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DNA barcoding of springsnails (Mollusca, Gastropoda, Caenogastropoda) endemic to the Trans-Pecos region of Texas (USA)

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Abstract. In desert environments, unique communities depend on groundwater from springs, including a diverse radiation of small (<5 mm) snails found in the desert across the southwestern United States. Nearly all springsnail species are considered critically imperiled with their existence depending on maintenance of spring-flows in regions of declining water availability. Extant, endemic springsnails in the Trans-Pecos region of Texas include one species of *Pseudotryonia* Hershler, 2001, five nominal *Tryonia* W. Stimpson, 1865 (Cochliopidae) and seven *Pyrgulopsis* Call & Pilsbry, 1886 (Hydrobiidae). Four of these are classified as endangered under the US Endangered Species Act. Surveys for springsnails were conducted at 128 sites, including 13 sites that were previously reported localities, and 115 previously unsampled spring sites were also searched for new springsnail populations. Sequences of the DNA barcoding region were used to establish a database of known sequences from the named species and confirm identifications of new populations encountered. We report eight new springsnail populations, including new records for *T. metcalfi* Hershler, Liu & Landye, 2011, *T. cheatumi* (Pilsbry, 1935), *P. ignota* Hershler, Liu & Lang, 2010, *P. metcalfi* (D.W. Taylor, 1987), and *P. texana* (Pilsbry, 1935). We were not able to recollect *Juturnia brunei* (D.W. Taylor, 1987), *T. oasiensis* Hershler, Liu & Landye, 2011, or *P. davisi* (D.W. Taylor, 1987). The DNA barcoding gap for *Tryonia* ranged from 1.56–4.47% and for *Pyrgulopsis* from 0.68–1.68%.

Keywords. DNA barcoding, ciénegas, Cochliopidae, conservation, groundwater, Hydrobiidae, narrow-range endemic

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Introduction

Freshwater is an increasingly scarce resource for human and animal communities (Boretti and Rosa 2019), and springs are experiencing increasing anthropogenic use of water, heightened by the effects of climate change (Seager et al. 2007; Taylor et al. 2013). Spring systems, particularly those in arid environments, are isolated patches of unique habitat with endemic diversity, including freshwater snails. Springsnails—e.g., *Pyrgulopsis* Call & Pilsbry, 1886, *Tryonia* W. Stimpson, 1865, and *Pseudotryonia* Hershler, 2001—are tiny (1–5 mm) aquatic snails found in groundwater-dependent spring habitats (Brown et al. 2008). Springsnails are highly abundant (e.g., >10,000/m^2) in their preferred habitat and graze on periphyton that grows on rocks, vegetation, and other aquatic substrates (Mladenka and Minshall 2001). The ecology of most species of springsnails is poorly known, but their abundance in many springs suggests they have important roles in these ecosystems. Many species have restricted distributions (i.e., a single-site or micro endemics) and occur in sensitive communities vulnerable to surface disturbance and declines in water quality and quantity (Hutchins 2018). Groundwater-dependent taxa in arid regions, including springsnails, face a suite of well-documented threats that has led to their prevalence as conservation targets (Johnson et al. 2013; Hershler et al. 2014).

Springsnails are difficult to identify using shell morphology due to their relatively "plain," unornamented shells. Additionally, some species display marked sexual dimorphism of shell size and shape (Hershler 2001). As a result, springsnails from previously undocumented localities can be impossible to identify using shell morphology, while dissection and characterization of penial anatomy of a 3 mm snail is outside the expertise of most researchers (Liu et al. 2018). Therefore, we developed a DNA barcoding library to allow rapid identification of springsnails in Texas. DNA barcoding in most animals relies on a short (~600 base pair) sequence of the cytochrome c oxidase subunit 1 (CO1) gene to provide a practical, species-level identification tool (Hebert et al. 2003a, 2003b). This method relies on a "barcoding gap" to recognize species-level distinctions. A frequently used standard is that interspecific variation is 10 times the mean intraspecific variation; however, a best practice is to estimate it independently in the group under study. The broad utility of DNA barcodes has been shown across plant and animal groups including snails (Grant and Linse 2009; Park et al. 2011; Siddall et al. 2012; Perez et al. 2014). While the limitations of barcoding and other DNA-based taxonomic methods are well known (Prendini 2005; Packer et al. 2009), this approach is recognized as a useful method for identifying species-level groups. Development of genomic identification resources can assist in understanding and conserving unique biodiversity.

We update the ranges and taxonomic status of the epigean springsnails endemic to Texas in the genera *Pseudotryonia*, *Tryonia*, and *Pyrgulopsis*. A few species were not reviewed because they are not endemic to Texas. For example, the range of *Cochliopina riograndensis* (Pilsbry & Ferriss, 1906) includes northern Mexico and *Assiminea pecos* D.W. Taylor, 1987 is also found in New Mexico. *Juturnia brunei* (D.W. Taylor, 1987) was not reviewed because we were unable to collect live snails or even dead shells from its reported habitat, and the spring run it reportedly occupied is now dry. Finally, "*Tryonia*" *diaboli* (Pilsbry & Ferriss, 1906) was not reviewed, as it is most likely not a member of the epigean springsnail fauna and requires generic reassignment (Diaz et al. 2020).

Due to their low dispersal ability and habitat requirements, springsnails primarily occupy headwater reaches of perennial spring systems (Hershler et al. 1999). In addition, *Pseudotryonia* and *Tryonia* primarily occupy thermal or highly mineralized springs (Hershler 2001). Sampling sites for this study are all in the Trans-Pecos region of west Texas, USA. We sampled 128 localities, including type localities of previously described taxa and a subset from a large list of previously unsampled springs that we generated using literature (Brune 2002), museum localities of spring fauna, and examination of imagery in Google Earth. From the list of springs, we pursued access on both public and private lands. Many sites were inaccessible for sampling due to the wishes of private landowners. In addition to the type localities, we sampled 115 springs that (to our knowledge) had not previously been surveyed for springsnails (Fig. 1).

Study Area

The Trans-Pecos is the portion of Texas lying west of the Pecos River and is fully contained in the larger Chihuahuan Desert ecoregion. It is largely characterized by sparse seasonal precipitation, diverse geology, and an increasing and unsustainable modern demand on regional groundwater resources. Mean annual precipitation (20–50 cm/y) decreases from east to west but is also highly dependent on elevation, with the Davis Mountains receiving the highest amounts and low elevations and far western Texas receiving the lowest amounts (PRISM 2023). Temperatures vary widely across elevation and season (PRISM 2023), from exceptionally high in summer at low elevations, to below freezing at higher elevations during the winter. Despite the aridity, the number and diversity of springs in the region is surprisingly high and reflects a high diversity in the types and sizes of groundwater systems. These range from relatively small, high-elevation, sky-island, fractured rock gravity-fed groundwater systems, to low-elevation, fault-controlled, deep aquifers with flow paths rising under artesian pressure (Nicot et al. 2022). Spring size varies widely across the region and depends on precipitation, aquifer size, and anthropogenic pressures. Some perennial springs are barely a trickle and support only a few square meters of ciénega vegetation, while others were once some of the largest springs in Texas and supported tremendous spring-fed wetland and riparian ecosystems before flows decreased or ceased due to groundwater extraction (Land and Veni 2018; Mace et al. 2020). In terms of their importance as springsnail habitats, the most important factor seems to be flow-permanence, which is not always associated with spring size. Relative to the rest of Texas, the region has a large amount of public land, including Big Bend National Park, Big Bend Ranch State Park, several State Wildlife Management and State Natural areas, and

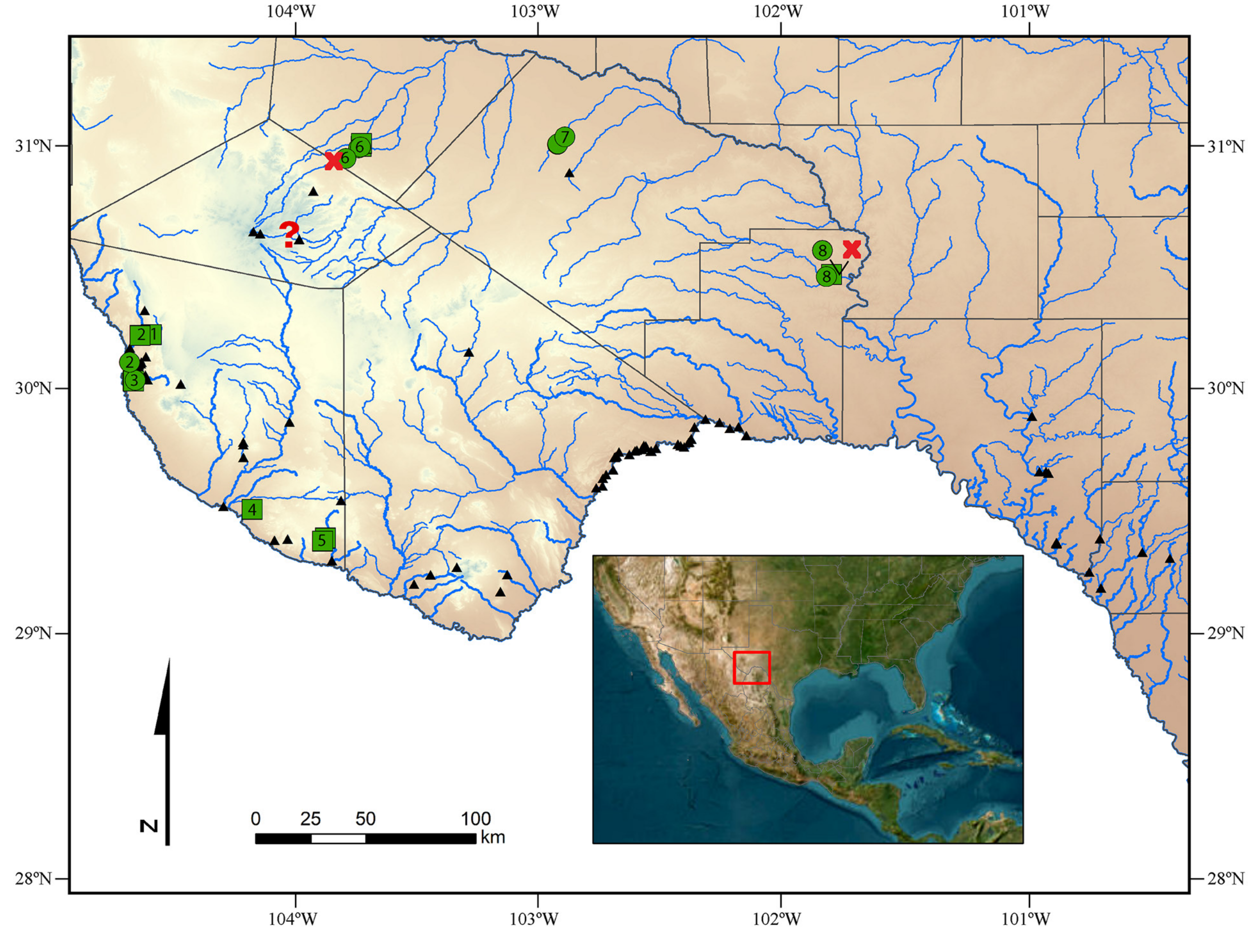

Figure 1. Sample locations for Trans-Pecos springsnails. Blue circles and squares = previously known (circles) and new (squares) locations where snails were successfully collected for molecular analysis. Small black triangles = sampled locations where snails were not detected and not historically known. Xs are sites sampled sites with extirpated taxa (western site = *Tryonia cheatumi* and *Pyrgulopsis texana* at Phantom Lake Spring, eastern site = *Tryonia oasiensis* at Caroline Springs). Question mark = historic location for *Pyrgulopsis davisi* that could not be visited because we were denied access. 1 = *Pyrgulopsis harrymilleri*, 2 = *Tryonia metcalfi*, 3 = *Pyrgulopsis metcalfi*, 4 = *Pyrgulopsis rubra*, 5 = *Pyrgulopsis madridensis*, 6 = *Tryonia cheatumi* + *Pyrgulopsis texana*, 7 = *Pseudotryonia adamantina* + *Tryonia circumstriata*, 8 = *Pyrgulopsis ignota*.

other smaller tracts of state, county, or municipal lands; many of these include springs and other groundwater-dependent ecosystems.

Methods

We used some or all of the following sampling methods, depending on site conditions such as water depth, flow, and sediment type: drift net sampling over spring openings (1–2 days), dip net sampling, Surber sampling (3× per site), Bou-Rouch sampling (3× per site), and searching by eye and hand-collecting snails. We found snails primarily by hand-collection, but at a few sites where the snails occurred in low abundances (Diamond Y Spring, Karges Spring, Naegele Spring, Capote Creek Crossing), they were only found by dip net or Surber sampling. Where they were visible, we hand-collected up to 60 individuals per site for DNA barcoding and species characterization. Voucher specimens for new and resampled sites were placed into either the Academy of Natural Sciences at Drexel University (ANSP) in Philadelphia, Pennsylvania or the Edwards Aquifer Research and Data Center, Aquatic Biodiversity Collections (ABC) in San Marcos, Texas. Some occurrence records were obtained from the National Museum of Natural History, Smithsonian Institution (USNM), Washington, D.C. For two occurrences, *T. cheatumi* (Pilsbry, 1935) from West Sandia Springs and *T. metcalfi* Hershler, Liu & Landye, 2011 from Capote Creek Crossing, the only available voucher materials were used for DNA work and museum vouchers are not available; these are indicated with collection numbers (FN) from the author's (KEP) collection. Collections were conducted under TPWD permit #SPR-0116-011 and USFWS permits #TE802211-2 and #TE802211-0.

Bulk samples were immediately preserved in 95% ethanol in the field, then ethanol was replaced after 24 h. Snails intended for DNA and taxonomic work were kept alive in cool spring water until processing the same day. Individuals for DNA work were killed by flash-boiling (Fukuda et al. 2008), followed by preservation in 95% ethanol. Individuals for morphological study were relaxed in mentholated water, then preserved in 70% ethanol. DNA was extracted using Qiagen DNAEasy Blood & Tissue Kit, followed by PCR using one of two primers for amplification of COI, the universal DNA barcoding primers (Folmer et al. 1994) or a derivative designed for springsnails (Liu et al. 2001). PCR was conducted using the Platinum SuperFi PCR Master Mix (Invitrogen) with the manufacturer's protocols for concentrations and temperature profiles, but annealing at 51 °C. Samples were cleaned using the Qiagen QIAquick PCR Purification Kit, quantified using a Qubit (Invitrogen), and sequenced by Eton BioScience, Inc.

Contigs were made and sequences were aligned using Geneious 10.2.6 (Biomatters). All available sequences from GenBank appearing in previous literature were included with new sequences generated during this study. GenBank sequences must be used with caution as misidentifications and outdated metadata are typical. Therefore, the GenBank sequences that were included are only those from the taxonomic literature that described these species, or from topotypic individuals. For type localities where we were not able to sample and sequence fresh materials, GenBank sequences were used to confirm species identity. For *Tryonia* the analysis was conducted with the Texas *Pseudotryonia* included, as these genera are closely related. Previous analyses have placed *Pyrgulopsis texana* (Pilsbry, 1935) distant from the rest of the Texas *Pyrgulopsis* and, therefore, that species was used to root the tree for analysis (Perez 2021). Sequences were aligned in Geneious R10 using the MUSCLE alignment algorithm (Edgar 2004). Alignments were deposited in Dryad (https://doi.org/10.5061/dryad.t76hdr85h). Phylogenetic analyses were conducted in IQTREE 1.6.12 (Minh et al. 2013; Nguyen et al. 2015; Hoang et al. 2018). Pairwise differences were calculated in MEGA 11 (Tamura et al. 2013) using the Kimura two-parameter model (Kimura 1980). The K2P model is the standard for DNA barcode studies, where distances are assumed to be relatively low (Hebert et al. 2003). To look for a barcoding gap, we compared the within- and among-species K2P distances within each genus.

Results

Springsnails were observed at 22 out of the 128 sites sampled (Supplemental Table S1). The COI alignment for *Tryonia* included 48 unique sequences: three identical sequences of individuals from the same population were removed from the analysis at this step, two from *Tryonia metcalfi*, and one from *Tryonia circumstriata* (A.B. Leonard & Ho, 1960). The COI alignment for *Pyrgulopsis* included 46 unique sequences: three identical sequences of individuals from the same population were removed from the analysis at this step, one each from *P. texana*, *P. rubra* Perez, 2021, and *P. madridensis* Perez, 2022.

Model testing, maximum-likelihood analysis, and 1000 bootstrap replicates were conducted using iqtree 1.6.12 (Thomas 1988). The *Tryonia* alignment contained 672 bases, and 80 were parsimony informative. The best fit model of evolution was HKY+F+I chosen according to the Bayesian information criterion. Base frequencies were: A: 0.244, C: 0.177, G: 0.188, T: 0.390. The highest likelihood tree was −1722.578. The *Pyrgulopsis* alignment contained 658 bases; 115 were parsimony informative. The best fit model of evolution was TPM2+F+G4 chosen according to the Bayesian information criterion. Base frequencies were: A: 0.252 C: 0.197 G: 0.184 T: 0.367. The highest likelihood tree was −1691.1518.

In both *Tryonia* (Fig. 2) and *Pyrgulopsis* (Fig. 3), the described species form well-supported clades, distinct from other nominal species. The average pairwise difference within species of *Tryonia* was 0.0063 (0.000–0.0156) with a value of 0.0156 between the two populations of *T. cheatumi*. Average pairwise difference among *Tryonia* species was 0.0686 (0.0447–0.0835). The "barcode gap" between intra and inter specific difference was between 0.0156 and 0.0447 for the *Tryonia* species examined. The average pairwise difference within species of *Pyrgulopsis* was 0.0009 (0.000–0.0068). Average pairwise difference among *Pyrgulopsis* species was 0.0937 (0.0168–0.1228). The "barcode gap" between intra- and interspecific difference was between 0.0068 and 0.0168 for the *Pyrgulopsis* species examined.

Family Cochliopidae
Genus *Pseudotryonia* Hershler, 2001

Pseudotryonia adamantina **(Taylor, 1987)**
Figures 2, 4A

Materials examined. USA – **Texas** • Pecos County, Diamond Y Spring (Main Spring); 31.0014°N, −102.9239°W; 19.XI.1995; Hershler R, Landye JJ leg.; USNM 892020.

Identification. *Pseudotryonia* is diagnosed as having a weakly pigmented distal portion of the penis, a large stylet, and nearly straight penial duct, and two papillae, one inner and distal. The other outer and medial (Hershler 2001). The shell is expected to be conical, up to 3.8 mm tall, sometimes weakly sculptured with spiral lines.

Comments. *Pseudotryonia adamantina* and *Tryonia circumstriata* have each been reported from both the Diamond Y and Euphrasia spring systems (letter from Landye Aug. 15, 2000 to USFWS), which are adjacent and connected by the Leon Creek drainage when water levels are high. In a series of gray literature documents, they have been reported to occupy separate springs but with the identification of which snail species was present shifting among survey reports. It is not clear if this is due to sampling error, the species shifting their occupancy, or misidentification. *Tryonia circumstriata* is reported to have an obvious pattern of sculpture on the shell (striations) that should distinguish the species. However, we dissected and sequenced completely smooth individuals that were identified by both methods as *T. circumstriata*, demonstrating how the supposedly diagnostic sculpture feature could have misled past identification. Currently these species cannot be reliably distinguished without examination of penial

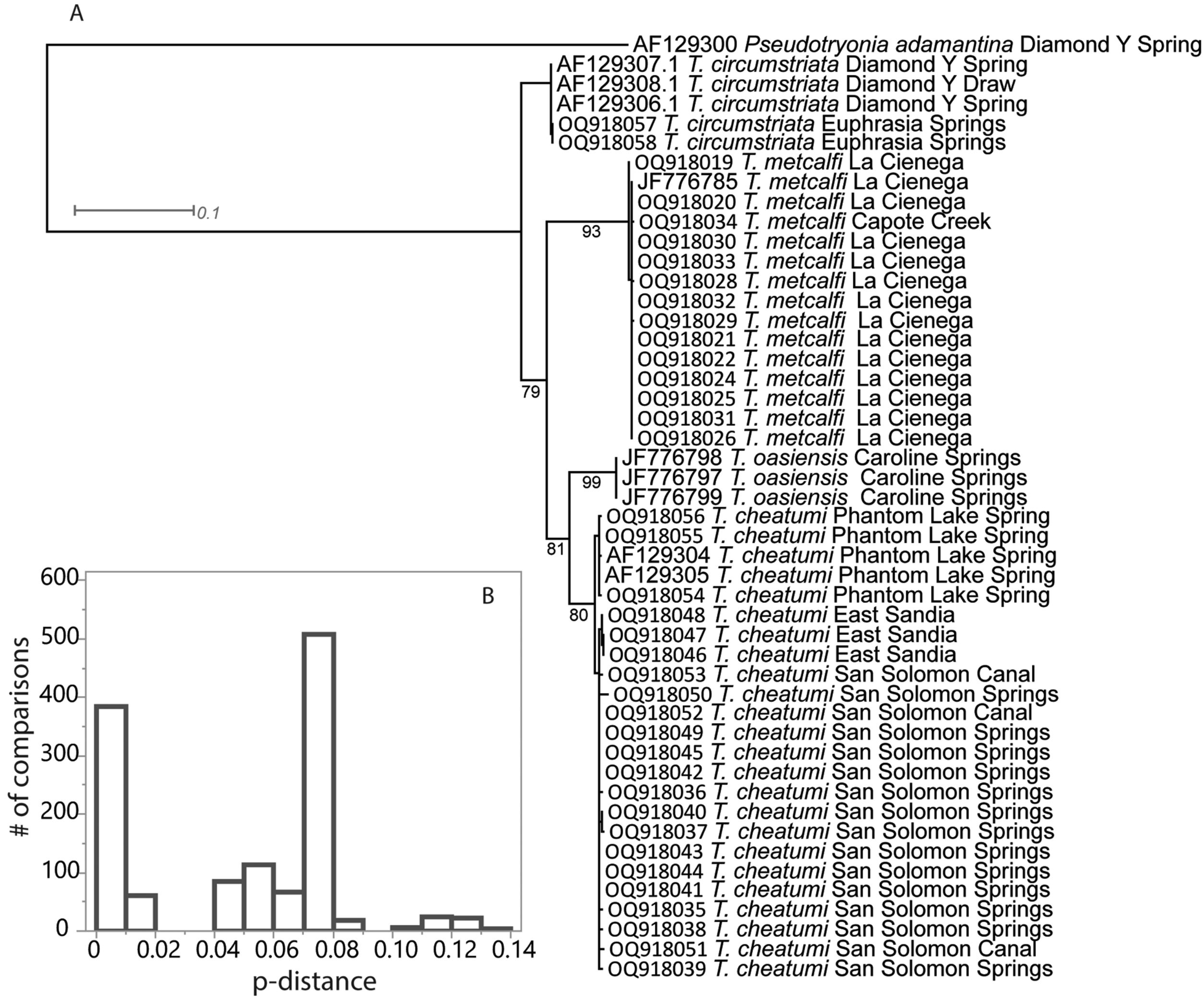

Figure 2. COI DNA barcoding results for *Tryonia* and *Pseudotryonia*. **A.** Tree with highest likelihood. **B.** Histogram of pairwise identity between individuals in the tree. Barcoding gap from 1.56–4.47%. Bootstrap values >70% shown below branches.

anatomy or DNA sequencing as both can appear to have similar, smooth shells. We report here only the site listed in the taxonomic literature, as our samples from Euphrasia springs and Diamond Y springs only found *T. circumstriata* and could not confirm the presence of *Pseudotryonia adamantina*. A concerted effort to document the occurrence of this federally endangered species is recommended.

Genus *Tryonia* W. Stimpson, 1865

***Tryonia cheatumi* (Pilsbry, 1935)**
Figures 2, 4B

Materials examined. USA – **Texas** • Reeves County, San Solomon Springs; 30.9441°N, –103.7885°W; 02.X.2020; Perez KE, Glover H, Chastain, R leg.; 4 spec., ABC 005605 • East Sandia Spring; 30.9909°N, –103.7291°W; 01.III.2017; Noreika N leg.; 5 spec., ABC 005606 • West Sandia Spring; 30.9868°N, –103.7364°W; FN 2043 • East Sandia Springs Road Crossing 1; 30.9924°N, –103.7266°W; 01.X.2020; Perez KE, Glover H leg.; ABC 005612 • East Sandia Springs Road Crossing 2; 31.0056°N, –103.7246°W; 02.X.2020; Perez KE, Glover H; ABC 005613. – Jeff Davis County • Phantom Lake Spring; 30.9350°N, –103.8496°W; 19.VI.2019; Perez KE, Diaz P leg.; 7 spec., ABC 005609.

Identification. *Tryonia cheatumi* is diagnosed by two distal papillae along the inner edge, and no basal papillae (Hershler 2001). The shell is expected to be conical, up to 4.2 mm tall, sometimes weakly sculptured with spiral threads.

Comments. Individuals sampled from Phantom Lake Spring formed a separate clade from the samples found in the other localities. These differed by 1.56% in the COI barcoding region, which is within the expected range of different populations within a single species. The Phantom Lake Spring population has a reproductive season that is distinct from the other localities (Perez et al. 2022b). In this region of declining groundwater levels and spring flows (Nunu and Green 2021), the spring flow supporting the population at Phantom Lake Spring had declined in May 2001 to the point of requiring a pump to maintain water in a small ciénega at the site (Ridgeway et al. 2004). In early 2023, the pump maintaining the ciénega failed and was not repaired and there are recent reports that the ciénega is completely desiccated; we assume this population is now extirpated.

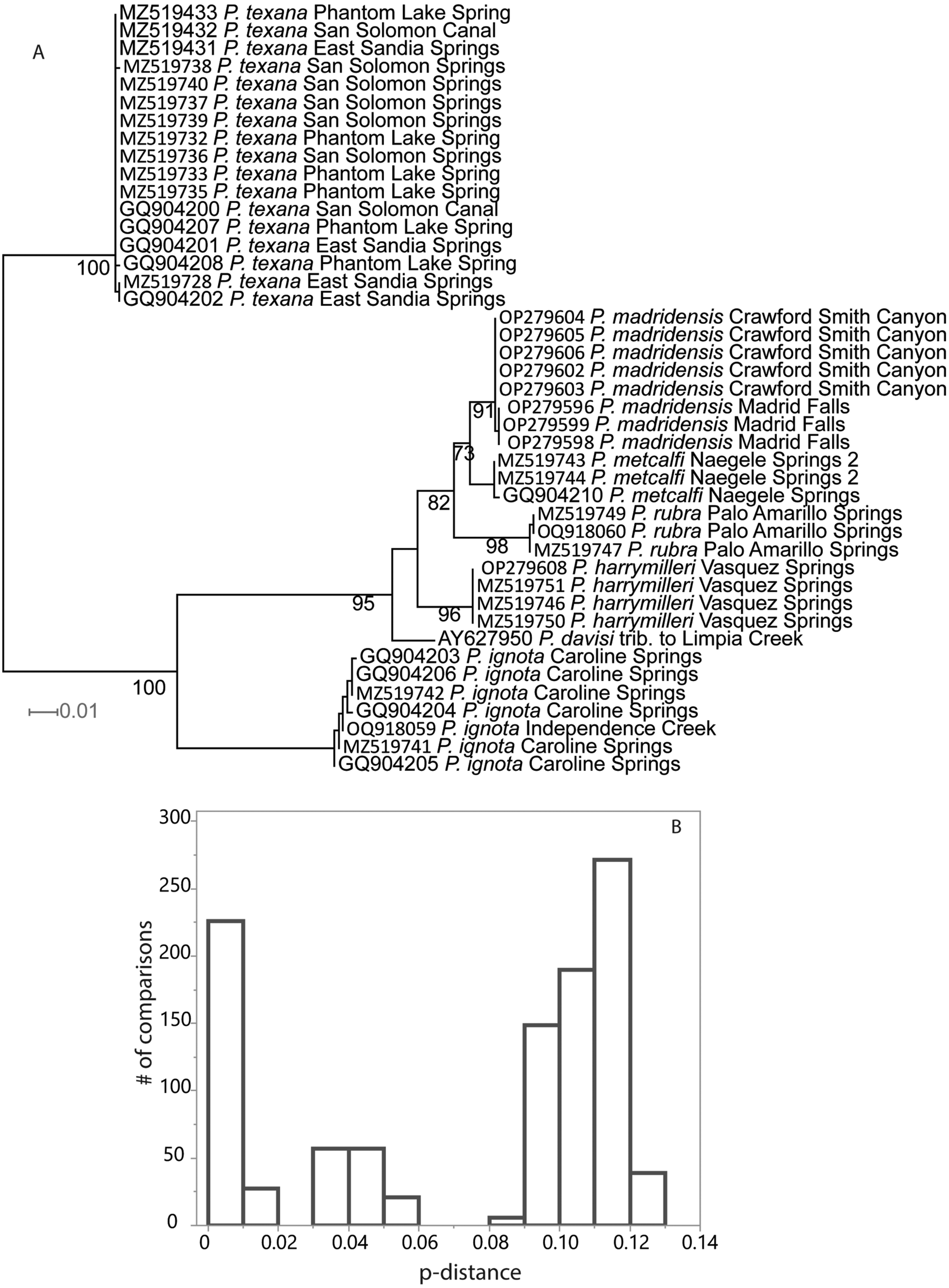

Figure 3. COI DNA barcoding results for *Pyrgulopsis*. **A.** Tree with highest likelihood. **B.** Histogram of pairwise identity between individuals in the tree. Barcoding gap from 0.68–1.68%. Bootstrap values >70% shown below branches.

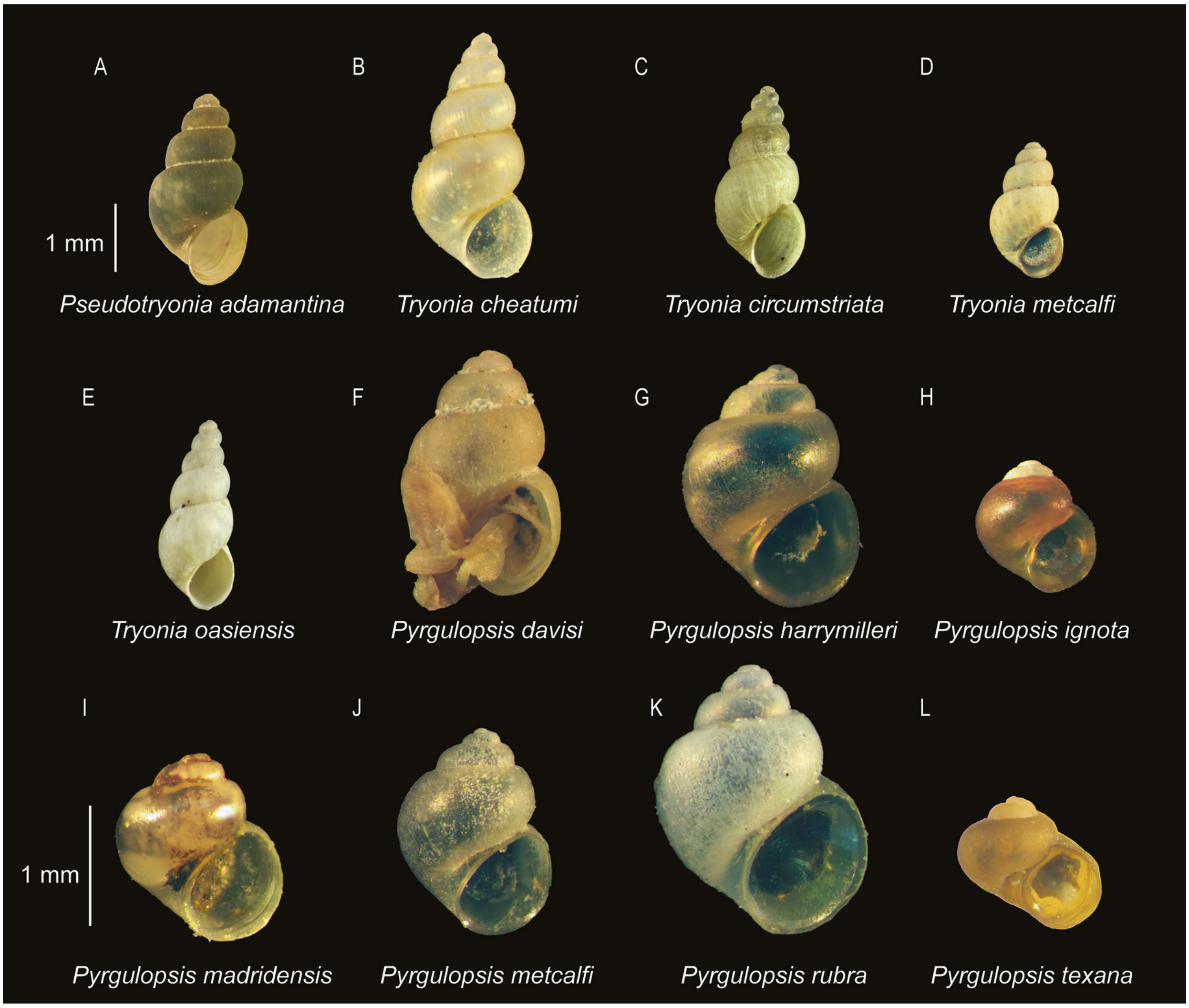

Figure 4. Species of Texas *Pseudotryonia*, *Tryonia*, and *Pyrgulopsis*. Shells of *Pseudotryonia* and *Tryonia* are set to the same relative scale with the scale bar on the upper row. Shells of *Pyrgulopsis* are set to the same relative scale with the scale bar on the lower row. Shells figured measure as follows: **A** = 2.82 mm, **B** = 3.90 mm, **C** = 3.10 mm, **D** = 2.11 mm, **E** = 3.11 mm, **F** = 2.35 mm, **G** = 2.38 mm, **H** = 1.12 mm, **I** = 1.35 mm, **J** = 1.91 mm, **K** = 2. 25 mm, **L** = 1.23 mm.

Two notable new records of *T. cheatumi* are from the road crossings downstream of East Sandia Springs. These sites are 0.5 km and ~1.5 km downstream from the groundwater source and highly disturbed (road with vehicle traffic passing through the stream channel, and under a multi-lane highway), but we found live individuals of *T. cheatumi* at both road crossings.

Tryonia circumstriata **(Leonard & Ho, 1960)**
Figures 2, 4C

Materials examined. USA – **Texas** • Pecos County, Euphrasia Spring; 31.0323°N, −102.8942°W; 01.X.2020; Perez KE, Glover H leg.; 3 spec., ABC-005610 • Diamond Y Spring (Main Spring); 31.0014°N, −102.9239°W; 16.VI.2000; Landye JJ leg.; USNM 1003871 • Diamond Y Spring (Karges Spring); 31.0034°N, −102.9227°W; 27.X.1984; Taylor DW leg.; USNM 854092.

Identification. *Tryonia circumstriata* is diagnosed by having a blunt, pigmented terminal portion of the penis, with pronounced swelling along inner edge, and an undulating penial duct. The shell is expected to be conical, up to 5.5 mm tall, smooth or sculptured with spiral lirae, sometimes also with collabral ribs (Hershler 2001).

Comments. This species has been previously reported from both Diamond Y and Euphrasia springs (see discussion above for *Pseudotryonia adamantina*). Compared with historical descriptions, water levels were very low at Diamond Y spring and draw (many previously wet sites were desiccated) and we only collected a few springsnails in dip net samples, However, we found springsnails in large numbers at Euphrasia springs in the headwater pool and spring run where both striated and completely smooth forms appeared to be *P. adamantina*. However, dissections and DNA sequences confirm the identity of *T. circumstriata* in both spring systems.

Tryonia metcalfi **Hershler, Liu & Landye, 2011**
Figures 2, 4D

Materials examined. USA – **Texas** • Presidio County, La Cienega; 30.1108°N, −104.6727°W; 06.III.2021; Perez KE, Glover H, Chastain R leg.; 6 spec., ABC 005604 •

Capote Creek Crossing; 30.2200°N, –104.6300°W; 07.III.2021; Perez KE, Glover H leg.; FN 1786.

Identification. *Tryonia metcalfi* is diagnosed as having a large penial stylet, two distal and two basal papillae along the inner edge of the penis (Hershler et al. 2011). The shell is expected to be ovate-conic, up to 2.6 mm tall, sculptured with strong growth lines.

Comments. *Tryonia metcalfi* was previously only known from the type locality: a small series of seeps that drain toward (but do not reach) the Rio Grande River. In this study, we report this species from one additional site, 13 km distant and, notably, in Capote Creek, which is a different drainage that does flow directly into the Rio Grande under episodic wet conditions.

***Tryonia oasiensis* Hershler, Liu & Landye, 2011**
Figures 2, 4E

Materials examined. USA – **Texas** • Terrell County, Caroline Springs, second pool of raceway; 30.4670°N, –101.8009°W; 07.12.2020; Perez KE, Glover H, Chastain R, Ortega C leg.; ABC 005611.

Identification. *Tryonia oasiensis* is diagnosed as having two distal papillae on the inner edge of the penis and a basal papilla on the outer edge (Hershler et al. 2011). The shell is expected to be narrowly conic, up to 3.4 mm tall, sculptured with strong growth lines and sometimes spiral threads.

Comments. This species was described based on a very small number of living individuals collected at Caroline Springs in 2009. The species was not found during two subsequent visits by the original author in 2011 (Hershler et al. 2011). In 2020 and 2021 we searched for *T. oasiensis* in the precise type locality described at Caroline Springs, including a full week (2021) of intensive sampling using multiple methods. Despite these efforts, we did not collect live individuals, although we did find numerous aged shells in the sediments.

Family Hydrobiidae
Genus *Pyrgulopsis* Call & Pilsbry, 1886

***Pyrgulopsis davisi* (D.W. Taylor, 1987)**
Figures 3, 4F

Materials examined. USA – **Texas** • Jeff Davis County, tributary of Limpia Creek about 5 mi. (8 km) northeast of Fort Davis; 30.6358°N, –104.1383°W; 15.V.1990; Hershler R leg.; USNM 873427.

Identification. *Pyrgulopsis davisi* is diagnosed as having a robust penis with a medium length penial filament and lobe, similar in length. The penis is heavily ornamented with an elongate, proximally bifurcate penial gland as well as three dorsal penial glands, a transverse terminal, and a ventral gland (Hershler 1994). The shell is expected to be ovate to narrowly conic, up to 3.9 mm tall, sculptured with moderate growth lines.

Comments. We could not confirm the presence of this species because we were unable to gain permission from private landowners to access the type locality or previously collected localities. We include it here using an available sequence from GenBank. We surveyed several permanent springs in the vicinity but did not find any species of *Pyrgulopsis*.

***Pyrgulopsis harrymilleri* Perez, 2021**
Figures 3, 4G

Materials examined. USA – **Texas** • Presidio County, Vasquez Springs; 30.2241°N, –104.5840°W; 08.V.2021; Schwartz B, Hutchins B, Pustka L leg.; ANSP A483286.

Identification. *Pyrgulopsis harrymilleri* is diagnosed as having a robust penis with a medium-length penial filament and lobe, with the filament slightly longer. The penis is heavily ornamented with an elongate, proximally bifurcate penial gland as well as three dorsal penial glands, a ventrally positioned terminal gland, and ventral gland that is basally located (Hershler 1994). The shell is expected to be globose to low-conic, up to 2.4 mm tall, sculptured with simple, distinct growth lines.

Comments. This species is known only from the type locality, which is a tiny, exposed spring on the steep western slopes of the southern Sierra Vieja Mountains. Habitat is a narrow, high-gradient spring run, 10–30 cm wide and 0.5–3 cm deep when we sampled it at and near the source. Two adjacent seeps did not have snails and the species was not detected at any other springs in the region. Habitat is moderately impacted by trampling and grazing, likely from non-native Aoudad (*Ammotragus lervia* (Pallas, 1777)).

***Pyrgulopsis ignota* Hershler, Liu & Lang, 2010**
Figures 3, 4H

Materials examined. USA – **Texas** • Terrell County, Caroline Spring, 2nd pool of raceway and (small spring near the main opening by concrete pavilion); 30.4670°N, –101.8009°W; 30.IIV.2009; Hershler R, Landye JJ leg.; USNM 1123757 • Independence Creek Highway crossing; 30.4578°N, –101.8285°W; 07.XII.2020; Perez KE, Glover H, Chastain R leg.; 8 spec., ABC 005601.

Identification. *Pyrgulopsis ignota* is diagnosed as having a gracile penis with a short penial filament. The ornamentation is limited to a large glandular pad on the dorsal surface of the penis (Hershler 1994). The shell is expected to be rounded, globose to trochoid, up to 1.5 mm tall, spire often eroded, sculptured with growth lines and weak spiral lines on later whorls.

Comments. *Pyrgulopsis ignota* is found in large numbers in the "raceway", which is the restored spring run downstream of the large concrete pool at Caroline Spring. A small population also occurs in a small spring near the main Caroline Spring near the concrete pavilion. Finally, we also found a new population of this species in Independence Creek, 7 km downstream from the springs. The individuals in Independence Creek did not have eroded spires and appeared more conical in shape, less globose. They were confirmed to be *P. ignota* by DNA sequencing.

***Pyrgulopsis madridensis* Perez, 2022**
Figures 3, 4I

Materials examined. USA – **Texas** • Presidio County, Madrid Falls Big Bend Ranch State Park; 29.3798°N, −103.8841°W; 06.X.2021; Schwartz B, Hutchins B, Pustka L leg.; ANSP A492826 • South Fork Crawford Smith Canyon Big Bend Ranch State Park; 29.3921°N, −103.8724°W; 07.X.2021; Schwartz B, Perez KE, Pustka L leg.; ANSP A492830.

Identification. *Pyrgulopsis madridensis* is diagnosed as having a robust penis with a medium-length penial filament and lobe, with the filament longer. The penis is heavily ornamented with an elongate penial gland as well as numerous dorsal penial glands, a terminal gland, and ventral gland that is basally located (Hershler 1994). The shell is expected to be low conical, up to 2.14 mm tall, sculptured with distinct growth lines.

Comments. This species is known from the type locality (Madrid Falls), which is a small spring-fed creek in a deep canyon on the southeast side of Big Bend Ranch State Park, and a second population in the southern fork of the nearby Crawford Smith Spring. Both springs are remote, rarely visited, and habitats are in good condition. *Pyrgulopsis madridensis* was found in high densities at both sites. Several other springs in the region were surveyed but none were found to have snails (Perez et al. 2022a).

***Pyrgulopsis metcalfi* (D.W. Taylor, 1987)**
Figures 3, 4J

Materials examined. USA – **Texas** • Presidio County, Naegele Springs #2; 07.V.2021; Perez KE, Schwartz B, Hutchins B, Glover H leg.; ANSP A483386 • Naegele Springs; 10.IIV.1989; Landye JJ leg.; USNM 873301.

Identification. *Pyrgulopsis metcalfi* is diagnosed as having a robust penis with a medium-length penial filament and lobe, with the filament slightly longer. The penis is heavily ornamented with an elongate, proximally bifurcate penial gland as well as three dorsal penial glands, a terminal gland, and ventral gland (Hershler 1994). The shell is expected to be globose to low-conic, up to 2.4 mm tall, sculptured with simple, distinct growth lines.

Comments. *Pyrgulopsis metcalfi* was described as occurring in numerous small springs that flowed from a rise, which together were called Naegele Springs. These springs have been modified to form an upper and lower pool, diverting the natural rivulets flowing from the hill. This species occurs in very low numbers in the concrete pools where they were not observed during hand-searches, but a few were collected in dip-net samples. Upon searching downstream (a dry arroyo when we visited) from the springs, we discovered an additional, nearby population of *P. metcalfi* at the confluence of an unnamed spring run (termed Naegele Springs #2 in our samples) and Naegele Creek. Other nearby springs where the snail could occur are on private land and appear to be heavily modified.

***Pyrgulopsis rubra* Perez, 2021**
Figures 3, 4K

Materials examined. USA – **Texas** • Presidio County, Palo Amarillo Springs, Big Bend Ranch State Park; 29.5093°N, −104.1716°W; 09.V.2021; Perez KE, Schwartz B, Glover H leg.; ANSP A483288.

Identification. *Pyrgulopsis rubra* is diagnosed as having a robust penis with a medium-length penial filament and lobe, with the filament longer. The penis is heavily ornamented with an elongate, penial gland as well as several dorsal penial glands, a subterminal gland that wraps from dorsal to ventral sides and ventral gland that is near the middle of the penis, similar to *P. metcalfi* (Hershler 1994). The shell is expected to be ovate conic, up to 2.5 mm tall, sculptured with strong growth lines.

Comments. *Pyrgulopsis rubra* has a tiny documented range with individuals found only in a single small, spring-fed cascade in Palo Amarillo Canyon.

***Pyrgulopsis texana* (Pilsbry, 1935)**
Figures 3, 4L

Materials examined. USA – **Texas** • Reeves County, San Solomon Springs; 30.9441°N, −103.7885°W; 02.X.2020; Perez KE, Glover H, Chastain R leg.; ANSP A483291 • East Sandia Spring; 30.9909°N, −103.7291°W • East Sandia Springs Road Crossing 1; 30.9924°N, −103.7266°W; 01.X.2020; Perez KE, Glover H leg.; ABC 005614 – Jeff Davis County, Phantom Lake Spring; 30.9348°N, −103.8497°W; 01.IIV.2017; Noreika N leg.; 7 spec., ABC 005602.

Identification. *Pyrgulopsis texana* is diagnosed as having a simple, medium-sized penis with a short, tapered penial filament and no penial lobe. There is no glandular ornamentation (Hershler et al. 2010). The shell is expected to be rounded, valvatiform to trochoid in shape, up to 2 mm tall, spire often eroded, sculptured with growth lines.

Comments. Individuals from East Sandia Springs had previously been found to have a distinct shell shape and number of gill filaments, but they are not genetically divergent from haplotypes found at all other locations (Hershler et al. 2010; this study). We assume the population at Phantom Lake Spring is now extirpated (for details see discussion of *T. cheatumi*).

Discussion

Many shells of the springsnails we studied are similar. For most, definitive identification requires dissection or DNA sequencing. Without sequences from topotypic specimens in a reference database, it is not possible to reliably identify most springsnail species or to distinguish unrecognized species. The largest contribution of this work is the production of a well-documented reference database of sequences submitted to GenBank, which provides the framework for reliable identification of springsnail populations in Texas. The DNA barcoding gap for *Tryonia* ranged from 1.56–4.47% and for *Pyrgulopsis* from 0.68–1.68%. While this is not a "genetic ruler," it can be used as a rough guideline for determining when newly encountered populations should be evaluated for specific status.

In this study, we collected and sequenced the DNA barcoding gene for most of the springsnail species found in Texas. However, in some cases we were unable to find the species in historical localities. In two cases (*Pyrgulopsis davisi* and *Tryonia oasiensis*) existing GenBank sequences were available from previous taxonomic work and were included in our study (Hershler et al. 2010, 2011). However, in one case, *Juturnia brunei* (D.W. Taylor, 1987), the species was described well before DNA barcoding was the norm. We were unable to locate *J. brunei* after searching at Phantom Lake Springs, the type locality. The sole water source the species relied on is no longer flowing and it is unlikely that the species will be found at the type locality in the future, making it likely extinct.

Most of the new records reported in this study are located relatively near previous records. For example, the new records for *T. cheatumi* and *P. texana* are in the downstream drainages of sites where they had been previously documented, road crossings of Sandia spring drainage; however, it is useful to note they are extant in these highly modified habitats. In the case of *T. metcalfi*, the new record, while only 13 km from the type locality, is particularly valuable as it is in a different drainage from the only previously known site (Hershler et al. 2011), adding redundancy to this species' distribution. The two new site records for *P. metcalfi* are quite near the known locality (Taylor 1987), expanding the range of this species by <2 km. However, since *P. metcalfi* has been reduced to low numbers at the type locality, any additional populations, even these heavily impacted ones, are important.

While we sampled 128 springs, we had at least twice that many potential sites in our database of springs, with most of those on private lands that we were unable to access. It is likely that additional springsnail populations and undescribed species remain to be discovered in the region. The discovery of new populations of *Tryonia* and *Pyrgulopsis* in the Big Bend region of Texas suggests that they are likely to also occur in the mountain ranges just to the south in Mexico, and that future collections there could add new species to each group. With increasing pressure on groundwater in this desert region, and springsnail species, particularly in *Tryonia* already extinct, increased protection for these species is necessary for their long-term survival.

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Author Contributions

Conceptualization: BH, BS, KP. Data curation: NSL, VS, BH, KP. Formal analysis: KP. Funding acquisition: KP, BS, BH. Investigation: NSL, KP, BS, BH, VS. Methodology: BS. Project administration: KP, BS. Supervision: KP. Visualization: VS, BH. Writing – original draft: KP, VS, NSL. Writing – review and editing: BH, BS, KP.

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Supplemental Data

Table S1. Table with 128 rows of all localities, with site name, county, coordinates, date, sampling method, springsnail species encountered, and their status reported in this article.