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MICROBIAL ASSOCIATIONS WITH METCALF'S TRYONIA, *TRYONIA METCALFI* (GASTROPODA: COCHLIOPIDAE), AN IMPERILED CIÉNEGA ENDEMIC

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Abstract—The Chihuahuan Desert swamps are a hotspot for imperiled organisms including freshwater springsnails and bacteria. Many of these taxa are endemic to the desert and to the individual waterbodies where they occur. Efforts to conserve diversity in these threatened areas must account for the life history of the organisms, but also interactions between organisms including microbes. We documented the microbial assemblage associated with *Tryonia metcalfi*, a critically imperiled freshwater snail endemic to a ciénega system in western Texas. We identified 14 bacterial families in our snail samples and determined a core assemblage of 19 bacterial taxa (4 of which represented novel lineages) that are likely dependent on the snail. Future conservation efforts involving *T. metcalfi* and its environment should therefore consider the microbial diversity associated with both the snail and the ciénegas.

Resumen—En las ciénegas del desierto de Chihuahua habitan muchos organismos en peligro, entre ellos caracoles de agua dulce y bacterias. Muchos de estos taxones son endémicos del desierto y de los específicos cuerpos de agua donde ocurren. Esfuerzos para conservar la diversidad en estas áreas amenazadas deben tomar en cuenta la historia de vida de los organismos junto con las interacciones entre ellos, incluyendo microbios. Documentamos el conjunto microbiano relacionado con *Tryonia metcalfi,* una especie de caracol de agua dulce en peligro de extinción, endémica a un sistema de ciénagas en el oeste de Texas. Identificamos 14 familias bacterianas en nuestras muestras de caracoles y determinamos un conjunto principal de 19 taxones bacterianos; cuatro representan nuevos linajes, que probablemente dependen del caracol. En el futuro, los esfuerzos para proteger a *T. metcalfi* y su ambiente en consecuencia deben considerarla diversidad microbiana asociada con esta especie de caracol y la ciénega.

The Chihuahuan Desert in the United States and Mexico is an imperiled freshwater diversity hotspot (Böhm et al., 2021). Waterbodies in the Chihuahuan Desert are often widely separated with poor hydrological connections between them, leading to unique assemblages within each waterbody (Sada et al., 2005; Murphy et al., 2015). This isolation may be a driving force in the speciation and endemism of freshwater groups (Tobler and Carson, 2010; Adams et al., 2018; Brown et al., 2020). Two groups that show high degrees of endemism in desert freshwater systems are springsnails and bacteria. Morphological and molecular studies on desert springsnails suggest that many species are limited to a handful of sites within regional spring systems; often, these species are endemic to single localities (Hershler et al., 2014; Czaja et al., 2020). Microbial diversity in desert waterbodies also shows high levels of endemism. For example, numerous endemic bacterial lineages have been described from the Cuatros Ciénegas basin of Mexico (Souza et al., 2006; Toribio et al., 2011).

Freshwater habitats and the species they support are highly imperiled in the Chihuahuan Desert. Urbaniza-

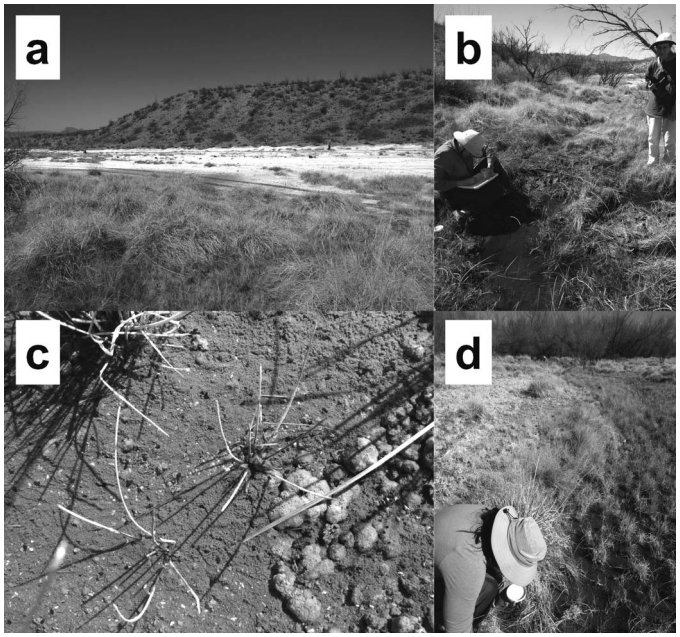

Fig. 1—Sites where *Tryonia metcalfi* are found: the type locality in Presidio County, Texas (a and b); and the collection site for this study in Presidio County (c and d).

tion, groundwater withdrawal, and other anthropogenic activities have led to habitat modification and loss (Garrett et al., 2021). Concurrent with these changes is declining biodiversity, with many endemic species and those with limited distributions becoming extirpated and extinct. In springsnails, Hershler et al. (2011) documented the extinction of multiple *Tryonia* species and populations presumably due to groundwater mining. Czaja et al. (2020) further noted that over 54% of imperiled freshwater gastropods across Mexico are springsnails, many of which occur in two conservation hotspots in the Chihuahuan Desert. Although the loss of microbial diversity in desert habitats is currently undocumented, freshwater bacteria likely face threats similar to other species where they co-occur (Souza et al., 2006; McFall-Ngai et al., 2013; Arocha-Garza et al. 2017).

Conservation efforts for desert freshwater endemics require integrated approaches that account for not only the species' specific environmental and life history needs but also those of taxa interacting with or living in and on the species (De la Maza-Benignos et al., 2014). This holistic approach may apply to both springsnails (Hershler et al., 2014) and bacteria (Trevelline et al., 2019) given the literature on snail–microbe interactions (e.g., O'Rorke et al., 2015; Huot et al., 2020). We sought an exemplar that encompassed endemism and conservation in the context of desert freshwater springsnails and snail–bacteria interactions. This led us to exploring the microbial diversity associated with the desert springsnail *Tryonia metcalfi*.

Metcalf's Tryonia, *T. metcalfi* (Cochliopidae), was described from a ciénega in Presidio County, Texas (Hershler et al., 2011). The species is small (2 mm shell length, 1 mm shell width) and can be distinguished from congeners by morphology of its shell and reproductive structures. The species' authors found *T. metcalfi* living in a small pool among mud and vegetation and on the undersides of rocks. They noted that livestock had heavily degraded the type locality environment. The species' distribution is restricted to a single ciénega system; it is listed as threatened in Texas (Texas Parks and Wildlife Department, https://tpwd.texas.gov/huntwild/wild/wildlife_diversity/nongame/tcap/sgcn.phtml) and carries G1 and S1 critically imperiled national and state designations respectively from NatureServe (NatureServe Explorer, https://explorer.natureserve.org).

We found *T. metcalfi* present in the type locality and in two nearby (within 0.5 km) small mineral, saline pools and runs in the La Ciénega system, Presidio County, Texas (Fig. 1). The La Ciénega system is a collection of permanent seeps and springs that are hydrologically disconnected from the Rio Grande River 1.8 km away under normal water conditions. The La Ciénega drainage has striking salt deposits, halophilic flora, and microbial mats throughout. We collected *T. metcalfi* for this study from a single pool in La Ciénega in March 2021. Water at the site was 25.2°C, pH 8.05, 4,740 µS/cm conductivity, and 76% oxygen saturation. We collected snails by hand and dip-net and stored them in 95% ethanol. We rinsed 12 snails under a stream of sterile water prior to using the DNeasy PowerSoil (Qiagen, Valencia, California) kit to extract microbial genomic DNA. Given their small size, we crushed each whole snail against the side of the tube using a sterile pipette tip prior to extraction and performed four extraction of three snails each. We sent the extracted DNA samples to Molecular Research LP (Shallowater, Texas) for amplification and sequencing of the near-complete 16S rRNA gene. Laboratory personnel used the 27F/1492R primer pair with barcode on the forward primer in a 35-cycle polymerase chain reaction (PCR; five cycles used on the PCR products) using the HotStar Taq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 90 s, after which a final elongation step at 72°C for 5 min was performed. After amplification, laboratory personnel checked PCR products on a 2% agarose gel to determine the success of amplification and the relative intensity of bands. They pooled samples together in equal proportions based on their molecular weight and DNA concentrations. Laboratory personnel then purified the PCR pool using Ampure PB beads (Pacific Biosciences, Menlo Park, California).

We prepared the SMRTbell libraries (Pacific Biosciences) following the manufacturer's user guide and performed sequencing at Molecular Research on a PacBio Sequel following the manufacturer's guidelines. After completion of initial DNA sequencing, each library

Table 1—Phylum-level bacterial abundance associated with *Tryonia metcalfi*.

Phylum	% Abundance
Acidobacteria	2.25
Actinobacteria	10.29
Bacteroidetes	5.06
Chloroflexi	0.28
Cyanobacteria	5.20
Deinococcus-Thermi	0.14
Firmicutes	12.48
Gemmatimonadetes	0.89
Ignavibacteriae	0.24
Planctomycetes	6.31
Proteobacteria	27.04
Spirochaetes	0.09
Tenericutes	23.99
Verrumicrobia	3.81
Unidentified	1.83

underwent a secondary analysis, circular consensus sequencing, using PacBio's algorithm. The circular consensus sequencing algorithm aligns the subreads individually from each template to generate consensus sequences thereby correcting the stochastic errors generated in the initial analysis. We then processed sequence data processed using Molecular Research's analysis pipeline. In summary, circular consensus sequencing data were depleted of barcodes and sequences shorter than 150 base-pairs and sequences with ambiguous base calls were removed. Sequences were then denoised and chimeras were removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence. We taxonomically classified the final OTUs against the National Center for Biotechnology Information (NCBI) prokaryotic 16S reference database (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/) using Geneious Prime (Biomatters, Auckland, New Zealand) using their provided tutorials (https://www.geneious.com/tutorials/metagenomic-analysis/). We used the "high sensitivity/medium" sequence classification setting with 100-base-pair overlap and minimum 97% overlap for the species level assignment and 95% overlap for all higher taxonomic assignments (Johnson et al., 2019). We calculated the relative abundance of bacteria at the phylum level and generated a "core" microbiome for *T. metcalfi* based on those OTUs that were present in every sample. If a core OTU was not identified to species, we used BLAST (Altschul et al., 1990) to compare it against both the NCBI microbial 16S reference database and the entire NCBI nucleotide database to determine whether it represented a novel taxon (no match at ≥97%).

We identified 380 OTUs from *T. metcalfi* (NCBI accessions OL862611-862982). Proteobacteria (27.04% relative abundance) and Tenericutes (23.99%) were the two most abundant bacterial phyla in our samples; eight other phyla had abundances ≥1% (Table 1). Nineteen OTUs composed the core microbiome associated with *T. metcalfi* (Table 2). Seven of the OTUs were identifiable down to genus and species at ≥97% similarity to bacterial reference sequences. The most abundant OTU was an unidentified Tenericutes comprising 47.72% of the core microbiome, followed by an unidentified *Erythromicrobium* species (10.03%) and a second unidentified Tenericutes (5.75%). The most abundant OTUs that were reliably identified to species in the core microbiome were *Variovorax boronicumulans* (4.22%), *Caedimonas varicaedens* (2.63%), and *Oscillatoria nigro-virdis* (2.15%). BLAST analyses of the 12 core OTUs that were not identified to species at ≥97% similarity indicated that all were undescribed taxa and that four were unrepresented in the NCBI complete sequence database.

Table 2—Core bacterial operational taxonomic units (OTUs) associated with *Tryonia metcalfi* ordered from the most to least abundant. Taxa with ≥97% similarity to National Center for Biotechnology Information (NCBI) reference sequences were identified to genus and species. Otherwise, OTUs were identified to their lowest reliable taxonomic level. OTUs marked with an asterisk had no ≥97% sequence matches in the NCBI complete database. RA = relative abundance relative to the core assemblage only.

Core OTU assemblage	% RA
Unidentified Tenericutes A*	47.72
Unidentified *Erythromicrobium*	10.03
Unidentified Tenericutes B*	5.75
Unidentified Xanthomonadales	4.88
Variovorax boronicumulans	4.22
Unidentified Oscillatoriophycideae	3.89
Unidentified Acidimicrobiales	3.23
Unidentified Rubrobacterales	3.12
Caedimonas varicaedens	2.63
Oscillatoria nigro-viridis	2.15
Algoriphagus aquatilis	2.02
Rhodobacter thermarum	1.99
Unidentified Burkholderiales A	1.97
Unidentified *Thalassiella*	1.92
Erythromicrobium sibricus	1.39
Unidentified Gemmatimonadales	1.27
Unidentified Burkholderiales B*	0.67
Unidentified Burkholderiales C*	0.62
Priestia megaterium	0.53

Desert waterbodies often represent diversity hotspots within relatively remote areas of low diversity. These freshwater systems are often home to unique species and taxon assemblages adapted to extreme environmental challenges (Brito et al., 2014). Chihuahuan Desert freshwater bodies are included among these oases of biodiversity (Hoyt, 2002). As one type of desert waterbody, ciénegas are ecosystems associated with freshwater seeps or springs, frequently containing shallow alkaline water, and harboring highly adapted plants along their edges

(Hendrickson and Minckley, 1985). Numerous springsnail and microbial species are endemic to Chihuahuan ciénegas and similar waterbodies, including multiple species of *Tryonia* (Hershler, 1985). Not only is *T. metcalfi* a threatened desert ciénega endemic, but our data suggest it lives in association with novel microbial lineages.

The phylum-level bacterial diversity and abundance associated with *T. metcalfi* was consistent with other freshwater snails including viviparids, *Pomacea canaliculata*, and *Potamopyrgus antipodarum* (Li et al., 2019; Bankers et al., 2020; North and Minton, 2021). The core microbiome of *T. metcalfi* was consistent with a benthic organism living in a desert freshwater environment. All core taxa were presumed aerobic save for Gemmatimonadales; members of the class can be aerobic or anaerobic. Gemmatimonadales, *Priestia megaterium*, and *Variovorax boronicumulans* were likely transferred or ingested from the sediment of the ciénega, while we predict the source of Xanthomonadales was the surrounding vegetation. Many core taxa (Acidimicrobiales, Burkholderiales, *Erythromicrobium*, *Oscillatoria nigro-virdis*, *Thalassiella*) were organisms known to occupy freshwater. The presence of *Algoriphagus aquatilis* correlated with the water alkalinity at the site, while Rubrobacterales and *Rhodobacter thermarum* were suggestive of the extreme temperatures and aridity of the desert (Liu et al., 2009; Chen et al., 2021). We were intrigued to find *Caedimonas varicaedens*, a bacterial endosymbiont of *Paramecium*, in the core microbiome (Schrallhammer et al., 2018). This suggests an incidental relationship between paramecium and *T. metcalfi* where the snail may be ingesting or carrying the protists (Maguire and Belk, 1967).

Twelve of the 19 core microbiome taxa were undescribed, and 4 of the 12 were unrepresented ($\geq$97% similarity) in the NCBI databases. Unidentified Tenericutes composed >53% of the core microbiome diversity. Preliminary phylogenetic analyses (data not shown) suggest that both Tenericutes A and B represent novel *Mycoplasma* species. Although *Mycoplasma* species are frequently associated with vertebrate hosts, many invertebrates including snails harbor them (Bolaños et al., 2019; Turgay et al., 2020; North and Minton, 2021). Similar preliminary analyses suggest that Burkholderiales B and C are novel lineages related to *Azohydromonas* and *Rhizobacter* respectively. If conservation efforts are to be as holistic and inclusive of all aspects of the species' biology and life history then host-associated microbial interactions should be considered (Amato, 2013; McKenney et al., 2018). Our data suggest *T. metcalfi* hosts two unique *Mycoplasma* species, and conservation measures for *T. metcalfi* need to consider these host–microbe relationships (Redford et al., 2012; Bahrndorff et al., 2016). *Mycoplasma* can be pathogenic or mutualistic, so future efforts should include determining their ecological role in association with *T. metcalfi* (Brown et al., 2007; Lian et al., 2020). Habitat conservation efforts for *T. metcalfi* would also affect environmental lineages such as the novel Burkholderiales we identified. A better understanding of the microbial community of La Ciénega may provide additional data that can be employed to preserve ciénega ecosystems and the organisms that inhabit them (Bodelier, 2011).

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