPM3u+F+I+G4, and 3rd positions: HKY+F+G4. For the LSU alignment (23 terminals) HKY+F+I+G4 was identified as the best fit model. Mega 11 was used to calculate P-distance (Tamura et al. 2013).

Anatomical terms follow recent works (Hershler and Longley 1986b; Hershler and Ponder 1998) on the Cochliopidae or Hydrobiidae. For anatomical examination, the calcareous portion of the shells were dissolved in 50% hydrochloric acid, then remaining proteinaceous material was removed by hand. Dissections were conducted in 70% ethanol and/or with the addition of Bouin's solution or 10% Eosin to allow visual contrast and hardening of tissues, resulting in a yellow color in anatomical photos. The unstained tissue of the species examined here are unpigmented and appear white after preservation in ethanol. Dissections and examination of shells and anatomical features were carried out using a Leica S9i, and the Leica LAS X software. Images were stacked using Helicon Focus. Shell measurements were taken using either Leica LAS X or AmScope calibrated with a S78-StageMicrometer 1mm/0.01 Div. Whorls were counted according to Burch (1989).

Scanning electron micrograph (SEM) images of *Vitropyrgus lillianae* gen. et sp. nov. were acquired using the methods of Perez et al., (2022) with a working distance of 10 mm and spot size between 48 and 52. Materials for SEM images of *Phreatodrobia bulla* sp. nov. were prepared with 75 angstroms of gold palladium alloy using a Quorum Sputter coater and imaged using a Zeiss EVO LS10, in high vacuum, at 20–100k magnification. The usual working distance was 4.5–5 mm, spot size was 242, and accelerating voltage was 10.94 kv.

Results

Phreatic snails were examined from 35 sites for this study (Suppl. material 1) and localities of the proposed new species are shown in Fig. 2.

An alignment of 74 COI sequences with 657 characters, 264 parsimony-informative, was analyzed using maximum likelihood. The ML tree (Fig. 3; best score = -7748.825) showed a close relationship between *Vitropyrgus lillianae* gen. et sp. nov. and a clade of epigean fresh and brackish water genera including *Onobops* F.G. Thompson, 1968 and *Heleobia* W. Stimpson, 1865. This relationship was not strongly supported with a bootstrap value of 36. *Phreatodrobia bulla* sp. nov. was sister to *Phreatoceras taylori* (Hershler & Longley, 1986) from the same region, but within the clade of *Phreatodrobia* species. P-distances calculated from COI were used to compare sequence divergence among genera, species, and populations in this group. COI was used because it is the DNA barcoding region, potentially allowing comparison with other phreatic Cochliopidae or Hydrobiidae. Intergeneric sequence divergence averaged 16.54% (range 5.19–25.37). Sequence divergence between *Vitropyrgus* gen. nov. and other genera in the clade (e.g., *Onobops* and *Heleobia*) averaged 16.0% (range 15.5–16.6). Interspecific sequence divergence averaged 10.7% (range 4.1–14.2) across all comparisons. Intraspecific sequence divergence averaged 2.5% (range 0–12.3) across all comparisons. Some species exhibited notably higher intraspecific divergence: 3.8% (range 3.8–5.7) for *Phreatodrobia nugax* and 12.2% (range 12.2–12.3) for *Balconorbis uvaldensis*. *Phreatodrobia bulla* sp. nov. was placed sister to *Phreatoceras* Hershler & Longley, 1986 (average sequence divergence between the species = 6%, range 6.2–5.7) with weak bootstrap support, and both species were embedded in the clade with other *Phreatodrobia* species. A small amount of sequence divergence exists between two subclades of *P. bulla*, with individuals from Tahuaya and Anderson springs more closely related to each other (average p-distance 0.9%, range 1.2–0.2) than to individuals from Solana Ranch springs (average p-distance 2.1%, range 2.3–1.9).

The LSU alignment had 23 sequences, 947 characters, with 224 parsimony-informative. A single ML tree with a score of -3416.312 was found (Fig. 4). Analysis

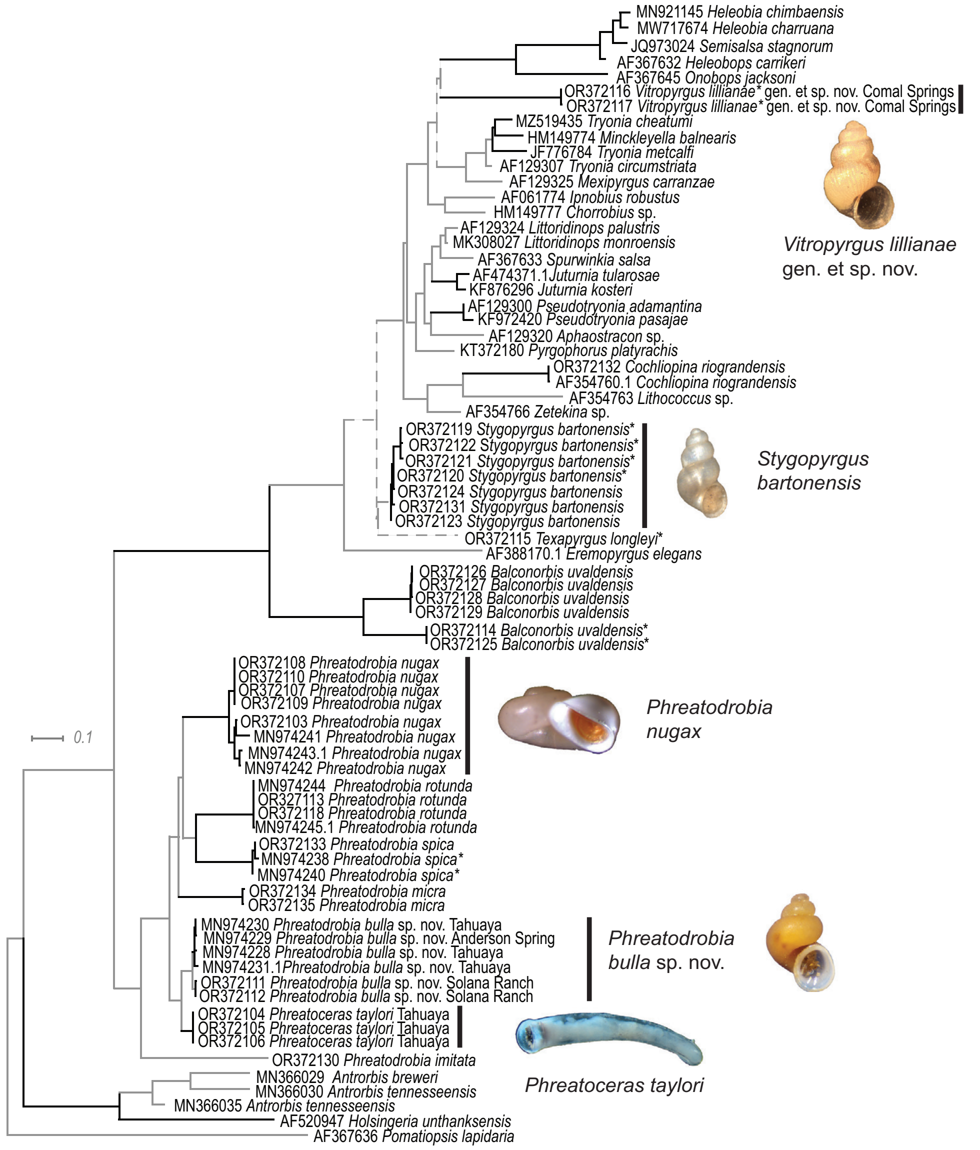

Figure 3. Highest likelihood trees resulting from maximum-likelihood analysis of COI alignment. Ultrafast bootstrap values >95% are shown with black branches, 50–94% with gray branches and <50 with dashed gray branches. Terminals are labeled with Genbank voucher numbers, species, and sampling locality for our focal taxa. Type localities are indicated with an *. The new species and select relatives are figured.

recovered each of the proposed new species as distinct monophyletic groups. Relationships are not entirely congruent with the COI tree, with the placement of *Tryonia* closer to *Stygopyrgus* than *Vitropyrgus*, however, there is relatively little support for these relationships in either gene tree.

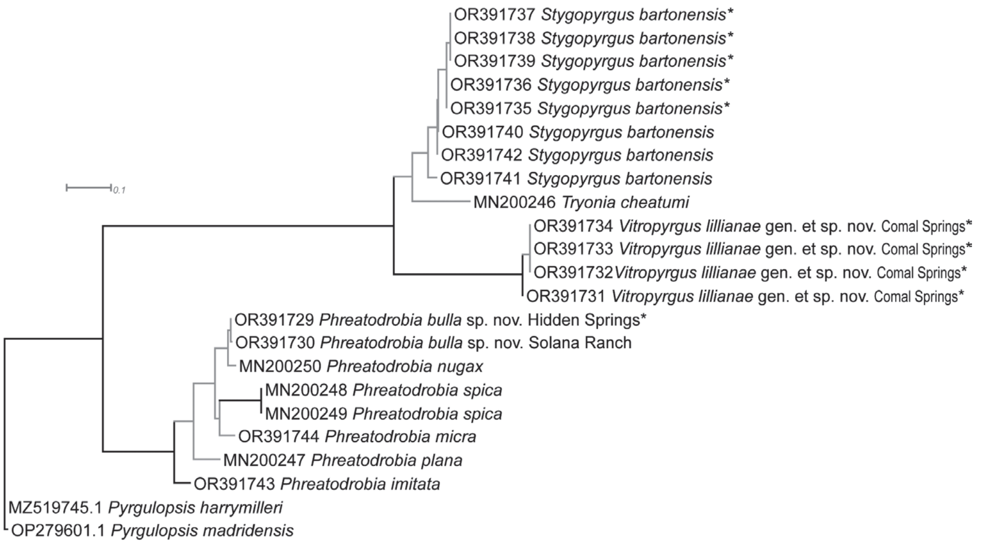

Figure 4. Highest likelihood trees resulting from maximum-likelihood analysis of LSU alignment. Ultrafast bootstrap values >95% are shown with black branches and 50 –94% with gray branches. Terminals are labeled with Genbank voucher numbers, species, and sampling locality for our focal taxa. Type localities are indicated with an *.

Systematics

Class Gastropoda Cuvier, 1795
Subclass Caenogastropoda Cox in Moore, 1960
Order Littorinimorpha Golikov & Starobogatov, 1975
Superfamily Truncatelloidea Gray, 1840
Family Cochliopidae Tryon, 1866

Genus *Vitropyrgus* Perez & Guerrero, 2023, gen. nov.
https://zoobank.org/4E6DE6B7-1891-4960-8211-AC921C8171D0
Figs 5, 6

Type species. *Vitropyrgus lillianae* gen. et sp. nov.

Diagnosis. Minute shell with spiral and collabral sculpture on teleoconch that extends to sutures. Embryonic whorl distinctively sculptured with wrinkles giving a malleated appearance. Aperture ovate to round, with slightly reflected lip near umbilicus. Umbilicus open. Animal eyeless and unpigmented. Penis attached behind right eye position, simple in glandular structure. The single known species of *Vitropyrgus* is a quarter of the size of related epigean taxa and is adapted to a subterranean environment (e.g., lacking pigmentation, eyes, and ctenidia). Simple penial morphology lacking the papillae or apocrine glands that define other members of Cochliopidae. Finally, the

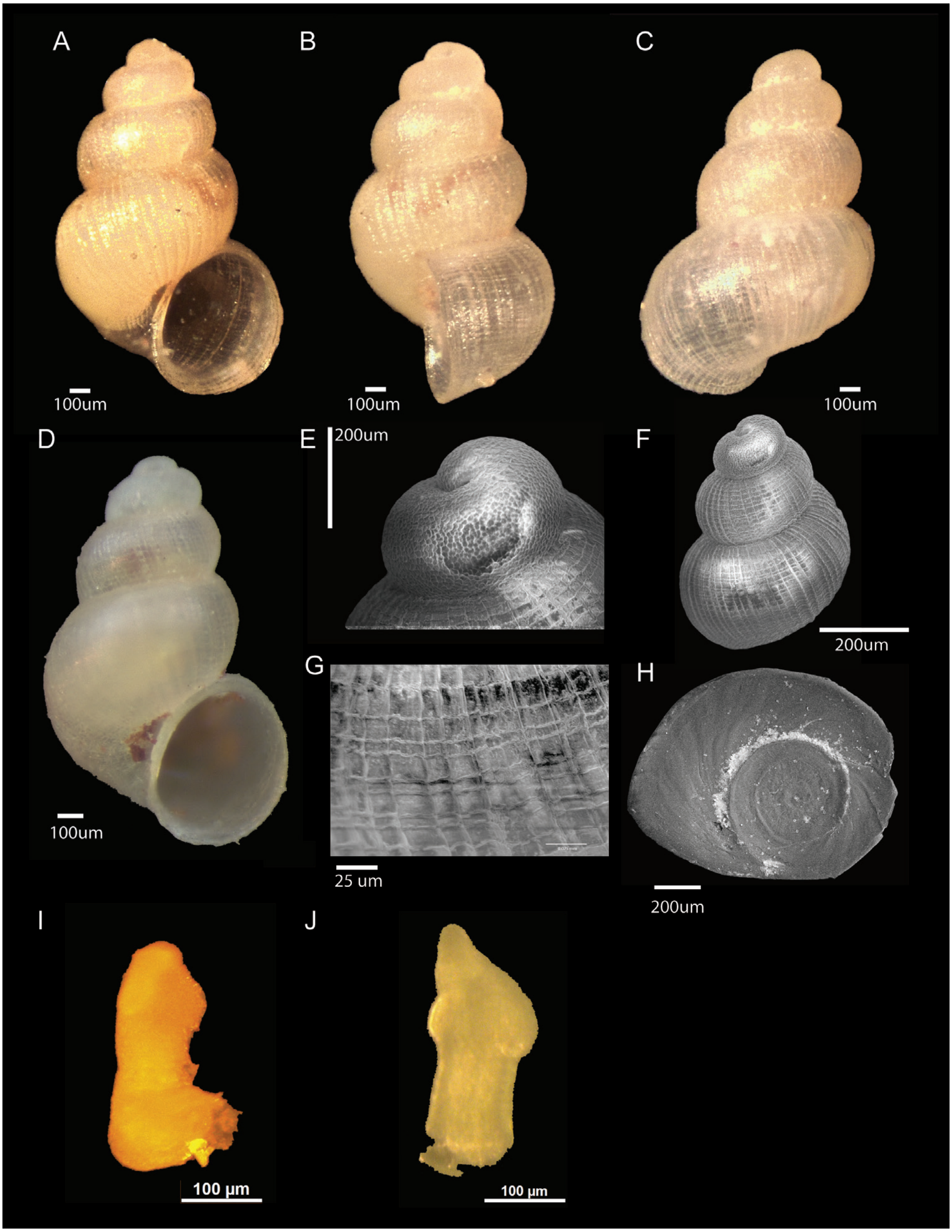

Figure 5. Shells and anatomical features of *Vitropyrgus lillianae* gen. et sp. nov. All localities in Texas **A–C** holotype, Comal Springs, Comal County, ANSP 494654 **D** shell frontal view of individual from Fessenden Springs, Kerr County, ABC 005622 **E** SEM of embryonic whorls to detail sculpture **F** SEM of rear shell and sculpture **G** SEM of teleoconch sculpture **H** SEM of operculum, outer view **I** penis, ventral view **J** penis, dorsal view. The yellow coloration in I and J is caused by immersion in Bouin's solution, the tissues are white, unpigmented in life.

shell has a distinctive clear and glassy appearance, lacking the tan color of *Tryonia* or *Stygopyrgus* Hershler & Longley, 1986 or the usual translucency of *Phreatodrobia*.

Taxonomic remarks. The most recent review of Cochliopidae (Hershler and Thompson 1992) divided the family into three subfamilies, Cochliopinae Tryon, 1966, Littoridininae Thiele, 1928, and Semisalsinae Giusti & Pezzoli, 1980 largely distinguished by glandular features of the male reproductive anatomy, including “Tribe” *Heleobia* Thompson, 1968 (Hershler and Thompson 1992; Liu et al. 2001) diagnosed by apocrine penial glands. Cochliopinae (e.g. *Cochliopina* W. Stimpson, 1865) is diagnosed by a simple, non-glandular penis with a long filament distinct from the wrinkled or folded base and Littoridininae (including *Stygopyrgus*, *Pyrgophorus*, *Mexipyrgus*, and *Tryonia*) is characterized by a long sperm duct and often with numerous glandular papillae. A subsequent molecular phylogenetic analysis broadly supported these groupings (Liu et al. 2001).

We do not attempt to place this genus among the subfamilies of Cochliopidae. The COI phylogeny has limited resolution at this level, we have limited sampling in LSU for placement among subfamilies. The COI tree places *Vitropyrgus* close (with no support) to a clade that included *Heleobia* (Semisalsinae) and *Onobops* (Littoridinae). Members of Semisalsinae are diagnosed by penial papillae or apocrine glands (Liu et al. 2001), which *Vitropyrgus* lacks. *Onobops* is one of several cochliopid genera that have a simple penis with no papillae or apocrine glands, resembling *Vitropyrgus*. *Onobops* are epigean, brackish water species from North America. The subfamily placement of this genus is best defined as uncertain along with many other genera in Cochliopidae.

Vitropyrgus is proposed as a new genus with the following rationale. First, it was found by COI and LSU phylogenies sister to epigean taxa. In the COI phylogeny, *Vitropyrgus* is most closely related to members of the *Heleobia* and *Onobops*. Divergence in COI between *Vitropyrgus* and other members of that clade averaged 16.0% with a range from 15.5–16.6. Intergeneric comparisons in our dataset averaged 16.54% with a range from 5.19–25.37. This places the level of divergence between *Vitropyrgus* and its closest known relatives within the range of intergeneric divergence and just under the average for the Texas genera. In other groups of subterranean hydrobioids the range of 14.5–16.7% has been used to justify genus level distinction (Delicado et al. 2019; Delicado and Gürlek 2021).

Vitropyrgus lillianae **Perez & Guerrero, 2023, sp. nov.**

https://zoobank.org/DB9F6F2C-749B-45C1-B496-8BB603C7BAF4

Figs 5, 6

Stygopyrgus bartonensis, Hutchins 2018, suppl. material 1: table S1.
Stygopyrgus bartonensis, Hutchins et al. 2021, suppl. material 1: table S2.

Type locality. USA, Texas. Comal County, New Braunfels, Comal Spring Upwelling #7, (29.7135, -98.1370).

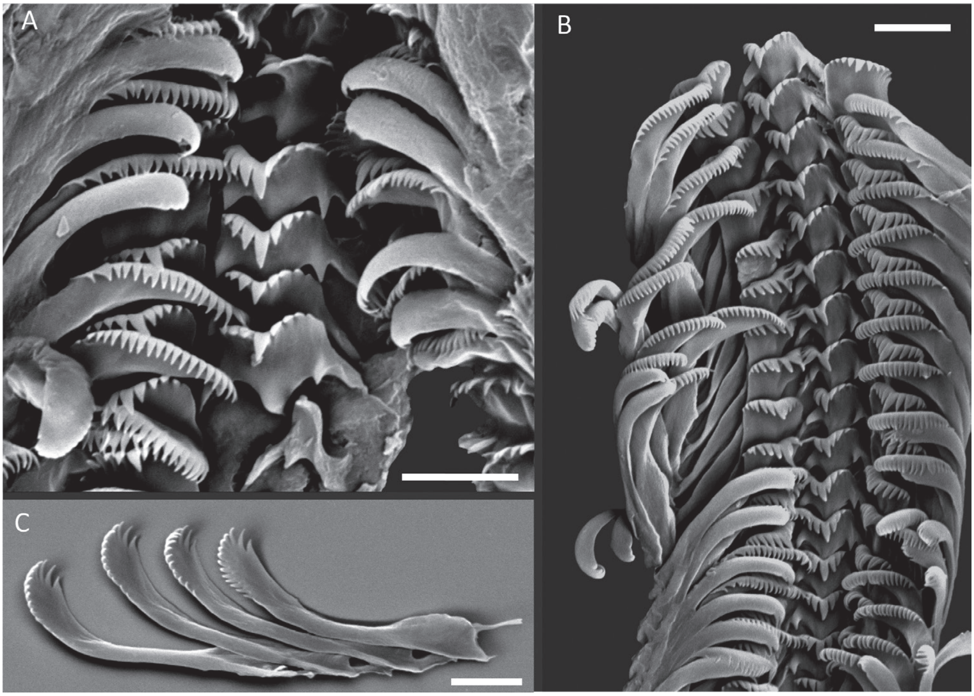

Figure 6. SEM of *Vitropyrgus lillianae* gen. et sp. nov. radula **A** view of central radula teeth **B** radula ribbon showing details of inner and outer marginals **C** outer marginal teeth.

Material examined. All sites are in Texas, USA. ***Holotype*** – Comal County, Landa Park, New Braunfels, Comal Spring Upwelling #7, (29.7135, -98.1370), drift net, collected by Randy Gibson, 2 May 2019 (ANSP 494654). ***Paratypes*** – Comal County, Landa Park, New Braunfels, Comal Spring Upwelling #7, (29.7135, -98.1370), drift net, collected by Randy Gibson, 1–5 June 2018 (ANSP 494656).

Additional material examined. – Kerr County, Fessenden Spring, near Heart of the Hills Fisheries Science Center (30.1670, -99.3427), drift net, collected by K.E Perez, D. Deshommes, N. Loveland, 4–6 November 2020 (ABC 005622).

Diagnosis. Minute shell with glassy appearance, with distinctive spiral and collabral sculpture on teleoconch that extends to sutures. *Vitropyrgus lillianae* differs from similar species in the region by shell shape, sculpture, or shell color. *Stygopyrgus bartonensis* has a taller, more columnar, and less heavily sculptured shell. The shell of *S. bartonensis* also has a pale brown tint in fresh shells that is not present in *V. lillianae*. The animals most easily confused with *V. lillianae* are very juvenile individuals of *Pyrgophorus spinosus* (Call & Pilsbry, 1886). While their sculpture can appear quite similar, juvenile *P. spinosus* are much larger, have a white base color and the aperture forms an oval, completely appressed to the body whorl. *Pyrgulopsis spinosus* shells have a more steeply tapering spire than *V. lillianae*. Dissection and comparison of penial anatomy will readily distinguish *V. lillianae* due to its simple structure with no papillae or apocrine glands.

Description. Shell very small, clear, glassy, heavily sculptured, ovate-conic with rounded whorl outlines (Fig. 5A–D). Average shell measurements for adults (n = 20): shell height = 0.737 mm (SD = 0.25), shell width = 0.470 mm (SD = 0.17), aperture height = 0.333 mm (SD = 0.11), aperture width = 0.268 mm (SD = 0.09), number of whorls = 4.5 (SD = 0.20).

First whorl of protoconch slightly elevated, separated from subsequent whorls (Fig. 5E, F). Protoconch surface heavily sculptured by wrinkles that form irregularly shaped shallow depressions or pits. Teleoconch sculpture includes finely spaced collabral ribs dissected by spiral lines (Fig. 5F, G). Ribs slightly more elevated, spaced 20–23 µm apart. Aperture ovate, slightly pulled away from body whorl, only lightly touching body whorl at parietal corner. Lip reflected on basal and umbilical portions in larger individuals. Outer lip straight, simple, slightly tilted forward (prosocline). Umbilicus open. Operculum clear, extremely thin, pliable, fragile (Fig. 5H). Shape elongate ellipsoidal, nucleus submarginal, spiral weakly convex. Growth lines not distinct or frilled.

Unpigmented body visible through shell. Snout nontapered, about as long as wide, with strong distal lobation. Foot short, anterior portion rounded, anterior edge indented, without lateral wings. Cephalic tentacles tapered, rounded, unpigmented, with no visible cilia, about 5 times as long as wide. Mantle tissue unpigmented. No visible eyes and no visible pigment at base of eyestalks. No ctenidium observed, osphradium rounded.

Intestine uncoiled, mostly filled with rounded fecal pellets, rectum exiting in pallial cavity, near mantle edge on right side of head. Esophagus entering stomach below, smaller posterior chamber with large digestive gland aperture and larger anterior chamber. Stomach speckled with dark flecks of pigment. Caecum not observed.

Penis large relative to body size tapering, attached well behind right eye, with an expanded, muscular base, narrow body segment, tapering to a distal tip (Figs 5I, J). Penis base with moderate folding along inner curvature. Distal portion tapered, inner and outer curves with aglandular curving lobes nearly opposite each other, giving a blunt, asymmetrical "arrowhead" shape to distal portion of penis. Neither apocrine glands or papillae present on examined individuals. Cilia not observed on distal penis.

Central radular tooth with indented dorsal edge (Fig. 6A); lateral cusps 4 on each side; central cusp ~1/3 longer than adjacent cusps, pointed but tapering at the end and at the base, wider in the middle, singular basal cusps pointed, with small buttress, paddle shaped, not needle-like, basal process triangular in shape; deep basal socket. Lateral tooth rectangular, narrowing slightly upon reaching the outer wing; outer wing tapering; central cusp longer than lateral cusps, 5–6 cusps outer and 5 cusps inner direction, decreasing in size distally. Inner marginal teeth with broad outer wing with basal notch, 17–19 cusps, mostly similar in length except two outermost cusps shorter, triangular, wide at base. Outer marginal teeth broad and curved at end, with 14–16 cusps. Cusps along inner edge longer; tooth face tapering to outer wing which then broadens again at base (Fig. 6B, C).

Etymology. We use the generic name "*Vitropyrgus*" reflecting the glassy appearance of the shell of this phreatic snail compared to related groups. The specific epithet "*lillianae*" is in honor of Dr. Lillian E. Perez, the first author's mother. We propose the common name "glass cavesnail".

Ecology. This new snail species is found among other phreatic snail fauna in Comal Springs including: *Phreatodrobia nugax* (Pilsbry & Ferriss, 1906), *Phreatodrobia plana* Hershler & Longley, 1986, *Phreatodrobia spica* K. E. Perez & Alvear, 2020, and *Phreatodrobia rotunda* Hershler & Longley, 1986. Other members of this unique subterranean fauna include the federally endangered amphipod *Stygobromus pecki* (Holsinger, 1967), the federally endangered dryopid beetle *Stygoparnus comalensis* Barr & Spangler, 1992, an undescribed stygobiontic salamander, and many other invertebrates (Hutchins et al. 2021). Federally endangered epigean fauna at Comal Springs include the riffle beetle, *Heterelmis comalensis* Bosse, Tuff, & Brown, 1988, fountain darter, *Etheostoma fonticola* (Jordan & Gilbert, 1886), and Comal Springs salamander, *Eurycea neotenes* Bishop & Wright, 1937.

Habitat. This species is known from two localities in the karstic Edwards and Edwards-Trinity Aquifers, separated by ~125 km. Comal Springs is the largest spring in Texas (mean annual discharge = 8.4 m^3/s, (USGS 2023)) and discharges water from the deep confined portion of the regional San Antonio segment of the Edwards Aquifer. The spring is a complex of openings discharging on and along a normal fault that divides the deep confined and recharge zones of the Edwards Aquifer, and the springs integrate a mix of species found in one or both aquifer zones.

Fessenden Spring on Johnson Creek is a smaller spring that is part of the large regional Edwards-Trinity Aquifer system. Fessenden Spring discharges from the base of the Edwards Limestone in the central Texas Hill Country and is one of many Edwards-Trinity springs that support baseflows in the headwater reaches of the Guadalupe River. Across much of the southeastern portion of this aquifer, springs discharge into streams and rivers in the contributing zone for the Edwards Aquifer. The Edwards-Trinity system is hydrologically connected to the Edwards Aquifer along the Balcones Fault zone through both groundwater and surface-water linkages. The Guadalupe River is the only river in the contributing zone to not consistently lose much or all its flow to the Edwards Aquifer as it crosses the aquifer recharge zone (Ockerman and Slattery 2008; Wehmeyer et al. 2013). Instead, it consistently gains discharge via Comal Spring, Hueco Spring, and several other springs discharging from both the Edwards and Edwards-Trinity aquifers.

In the boundary zone between the two aquifer systems, movement of organisms across blurry hydrologic boundaries between the aquifers is possible. Additionally, there is increasing evidence that the hyporheic zone along river corridors can provide important habitat and connectivity for a variety of Texas groundwater taxa, including snails (Hutchins et al. 2020; Sparks 2023). Because the Edwards Limestone is continuously exposed across the upper and middle Guadalupe River watershed between Fessenden and Comal Springs, it is likely that *Vitropyrgus lillianae* gen. et sp. nov. is more widespread than the localities reported here. More occurrences will likely be discovered once the species is characterized, and additional samples are collected across this watershed. However, given the prevalence of restricted range size in most (though not all) Texas groundwater snails (Alvear et al. 2020a), it is unlikely that the range for *Vitropyrgus lillianae* gen. et sp. nov. will be expanded considerably. With only two populations currently known, the species is classified as critically imperiled (G1S1) using NatureServe methodology and considering distribution data only.

Taxonomic remarks. The species superficially resembles *Stygopyrgus bartonensis* in overall shell form and sculpture and was initially identified as that species (e.g. Hutchins 2018, suppl. material 1: table S1, identification by R. Hershler, and Hutchins et al. 2021, suppl. material 1: table S2). Here we examine the relationship with both COI and LSU data of *V. lillianae* to several populations of *S. bartonensis*, including the type locality. In both analyses, while it is not certain which members of the Cochliopidae *V. lillianae* are closely related to, this species is not supported as closely related to *S. bartonensis.*

Class Gastropoda Cuvier, 1795
Subclass Caenogastropoda Cox in Moore, 1960
Order Littorinimorpha Golikov & Starobogatov, 1975
Superfamily Truncatelloidea Gray, 1840
Family Cochliopidae Tryon, 1866
Genus *Phreatodrobia* Hershler & Longley, 1986

***Phreatodrobia bulla* Perez & Castañeda, sp. nov.**
https://zoobank.org/5393D9DA-DCC8-49B0-BC2C-4C45FC2E45F4
Figs 7, 8

Phreatodrobia cf *imitata* Perez et al., 2020, pp. 7.
Phreatodrobia conica Gibson et al., 2021b, pp. 33.

Type locality. USA, Texas, Bell County, Hidden Springs (30.9382, -97.6044).

Material examined. All sites are in Texas, USA. ***Holotype*** and ***Paratypes*** – Bell County, Hidden Springs, collected by Peter Diaz (30.9382, -97.6044), 27 October 2021 (ANSP 494658, 494660).

Additional material examined. – Bell County, Salado Springs Complex, Anderson Spring (30.9441, -97.5347); Stagecoach Inn Cave, Salado (30.9432, -97.5375), 1 May 2020, P. Diaz (ABC 005618); Copperhead Spring Cave, Ft. Cavazos (confidential location); Bent Oak Spring (30.8916, -97.7092), 17 August 2022 (ABC 005616); Gault Archaeological Site Spring (30.8916, -97.7095), 8 June 2019 (ABC 005615); Robertson Springs, Creek Springs (30.9445, -97.5413); Solana Ranch Spring (30.8997, -97.6390), 25 March 2020 (ABC 005620), P. Diaz; Spicewood Creek Pipe Spring (confidential location); Spring 23-398, Ft. Cavazos (confidential location); Camp Tahuaya, Tahuaya Spring Pool (31.0087, -97.5093). – Williamson County, PC Spring (30.4818, -97.7419), 23 March 2023 (ABC 005617).

Diagnosis. Shell translucent, conical, with nearly smooth teleoconch, dome-shaped protoconch with wrinkles. Aperture round to slightly ovate, usually separated

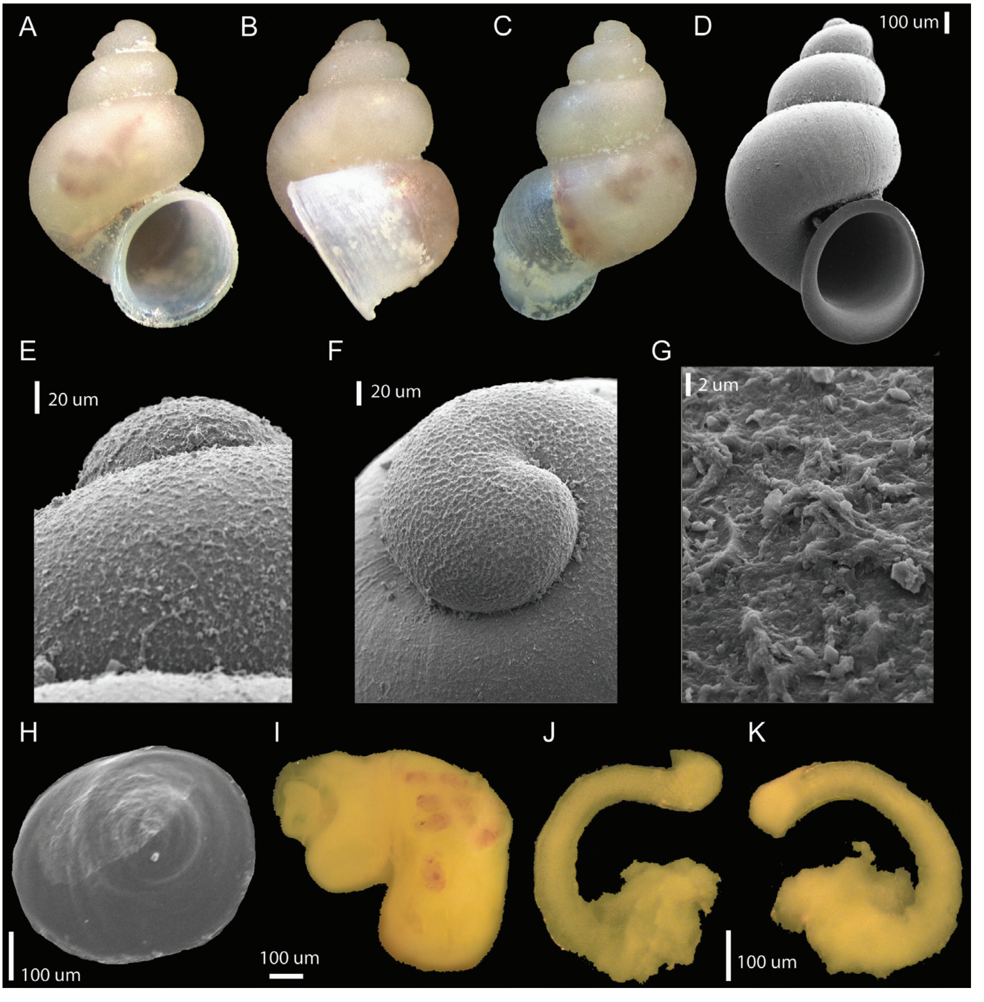

Figure 7. Shells and anatomical features of *Phreatodrobia bulla* sp. nov. All sites are in Texas **A–C** holotype, Hidden Springs, Bell County, ANSP 494658 **D** SEM of individual from Hidden Springs, Bell County ANSP 494660 **E–G** SEM of embryonic shell sculpture **H** SEM of operculum, inner surface **I** dorsal view of body **J** ventral view of penis **K** dorsal view of penis. Scale bars: 100 µm (**A–E**); 100 µm (**J, K**).

from body whorl in adults. Mantle tissue white, unpigmented. Sharply pointed median cusp of central radular teeth with small basal cusp. Penis long, equal width most of the length, tapering sharply near tip, loosely to tightly coiled, length 2–3 times length of snout.

Description. Shell translucent, usually pale tan, conical with 3.5–4.5 well rounded whorls (Fig. 7A–D). Shell height ranges from 1.1–2.39 mm. Average shell measurements (n = 14 adult individuals): height = 1.86 mm (SD = 0.5), width = 1.22 mm (SD = 0.3), aperture height = 0.78 mm (SD = 0.2), aperture width = 0.77 mm (SD = 0.2), number of whorls = 4.0 (SD = 0.5). Sutures deeply impressed giving whorls a very rounded aspect. Body whorl wider than others, which taper steeply to a dome-shaped embryonic whorl. Spire with distinctive "bubble" or dome shape. Dome-like embryonic whorl sculptured with irregular granules and wrinkles (Fig. 7E–G), teleoconch smooth, without visible sculpture, except under high-magnification. Regular growth lines visible on recent shells. In most individuals, aperture fully detached from previous whorl (appressed only at top of aperture in some smaller individuals). Aperture ovate, with simple, prosocline, reflected lip that flares at base. Umbilicus present.

Operculum round to broadly ovate, concave, amber in color, deeply concave, with narrow band of thinner material on outer margin, tapering to a point but without nuclear peg (Fig. 7H). Opercular growth lines vague, simple. Nucleus slightly eccentric, central, paucispiral. Muscle attachment scar distinct and thickened toward edges, with undifferentiated edges. Attachment region callus thin.

Body visible through translucent shell. No eyes present. Ctenidium composed of triangular filaments, approximately as broad as high, stretching from posterior portion of pallial cavity nearly to mantle edge. Osphradium oval shaped, elongate, positioned opposite posterior portion of ctenidium, occupying ~25% of pallial cavity. Pallial portion of intestine with loops in posterior portion of pallial cavity similar to *P. imitata*. Fecal pellets in the coiled intestine usually clearly visible through the shell, bright orange, oval-elongate in shape (Fig. 7I). Rectum ends just before mantle edge.

Snout narrow, longer than wide, deeply lobate distally, with folds along sides. Tentacles tapered, with scattered granules or pigmentation at base, length equal to snout. No eye visible. Foot rounded anteriorly, with lateral wings. Penis base well behind right tentacle, slightly wider and with deeper folds near base, tapering quickly to a consistent length until sharply tapering at tip. Slight folds continue until midway along penis length. Penis long, loosely to tightly coiled, curved and 2–3 times longer than snout (Fig. 7J).

Central radular tooth with deeply indented dorsal edge; central cusp longer than adjacent cusps; lateral cusps 5–6 on each side, evenly decreasing in width towards tip, sharply pointed; basal cusps small, triangular; basal socket deep, v-shaped. Lateral tooth rectangular, with a longer central cusp and 4 (inner) – 7 (outer) cusps on either side. Some laterals with wide deposit down mid-line. Base of lateral tooth with triangular, well excavated ventral process, tapering to wing. Inner marginal teeth with ~25–30 cusps, similar in length, decreasing slightly in outermost cusps. Tooth surface tapering towards outer wing with narrow neck before flaring smoothly towards the base. Outer marginal teeth rounded, spoon-shaped, wide at top, smoothly curving, with 12–20 small cusps, tapering slightly to short neck.

Etymology. We use the specific epithet "*bulla*" from the latin for "bubble", referring to the rounded appearance of each whorl, particularly the rounded spire. We propose the common name "Brown's cave snail" in honor of Mr. Tim Brown, a Bell County native and former Commissioner who has worked extensively to promote conservation of archaeological and groundwater resources in the region.

Ecology. This new species is part of a diverse aquifer community. Relative abundance varies across the range of the species. At Creek Springs (part of the Robertson Springs Complex), as many as 200 snails can be captured over a couple of days of drift

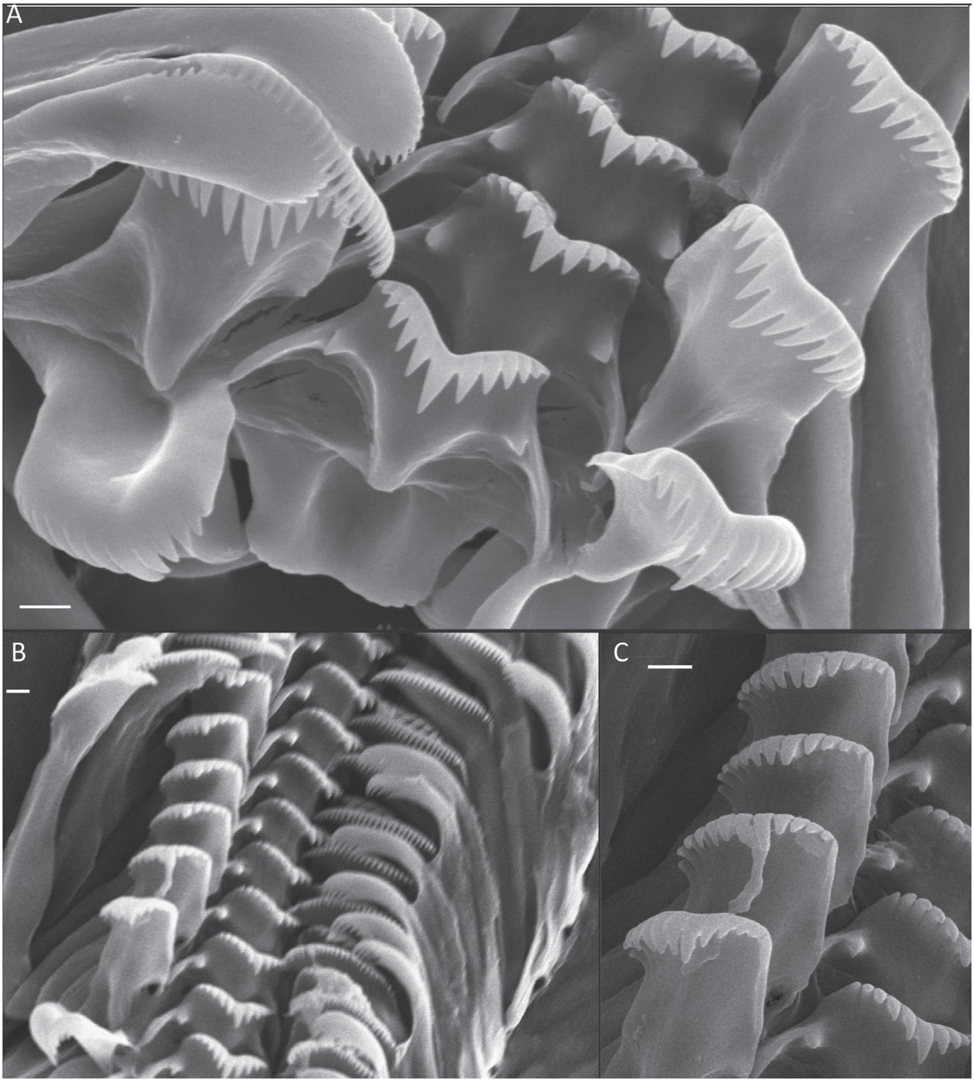

Figure 8. SEMs of radula of *Phreatodrobia bulla* sp. nov. Hidden Springs, Bell County **A** central and marginal radular teeth **B** portion of radular ribbon **C** lateral teeth. Scale bars: 2 μm.

net collection, whereas at PC Springs, similar sampling effort yields only one or a few specimens. *Phreatodrobia bulla* sp. nov. is often collected with other phreatic snails: *P. nugax*, *P. micra* (Pilsbry & Ferriss, 1906), and *Phreatoceras taylori* (Diaz and Warren 2018). Depending on site, *P. bulla* sp. nov. may also occur with several crustaceans *Lirceolus* sp., *Stygobromus bakeri* Gibson et al. 2021, *Parabogidiella americana* Holsinger, 1980, and undescribed Bathynellacea and Microcerberidae. They also occur with the federally threatened Salado salamander (*E. chisholmensis* Chippindale, Price, Wiens & Hillis, 2000) and Jollyville salamander (*E. tonkawae* Chippindale, Price, Wiens & Hillis, 2000).

Habitat. All known localities for *Phreatodrobia bulla* sp. nov. are springs or hyporheic samples taken near springs discharging from the northern segment of the karstic Edwards Aquifer (north of the Colorado River). The northern segment lies adjacent to, but is disconnected from, the Barton Springs segment of the Edwards Aquifer, with the Colorado River a topographic low that forms the boundary between the two segments. Faults, erosion, and other geologic and geomorphic factors in the northern segment have resulted in groundwater basins that are relatively smaller and more dissected than in the Barton Springs and San Antonio segments to the south (Jones 2003). More and smaller springs in the region, combined with relatively intensive sampling at many of those springs, are likely factors contributing to the higher number of known occurrences for this species, and it is likely that additional sampling in Bell, Williamson, and northern Travis Counties will result in more localities, particularly in the 45km gap between PC Spring (the southernmost location) and Kings Garden Spring. In particular, very little hyporheic sampling has been performed along streams and rivers in the region and this has been a productive method for sampling groundwater snails in other parts of Texas. Nevertheless, for the same reasons discussed for *V. lillianae* gen. et. sp. nov., it is unlikely that additional work will substantially expand the known range of *P. bulla* sp. nov.

Currently, *P. bulla* sp. nov., is known from 12 sites across a range of approximately 680 km^2. Occurrence at multiples sites provides some security against catastrophic events (redundancy, sensu Shaffer and Stein 2000). Nevertheless, Bell and Williamson Counties are among the fastest growing counties in Texas, resulting in substantial pressure on groundwater resources. Eleven of the 12 locations occur within the Clearwater Underground Conservation District, which is tasked with developing and implementing a groundwater management plan for the Edwards and Trinity aquifers in Bell County (Clearwater Underground Water Conservation District 2020). The desired future condition adopted by the conservation district, which provides a basis for some permitting and regulation of groundwater extraction, is preservation of a minimum acceptable springflow of 1.66 cfs at the Salado Springs complex (which includes Anderson and Creek springs) during hydraulic conditions equal to the 1950s drought of record. That is approximately 10% of average flows during the 1980s (Brune 1995). Currently, several spring orifices in the region go dry during drought periods (Diaz et al. 2015), illustrating that groundwater availability is the central conservation concern for *P. bulla* sp. nov. The sites where this species has been encountered are restricted to springs and spring-run hyporheic habitats, with sampling of wells or caverns in Bell and Williamson counties needed to determine its' full extent. Without quantifying the severity and scope of threats, *P. bulla* sp. nov. is ranked as imperiled (G2S2) using NatureServe methodology.

Taxonomic remarks. Intraspecific and interspecific sequence divergence averaged 2.45% and 10.73%, respectively, in our dataset of Texas phreatic snails. *Phreatodrobia bulla* has an average sequence divergence of 10.34% with the other members of *Phreatodrobia* and *Phreatoceras*, and 6% divergence with its sister *Phreatoceras taylori*. Interspecific variability in COI has been examined in several groups of subterranean hydrobioid gastropods inhabiting karstic environments. In *Belgrandiella* A. J. Wagner, 1928 "species" (Hydrobiidae) COI divergence ranged from 5.2–9.9% (Jaszczyńska et al. 2022). An analysis of *Bythinella* Moquin-Tandon, 1856 (Bythinellidae Locard, 1893) from a karstic region of France, which included epigean and cave species, found that maximum species-level divergence was 1.5% (Bichain et al. 2008). In *Kerkia* Radoman, 1978 (West Balkans), a group of snails that resembles *Phreatodrobia* in habitat and morphology, interspecific genetic divergence ranged from 4.2%–14.7% (Hofman et al. 2022) and similar values were found in *Balkanica* Georgiev, 2011 and related lineages (Hydrobiidae, 7.8%–11.8%) in Bulgaria (Osikowski et al. 2017). Thus, gene flow seems to vary by group and may be relatively low within some taxa or high, possibly facilitated by movement through routes such as the hyporheic or phreatic rhizosphere (Haase et al. 2021). While there is not a molecular ruler denoting species-level distinction among subterranean species, *Phreatodrobia bulla* has sequence divergence comparable to other species of *Phreatodrobia* and greater than average species level divergence relative to most subterranean gastropods.

Lacking circumscription, *Phreatodrobia bulla* has been previously identified in recent literature (Alvear et al. 2020a; Gibson et al. 2021b) as *P. conica* or *P.* cf *imitata* as it resembles these species in some aspects of shell morphology, the basis for those identifications. When we consider internal anatomy or DNA, these species are diagnosably different from *P. bulla*. The shell of *Phreatodrobia conica* is described (Hershler and Longley 1986b) as having a simple aperture and a varix (ridge behind the aperture marking previous aperture position) near the end of the body whorl. It also has a distinctive teleoconch sculpture with numerous ridges. Its internal anatomy is distinguished by the lack of a ctenidium and a square-shaped central radular tooth. *Phreatodrobia bulla* in contrast has a flared and reflected aperture in adults with no sign of a varix in any material examined. The teleoconch sculpture is smooth without ridges and with a few collabral growth lines near the aperture, however, these are not elevated as described in *P. conica*. Finally, *P. bulla* has a robust ctenidium and the usual V-shaped central radular tooth both in contrast to what is described for *P. conica.* Access to the type locality of *P. conica* has not been possible during this study, preventing collection of tissues for DNA data collection. Even in the absence of DNA data, however, the anatomical distinctions between these species are sufficient to describe *P. bulla* as distinct from *P. conica*.

Phreatodrobia imitata and *P. bulla* share the same general shell shape and highly flared aperture (Fig. 9). However, the shells are readily distinguished. *Phreatodrobia imitata* has a translucent or clear shell which is heavily sculptured shell with collabral costae (ribs) and spiral lines (running opposite the ribs) while the teleoconch of *P. bulla* is unsculptured. Even though these sculptural features appear to consistently distinguish *P. imitata* and *P. bulla*, sculptural characters alone are insufficient to distinguish these species as ribs are polymorphic among *Phreatodrobia* and other freshwater snails. There are more pronounced differences found in the radula and DNA. The central radular tooth of *P. imitata* has a very narrow central cusp, 6–7 cusps on either side, and it lacks a basal cusp. The central radular tooth of *P. bulla* has a wider central cusp, ~ 5 cusps on

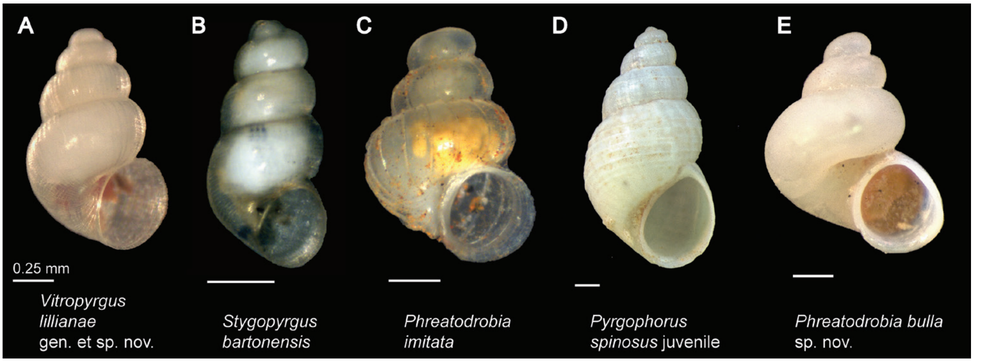

Figure 9. Shells of the species described here compared to shells of similar species in the region. All localities are in Texas **A** *Vitropyrgus lillianae* gen. et sp. nov. Comal Springs, Comal County **B** *Stygopyrgus bartonensis*, Barton Springs, Travis County **C** *Phreatodrobia imitata*, Verstræten Well, Bexar County **D** *Pyrgophorus spinosus* juvenile, San Marcos River, near Martindale, Guadalupe/Caldwell County Line **E** *Phreatodrobia bulla* sp. nov. PC Spring, Williamson County.

either side, and it possesses a distinct basal cusp. We obtained *P. imitata* individuals for DNA analysis from Aldridge Well near the type locality (Verstræten Well), and both COI and LSU phylogenies have strong support for placement of *P. imitata* as the sister lineage to other species of *Phreatodrobia*, not part of the *P. bulla* clade.

Previous classification efforts have not determined the placement of *Phreatodrobia* and *Phreatoceras* within a subfamily of Cochliopidae (Hershler and Thompson 1992; Liu et al. 2001). They are both found in the Edwards and Edwards-Trinity Aquifers and share features such as a minute, colorless, translucent shell and pitted protoconch microsculpture (Hershler and Longley 1986b; Hershler and Longley 1986a). *Phreatoceras* is diagnosed primarily by its unique, uncoiled, horn-like shell (see Fig. 3), and some features that are shared with various *Phreatodrobia* species such as a smooth teleoconch, loss of ctenidium, long central cusp of the central radular tooth. In the COI phylogeny, *P. bulla* is found sister to *Phreatoceras taylori* from the same springs in Bell County, Texas, but with weak support, and both are embedded within a clade of *Phreatodrobia* species. When nine anatomical characteristics of the two genera are compared (Table 1), *P. bulla* shares one distinctive characteristic with *Phreatoceras*, four characteristics with both genera, and five characteristics with *Phreatodrobia*. As this proposed new species shares more morphological characteristics with *Phreatodrobia*, is found by the DNA data within a clade of *Phreatodrobia* and does not have the distinctive trumpet shaped shell of *Phreatoceras*, we place it in *Phreatodrobia*.

We propose two potential explanations for the sister relationship of *P. bulla* and *Phreatoceras taylori*. In this study, we have not sampled the type locality of *Phreatoceras*, so it is possible that snails with a trumpet-shaped shell that we sampled in Bell County are not *Phreatoceras taylori sensu stricto*, described from Real County, Texas, ~250 km distant. Alternatively, *Phreatoceras* may be better considered a morphologically divergent member of *Phreatodrobia* rather than a separate genus. We have observed other species of *Phreatodrobia*, such as *P. nugax*, with a loosely coiled or partially uncoiled

Table 1. Comparison of morphological features of *Phreatoceras* and *Phreatodrobia* with *P. bulla* sp. nov.

Character	*Phreatoceras*	*Phreatodrobia* spp.	*P. bulla* sp. nov.
Shell shape	horn-like	planispiral, trochoid, conical	conical
Protoconch sculpture	pitted/wrinkled microsculpture	pitted microsculpture	wrinkled
Teleoconch sculpture	smooth	variable	regular collabral growth lines
Aperture	simple	flared	flared
Operculum	near circular, concentric, multispiral, large ventral central process.	Round to oval, non-concentric, nucleus subcentral, sometimes with central knob	Oval, non-concentric, nucleus subcentral with ventral mound
Ctenidium	absent	absent, nearly absent, present	present
Radula - central tooth	central cusp long, basal tooth long at origin of lateral angle	central cusp long or not, basal cusp present or not at origin of lateral angle	central cusp long, basal tooth long at origin of lateral angle
Penial morphology	tight coil	coil or uncoiled	coiled

shell in some individuals, lending some observational support to this possibility. Examination and sequencing of *Phreatoceras taylori* from the type locality, is needed to resolve its relationship to *Phreatodrobia*.

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Supplementary material 1

List of specimens, including their sampling localities and voucher information

Authors: Kathryn E. Perez

Data type: xlsx

Explanation note: *denotes type localities. Alphanumeric identifiers correspond to Genbank accession numbers. USNM = United States National Museum, Smithsonian Institution; ANSP = Academy of Natural Sciences of Philadelphia at Drexler University, TMM = Texas Memorial Museum, ABC = Texas State University Aquifer Biodiversity Collection.

Link: https://doi.org/10.3897/subtbiol.47.113186.suppl1

Supplementary material 2

Photo-vouchers of individuals sampled for DNA or radula, *Vitropyrgus lillianae* gen. et sp. nov. and *Stygopyrgus bartonensis*

Authors: Kathryn E. Perez

Data type: tif

Explanation note: Shells are destroyed during both procedures. Photo vouchers are supplied here and paravouchers deposited in museum collections. All localities are in Texas. *Vitropyrgus lillianae* gen. et sp. nov., Comal Springs, Comal County, TX **A** OR372116 **B** OR372117, OR391734 **C** OR391731 **D** OR391732 **E** OR391733, Paravouchers (paratypes) = ANSP 494656; *Stygopyrgus bartonensis*, Parthenia Springs in Barton Springs Pool, Travis County, TX **F** OR391735 **G** OR372120 **H** OR372121, OR391737 **I** OR391738 **J** OR372122, OR391739 Paravouchers = ABC 003350; Old Mill Springs, Travis County, TX **K** OR391740 **L** OR391741; **M** OR372123, OR391742 **N** OR372124, Paravouchers = ABC 003357; ABC 003358**;** Camp Aransas Springs, Travis County, TX **O** OR372131, Paravouchers = ABC 005621. Scale bar: 0.5 mm.

Link: https://doi.org/10.3897/subtbiol.47.113186.suppl2

Supplementary material 3

Photo-vouchers of individuals sampled for DNA or radula, *Phreatodrobia bulla* sp. nov.

Authors: Kathryn E. Perez

Data type: tif

Explanation note: Shells are destroyed during both procedures. Photo vouchers are supplied here and paravouchers deposited in museum collections. All localities are in Texas. *Phreatodrobia bulla* sp. nov. Hidden Springs, Bell County, TX **A–N** Hidden Springs, Bell County, TX, Paravouchers = USNM 157128, radula specimens. Solana Ranch, Bell County, TX, Paravouchers = ABC 005620 **O** OR372111 **P** OR372112, OR391730 **Q** OR391729 **R** Anderson Spring, Bell County, TX MN974229. Paravouchers = USNM 1571284. Locality information in Suppl. material 1. Scale bar: 1 mm.

Link: https://doi.org/10.3897/subtbiol.47.113186.suppl3

Supplementary material 4

Photo-vouchers of individuals sampled for DNA

Authors: Kathryn E. Perez

Data type: tif

Explanation note: All localities are in Texas. *Balconorbis uvaldensis*, Uvalde Fish Hatchery, Uvalde County **A** OR372125; Sycamore Creek at HWY 277, Uvalde County **B** OR372126 **C** OR372127 **D** OR372128 **E** OR372129; *Cochliopina riograndensis*, Lake Amistad National Recreation Area, small spring 25 m W of Indian Springs, Val Verde County **F** OR372132; *Phreatodrobia imitata*, Aldridge Well, Bexar County **G** OR372130; *Phreatodrobia micra*, San Marcos River at Scull Road Crossing, Hays County **H** OR372134 **I** OR372135, OR391744; *Phreatodrobia nugax*, Little Hunt Spring, Travis County **J** OR372107 **K** OR372108 **L** OR372109 **M** OR372110, Paravouchers = TMM 12388; *Phreatodrobia rotunda*, Comal Springs, Comal County **N** OR372113 **O** OR372118; *Phreatodrobia spica*, Garden Ridge Well, Comal County **P** OR372133; *Texapyrgus longleyi*, Lake Amistad National Recreation Area, small spring 25 m W of Indian Springs, Val Verde County **Q** OR372115. Locality information in Suppl. material 1. Scale bar: 0.5 mm.

Link: https://doi.org/10.3897/subtbiol.47.113186.suppl4