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Population genetics and conservation of recently discovered springsnails in Arizona

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ABSTRACT

Establishing baseline geographical distributions of extant genetic diversity is increasingly important for future conservation efforts of freshwater species. We analyse the mitochondrial cytochrome *c* oxidase subunit I (COI) gene to taxonomically characterize 233 samples from recently discovered springsnail populations throughout 17 sites in Arizona, USA. A total of 28 unique COI haplotypes were recovered, with the number of haplotypes ranging from 1 to 4 by population in Arizona. Phylogenetic analyses resulted in haplotypes from 13 of 17 locations in Arizona being successfully identified to species, with five described and three undescribed species in the genus *Pyrgulopsis* (Hydrobiidae). Future work will require in-depth morphological work to clarify the taxonomic status of these putatively novel species. Importantly, among recovered species, we identified haplotype diversity of the critically endangered Three Forks springsnail, *Pyrgulopsis trivialis*, in the eastern Gila watershed, which will inform wildlife managers in deciding which source populations to use in reintroduction efforts. We discuss possible causes for observed population structure of Arizona's springsnail populations, with suggestions for the future sampling schemes necessary for the conservation of this uniquely important freshwater mollusc.

INTRODUCTION

Specialized and/or small aquatic populations are often vulnerable to extinction (Davies, Margules & Lawrence, 2004) and continue to be so as these habitats become increasingly fragmented and manipulated (Crook *et al.*, 2015; Borgwardt *et al.*, 2019). However, due to their biogeography, organisms found in fragmented and/or specialized habitats often lend themselves to a sweep of rapid allopatric and/or adaptive speciation events (Warren *et al.*, 2015; De Meester *et al.*, 2016). Thus, careful molecular analyses determining the taxonomic diversity comprising taxa are important in conserving existing populations and re-establishing populations that have become extirpated (Keller *et al.*, 2015; Ralls *et al.*, 2018). Here, we analyse the mitochondrial cytochrome *c* oxidase subunit I (COI) gene to taxonomically characterize recently discovered populations of the springsnail genus *Pyrgulopsis* Call & Pislbry, 1886 (Caenogastropoda: Hydrobiidae) throughout Arizona. In doing so, we identify the haplotype diversity of extant populations of an endangered springsnail in eastern Arizona.

There are 13 described species of *Pyrgulopsis* in Arizona (Hershler & Liu, 2017): *P. arizonae* (Taylor, 1987), *P. bacchus* (Hershler & Landye, 1988), *P. bernardina* (Taylor, 1987), *P. conica* (Hershler & Landye, 1988), *P. deserta* (Pilsbry, 1916), *P. glandulosa* (Hershler & Landye, 1988), *P. hualapaiensis* (Hershler, Liu & Stevens, 2016), *P. montezumensis* (Hershler & Landye, 1988), *P. morrisoni* (Hershler & Landye, 1988), *P. simplex* (Hershler & Landye, 1988), *P. sola* (Hershler & Landye, 1988), *P. thompsoni* (Hershler & Landye, 1988) and *P. trivialis* (Taylor, 1987). These species are distributed across the state from the south-eastern sky islands and drainages along the US–Mexican border, following the Apache Highlands in the central part of the state and the Arizona–New Mexico Mountains to the east, up to the Great Basin Desert springs in the northwest corner of Arizona. Springsnail populations are often exceptionally vulnerable to extinction due to habitat specificity for cold water springs and sometimes the requirement for high concentrations of dissolved CO_2 and calcium (Hurt, 2004; Hershler, Mulvey & Liu, 2005; Hershler, Liu & Sada, 2007; Hershler, Liu & Howard, 2014; Hershler *et al.*, 2016). Moreover, because the wetlands and springs that springsnails prefer tend to be small and disjointed in nature, there is a high probability of localized extirpation from continuous perturbations in water quality (e.g. due to livestock use and post-wildfire flooding; Hershler *et al.*, 2014; NMDGF, 2016). Finally, for genera characterized as poor dispersers (Hershler *et al.*, 2014), springsnails exhibit high rates of endemism and cryptic species diversity (Hershler *et al.*, 2014), as movement between often disjointed habitats is unlikely. Additionally, the inability for springsnails to move between habitats limits the potential for recolonization after a localized extirpation event (Malcom, Radke & Lang, 2005). Thus, the loss of a single spring habitat type can result in the extinction of a uniquely adapted organism.

Information on habitat, population structure and taxonomy is essential for the management of endangered species, particularly

those that have high rates of local endemism. Molecular studies of springsnails range from evolutionary perspectives (Liu & Hershler, 2005) to regional biogeography (Hurt, 2004; Liu & Hershler, 2005). Here, we expand on these efforts by targeting the mitochondrial gene COI to characterize the genetic diversity and taxonomy of 17 populations of springsnails found across Arizona. In addition to geographically extending previous attempts to describe the variation in extant populations within Arizona (Hurt, 2004), our work is nearly a decade and half after the last assessment, allowing us to update locality records of species studied and possibly to identify localized extinction and colonization events. Understanding extant diversity and population structure across the species' ranges will inform future conservation efforts of these springsnails as it will be possible to identify which populations can be used during reintroduction.

MATERIAL AND METHODS

A total of 233 snails were collected at 17 springs and wetland locations in Arizona between 2017 and 2018 (Fig. 1; Table 1; Supplementary Material Table S1). In short, snails were picked from rocks and leaf litter using soft entomological forceps, and were preserved in sample vials of absolute ethanol until DNA processing. Total genomic DNA was extracted using a DNeasy Blood & Tissue Kit and following the manufacturer's protocols (Qiagen, Valencia, CA). Extractions were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) to ensure a minimum concentration of 0.02 μg/μl.

Next, *c.* 650 bp of the mitochondrial gene COI was amplified across samples using PCR with the universal primers LCO-1490 and HCO-2198 (Vrijenhoek, 1994). Due to poor amplification for several populations, new internal primers COIpl-F (5′-ATTGTYACTGCTCAYGCTTTTGT-3′) and COIpl-R (5′-AAGCRGTRTTRAAGTTTCGATCTGTTA-3′) were designed to target 414 bp of the COI gene. An optimized PCR reaction was developed; this included 3 μl of template DNA ($\geq$10 ng/μl), Phusion High-Fidelity PCR Master Mixes (Thermo Scientific) and 0.5 nM of each primer, in a total volume of 50 μl. PCR was conducted using an Eppendorf Mastercycler (ep*gradient*) under the following conditions: denaturation at 94 °C for 7 min; 35 cycles at 94 °C for 1 min (denaturation), 50 °C for 1 min (annealing) and 72 °C for 2 min (extension); and a final extension step at 72 °C for 7 min. Amplification was verified using gel electrophoresis with a 1.5% agarose gel and PCR products were purified for Sanger sequencing using ExoSAP-IT (Thermo Fisher), following the manufacturer's protocols. Sequencing was done on an ABI 3730 at the University of Texas at El Paso's Genomics Core. Sequences were aligned and edited using Sequencher v. 4.8 (Gene Codes, Inc.).

Relationship among samples

First, haplotype diversity per location was based on mere counting. Next, genetic distance across pairwise populations was calculated as the number of base substitutions per site from averaging over all sequence pairs between groups using an uncorrected p-distance, as implemented in the programme MEGA6 (Tamura *et al.*, 2013); standard error (SE) estimates were based on 1,000 bootstrap replicates.

Relationships among sequenced samples were first visualized by reconstructing a mitochondrial DNA (mtDNA) haplotype network using the median-joining algorithm in the program Network v. 4.5.1.0 (Bandelt *et al.*, 1999). Next, a Bayesian-derived individual gene tree was reconstructed using a single representative sequence for each recovered haplotype using MrBayes v. 3.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). In addition to sequences with high GenBank BLAST hits to recovered haplotypes, single representative sequences for each of Arizona's 13 described species of *Pyrgulopsis* (Hershler & Liu, 2017) were also included in the Bayesian tree analysis. By doing so, we not only provide species relationships among known *Pyrgulopsis* species but are more confident in our taxonomic assignments for recovered haplotypes. Prior to running analyses, substitution models were tested in MEGA6 and ranked based on Bayesian information criterion scores. The tree search comprised two concurrent runs of 2 million MCMC generations with sampling done every 2,000 generations until the average standard deviation of split frequencies was $\leq$0.01 and the effective sample size values across parameters $\geq$100. The first 25% of trees were discarded as burn-in and the final tree was summarized and viewed in FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

RESULTS

Although the COI gene was successfully amplified and sequenced across 109 specimens using the LCO-1490/HCO-2198 primer pair, 52 samples required the newly designed COIpl-F/COIpl-R internal primer pair (Supplementary Material Table S1). We note that nearly all individuals from sampling sites HU01, GV02, BYB1 and BYB4 required the COIpl-F/COIpl-R primer pair. Due to the smaller size fragment targeted by the COIpl-F/COIpl-R primer pair, a total of 414 overlapping base pairs were recovered across samples and used in all downstream analyses. Total sequence lengths are archived in GenBank (acc. nos MZ081852–MZ082084; Supplementary Material Table S1).

COI diversity and phylogenetic relationships

A total of 28 unique COI haplotypes were recovered across samples and sites (Fig. 2A; Table 1; Supplementary Material Table S2). The number of haplotypes ranged from 1 to 4 per population, with the BYB4 site having the highest number of haplotypes (Table 1). Calculated genetic distances ranged between 0 and 0.093, with the lowest calculated divergence being among BYB (range = 0.00–0.00071) and FC (range = 0.00016–0.0026) sites. For the pairwise population comparisons, the lowest genetic distances were recorded between BCU3 and SCOTIA4 (0.0037), COOL and UNION (0.0075), and HU01 and BH (0.00083); these were 10–100-fold lower than the rest of the pairwise comparisons (Table 2). The largest genetic divergence was calculated when compared against the LCC population (range = 0.069–0.093; Table 2).

The 28 haplotypes composed nine major lineages, as recovered in the mtDNA haplotype network (Fig. 2A). These include: (1) BYB1, BYB4 and BYB7; (2) SCOTIA4 and BCU3; (3) GV01 and GV02; (4) BH and HU01; (5) FC populations; and (6) UNION and COOL. The Bayesian gene tree was reconstructed using a Hasegawa–Kishino–Yano substitution model (Hasegawa, Kishino & Yano, 1985) with a gamma distribution across sites, which is the optimum model in MEGA6 (Supplementary Material Table S3). For our MrBayes analysis, all downloaded sequences used in phylogenetic analyses were trimmed to the 414-bp region amplified by our internal primers and sample HAP 6, identified as *Physella gyrina* (Table 1; Supplementary Material Table S2), was excluded. The gene tree (Fig. 2B) was rooted on the outgroup *P. deserta*. Sequences belonging to the 13 species of *Pyrgulopsis* formed three clades, including a sister group relationship between *P. landyei* and *P. arizonae*, which together formed a sister clade to *P. trivialis*. The clade comprising these three taxa was sister to a much larger clade in which the deepest nine branches were unresolved. In this larger clade, *P. bernardina* was sister to a clade comprising (1) *P. stearnsiana*, (2) *P. morrisoni*, (3) *P. bacchus*, (4) *P. montezumensis*, (5) the clade of *P. sola* + *P. intermedia* + *P. robusta* (with *P. sola* sister to the other two taxa), (6) an unknown *Pyrgulopsis* sp., (7) *P. simplex*, (8) *P. glandulosa* and (9) the clade of *P. conicus* + *P. hualapaiens* + *P. thompsoni* (with *P. conicus* sister to the other two taxa).

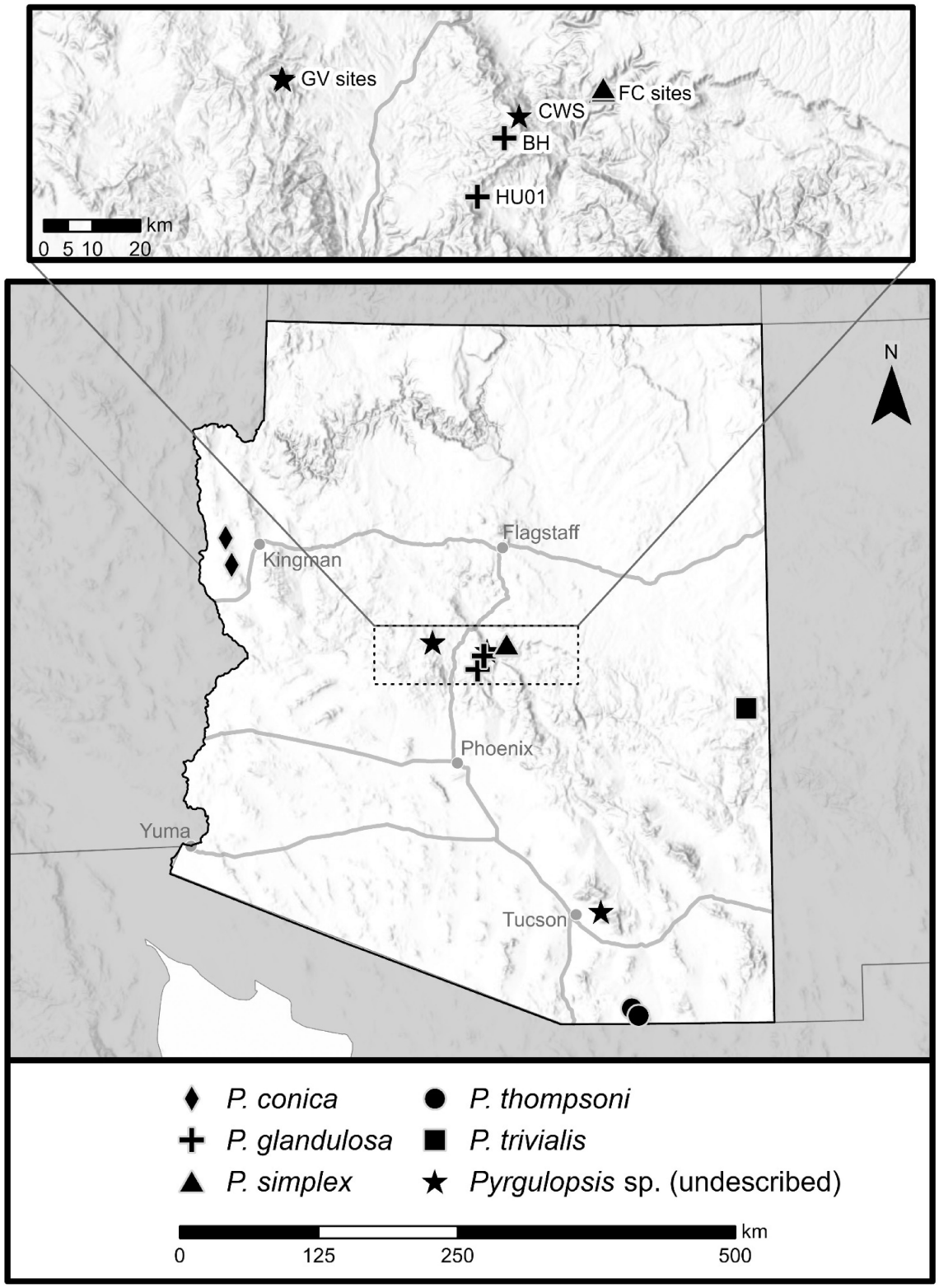

Figure 1. Map of all sampling localities and associated COI-based species assignments.

Table 1. Sample size and total number of COI haplotypes of *Pyrgulopsis* identified at individual sampling locations.

Sampling location	Sample size (*n*)	No. of haplotypes	Taxon
BH	15	2	*Pyrgulopsis glandulosa*
BYB1	21	1	*P. trivialis*
BYB4	18	4	*P. trivialis*
BYB7	18	1	*P. trivialis*
COOL	6	1	*P. conica*
CWS	20	2	*Pyrgulopsis* sp.
FC_{APS}	8	1	*P. simplex*
$FC_{Culvert}$	6	2	*P. simplex*
$FC_{Roadside}$	15	2	*P. simplex*
$FC_{Waterfall}$	13	1	*P. simplex*
GV02	16	3	*Pyrgulopsis* sp.
GV01	15	3	*Pyrgulopsis* sp.
HU01	14	3	*P. glandulosa*
LCC	8	2	*Pyrgulopsis* sp.
SCOTIA4	7	2	*P. thompsoni*
BCU3	13	2	*P. thompsoni*
UNION	20	3	*P. conica*

Taxonomy is based on BLAST hits (≥99% identity; Supplementary Material Table S2) and phylogenetic relationships (Fig. 1A).

Haplotypes belonging to 13 of 17 locations were successfully identified to species based on BLAST hits (Table 1; Supplementary Material Table S2) and Bayesian gene tree reconstruction (Fig. 2B). Haplotypes recovered at BH and HU01 locations formed a polytomy (Fig. 2B) and differed by a maximum of one mutation from the published *P. glandulosa* sequences; note that the major haplotype (Fig. 2A) recovered for BH and HU01 locations was identical to the published GenBank sequence AY485557 (Hurt, 2004). All but one haplotype recovered at BYB1, BYB4 and BYB7 locations formed a polytomy (Fig. 2B) and differed by a maximum of one mutation from the published *P. trivialis* sequences; note that the major haplotype (Fig. 2A) recovered at BYB1, BYB4 and BYB7 locations was identical to the published GenBank sequence AY485558 (Hurt, 2004). One haplotype from the BYB4 site was sister to and differed by 5 bp from the published sequence KT831388 that was identified as *P. gyrina* (Gordy *et al.*, 2016). Although snails were sorted into genera based on morphology prior to genetic analyses, this was a simple misidentification during specimen allocation as both *P. gyrina* and *P. trivialis* are known to co-occur at the BYB locations. Haplotypes recovered at the four FC sites formed a polytomy (Fig. 2B) and differed by a maximum of one mutation from the published *P. simplex* sequences; note, however, that the major haplotype (Fig. 2A) recovered at $FC_{Roadside}$ and $FC_{Waterfall}$ sites was identical to the published GenBank sequence AY485558 (Hurt, 2004). The single haplotype recovered at the COOL site (Fig. 2A) differed by a mutation of 1 bp from the published sequence of *Pyrgulopsis conica*, AY485546 (Hurt, 2004). Similarly, the three haplotypes recovered at UNION also clustered with and differed by 4–5 bp from the published sequence belonging to *P. conica* (Fig. 2B). Haplotypes recovered at SCOTIA4 and BCU3 (Fig. 2A) were identical to or differed by a mutation of 1 bp from the published sequences of *P. thompsoni* (Fig. 1A; Hurt, 2004). Although the BLAST identity suggested alternative species for the two haplotypes recovered at the CWS site (Fig. 1B; Supplementary Material Table S2), the Bayesian tree suggested a sister group relationship with a published sequence for *P. montezumensis*. We note, however, that the sequences differed by mutations of 10–11 bp from the used published sequence. Finally, haplotypes recovered at GV01 and GV02, as well as the LCC site, did not cluster with any of the 13 known species (Fig. 2B). Thus, while Bayesian tree reconstruction placed haplotype diversity recovered at CWS, GV01, GV02 and LCC sites within the genus *Pyrgulopsis*, we are not able to confidently identify these populations to species level (Table 1).

DISCUSSION

We provide an updated assessment of phylogenetic and population genetics for several species of Arizona's springsnails with priority management needs among state and federal natural resource agencies. We report all but one of the samples to be within the targeted genus *Pyrgulopsis* and were able to identify populations from 13 of 17 sampled sites to species level (Fig. 2; Table 1). For the successfully identified populations, we not only recovered the major haplotype to be the same as that published for the species nearly two decades ago (Liu, Hershler & Clift, 2003; Hurt, 2004; Hershler *et al.*, 2005), but also provide novel diversity for several of these (Fig. 2B; Supplementary Material Table S2). Importantly, COI haplotype diversity recovered in four sampling sites was not clearly assignable to any of the known species existing within Arizona. Although the two COI haplotypes recovered at the CWS site formed a clade with *Pyrgulopsis montezumensis*, they differed by 10–11 bp from the used *P. montezumensis* sequence. We also note that *P. montezumensis* was sister to a clade comprising the two haplotypes from CWS in the Bayesian tree (Fig. 2B). Together, these data indicate significant divergence between the CWS haplotypes and *P. montezumensis*, suggesting that these are different taxonomic units. Similarly, COI haplotype diversity recovered at GV01, GV02 and LCC sites did not show a high BLAST similarity to (Supplementary Material Table S2) and did not cluster with any single *Pyrgulopsis* species in the Bayesian gene tree (Fig. 2B). Moreover, CWS, GV01, GV02 and LCC sites had the largest calculated genetic divergences across pairwise location comparisons (Table 2). Consequently, these data suggest that the CWS, GV01, GV02 and LCC sites likely represent previously undescribed *Pyrgulopsis* species. Future work will require in-depth morphological work for snails present at the CWS, GV01, GV02 and LCC sites to clarify their taxonomic status.

Next, COI haplotype diversity ranged between one and four haplotypes, with most sampled sites harbouring only two haplotypes (Table 1). The limited COI diversity recovered here is similar to previous research (Hurt, 2004), and further exemplifies the low overall diversity but potentially high rate of endemism across the sampled sites. As compared to biparentally inherited markers, the smaller (i.e. one-fourth; Giles *et al.*, 1980) effective population size of mtDNA naturally results in higher rates of overall fixation, including increased effects of genetic drift during founder events on mtDNA as compared to the nuclear genome. Thus, rather than being the result of local adaption, the generally high rate of mtDNA differentiation (Table 2) between sites may simply be due to demographic processes that are impacted by dispersal events involving a few individuals and high rates of local extirpation due to constantly changing wetland and water habitats. Future research to determine extant molecular diversity, selective processes and gene flow/migration rates within springsnails will benefit from expanded genomic coverage. Importantly, such efforts will help determine accurate taxonomic relationships, as mtDNA and nuclear DNA can often provide discordant phylogenies due to inconsistent demographic and/or selective pressures across the genome (Humphries & Winker, 2011; Peters *et al.*, 2014; Bonnet *et al.*, 2017).

Finally, we provide an updated phylogeny of *Pyrgulopsis* springsnail species that inhabit Arizona. Our Bayesian tree recovered three major *Pyrgulopsis* groups (Fig. 2B) and these correspond well with previously published phylogenies (Liu *et al.*, 2003; Hurt, 2004; Hershler *et al.*, 2005). However, finer resolution was not achievable with the 414 bp of the COI gene used as relationships within the three major groups were largely unresolved. We note that whereas previous work using the larger *c.* 650-bp section of the COI gene appeared to provide better resolution, many of the

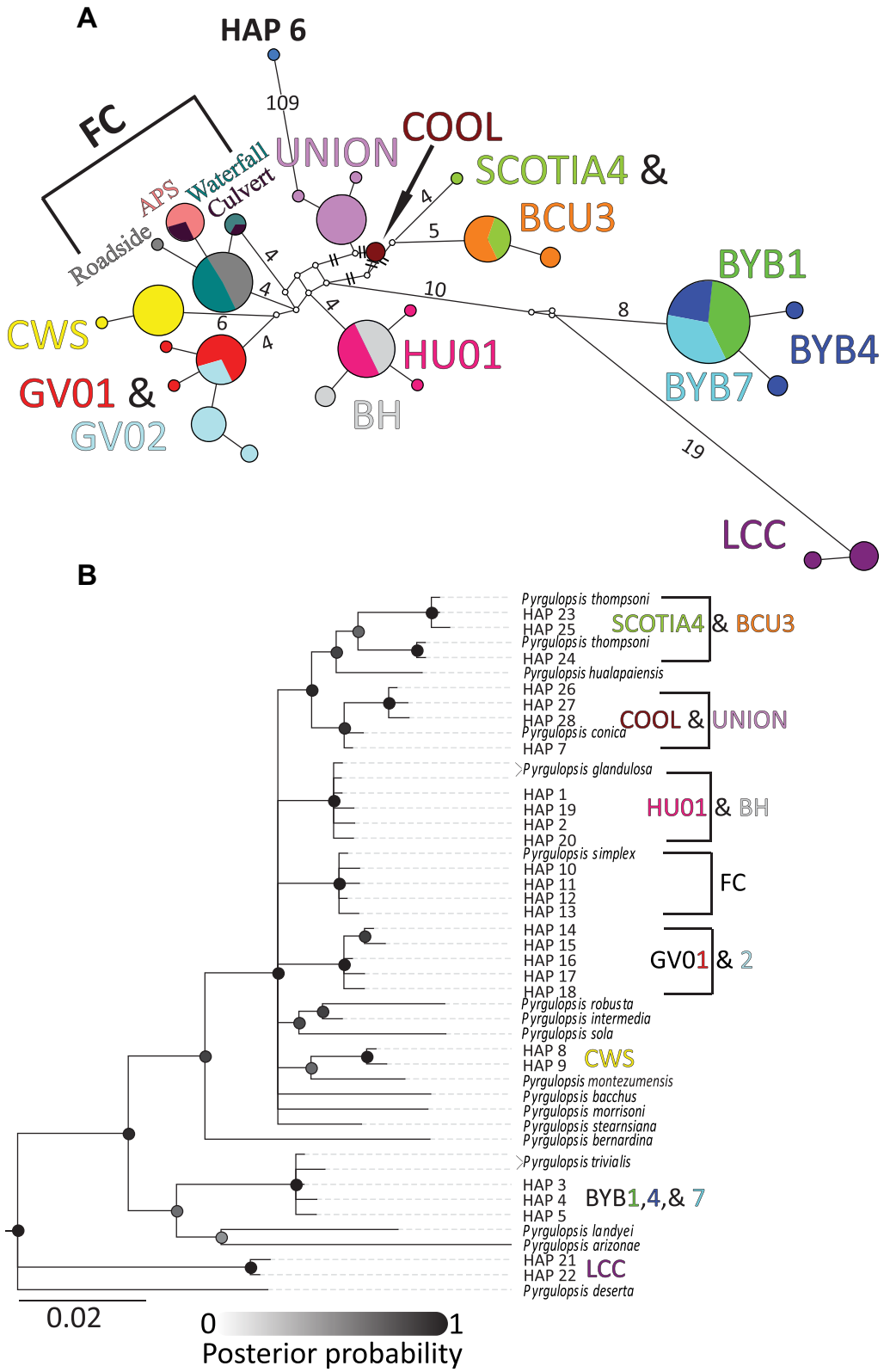

Figure 2. A. Median-joining mtDNA haplotype network (based on 414 bp of the COI gene), showing relationships across all sampled springsnails in Arizona. Circle size corresponds to the total number of individuals with a given haplotype and branch lengths are proportional to the number of mutations separating haplotypes. Branches associated with one or more mutations are shown by hash marks (for two mutations) or numerically (three or more mutations). The 17 populations are colour coded and site codes follow Table 1. **B.** Bayesian gene tree reconstructed from 414 bp of the COI gene, showing 28 haplotypes recovered across sampled springsnails in Arizona and at least one representative sequence for Arizona's 13 described species of *Pyrgulopsis* (Hershler & Liu, 2017). Branch support (Bayesian posterior probabilities) is shown by the grey scale.

Table 2. Pairwise genetic divergences for locations (below diagonal) and associated SEs estimated across 1,000 bootstraps (in italics and above diagonal).

	BH	BYB1	BYB4	BYB7	COOL	CWS	FC_{APS}	$FC_{Culvert}$	$FC_{Roadside}$	$FC_{Waterfall}$	GV02	GV01	HU01	LCC	SCOTIA4	BCU3	UNION
BH	–	*0.011*	*0.011*	*0.011*	*0.0066*	*0.0075*	*0.0073*	*0.0071*	*0.0068*	*0.0068*	*0.0075*	*0.0074*	*0.00054*	*0.013*	*0.008*	*0.0084*	*0.0069*
BYB1	0.058	–	*0.00047*	*0*	*0.011*	*0.01*	*0.011*	*0.011*	*0.011*	*0.011*	*0.012*	*0.011*	*0.011*	*0.012*	*0.011*	*0.011*	*0.011*
BYB4	0.059	0.00071	–	*0.00047*	*0.011*	*0.01*	*0.011*	*0.011*	*0.011*	*0.011*	*0.012*	*0.011*	*0.011*	*0.012*	*0.011*	*0.011*	*0.011*
BYB7	0.058	0	0.00071	–	*0.011*	*0.01*	*0.011*	*0.011*	*0.011*	*0.011*	*0.012*	*0.011*	*0.011*	*0.012*	*0.011*	*0.011*	*0.011*
COOL	0.022	0.056	0.056	0.056	–	*0.0069*	*0.0077*	*0.0072*	*0.0073*	*0.0073*	*0.0079*	*0.0077*	*0.0065*	*0.012*	*0.0064*	*0.0069*	*0.0039*
CWS	0.025	0.056	0.056	0.056	0.027	–	*0.0079*	*0.0076*	*0.0074*	*0.0074*	*0.0075*	*0.0073*	*0.0075*	*0.013*	*0.0086*	*0.009*	*0.0073*
FC_{APS}	0.025	0.058	0.059	0.058	0.027	0.029	–	*0.0016*	*0.0024*	*0.0024*	*0.0074*	*0.0073*	*0.0073*	*0.013*	*0.0083*	*0.0086*	*0.0082*
$FC_{Culvert}$	0.025	0.058	0.059	0.058	0.024	0.029	0.0024	–	*0.0016*	*0.0016*	*0.0072*	*0.007*	*0.007*	*0.013*	*0.008*	*0.0084*	*0.0077*
$FC_{Roadside}$	0.022	0.056	0.056	0.056	0.024	0.027	0.0026	0.0026	–	*0.00015*	*0.007*	*0.0069*	*0.0068*	*0.013*	*0.0079*	*0.0082*	*0.0078*
$FC_{Waterfall}$	0.022	0.056	0.056	0.056	0.024	0.027	0.0024	0.0024	0.0002	–	*0.007*	*0.0069*	*0.0068*	*0.013*	*0.0079*	*0.0082*	*0.0078*
GV02	0.027	0.064	0.065	0.064	0.029	0.026	0.026	0.026	0.024	0.024	–	*0.0016*	*0.0075*	*0.014*	*0.0091*	*0.0094*	*0.0082*
GV01	0.025	0.063	0.064	0.063	0.027	0.025	0.024	0.024	0.022	0.022	0.002	–	*0.0074*	*0.014*	*0.0089*	*0.0092*	*0.008*
HU01	0.00083	0.058	0.059	0.058	0.022	0.025	0.024	0.024	0.022	0.022	0.026	0.025	–	*0.013*	*0.0079*	*0.0083*	*0.0069*
LCC	0.087	0.069	0.07	0.069	0.079	0.086	0.082	0.079	0.079	0.079	0.093	0.092	0.086	–	*0.013*	*0.014*	*0.013*
SCOTIA4	0.034	0.062	0.063	0.062	0.021	0.038	0.034	0.034	0.032	0.032	0.04	0.039	0.033	0.087	–	*0.0012*	*0.0074*
BCU3	0.035	0.062	0.063	0.062	0.022	0.039	0.034	0.034	0.032	0.032	0.041	0.04	0.034	0.089	0.0037	–	*0.0078*
UNION	0.025	0.053	0.054	0.053	0.0075	0.029	0.029	0.027	0.027	0.027	0.031	0.03	0.025	0.082	0.029	0.03	–

deeper-level relationships were poorly supported, as was the case in our study. We conclude that although the dataset we analysed was smaller (414 bp), we did not lose much power in our phylogenetic analyses as compared to previous research because we also used the polymorphic region of the COI gene. Future attempts to resolve evolutionary relationships in the genus *Pyrgulopsis* will require increasing the number and size of markers used and should include comparisons of analyses based on nuclear DNA *vs* mtDNA. Moreover, species relationships can be better resolved through species tree reconstructions by coupling multiple individuals and loci in a coalescent framework (Drummond & Rambaut, 2007; Bryant *et al.*, 2012; Bouckaert *et al.*, 2014).

Phylogeography of Pyrgulopsis *in the North American Southwest*

Coupling phylogenetic and population genetics analyses of the COI gene provided important inferences into the taxonomic representation of springsnails across the sampled locations (Figs 1, 2). Moreover, although samples were sorted using morphological characters targeting the genus *Pyrgulopsis*, one additional genus was recovered (Supplementary Material Table S1). Specifically, a single sample from sampling site BYB4 was identified as *Physella gyrina*, which is known to co-occur with *P. trivialis* at this site; this was simply an error made during sorting. Nonetheless, the recovery of this additional genus demonstrates the importance of molecular methods in ensuring samples are properly identified.

Across the Arizonian samples identified as belonging to the genus *Pyrgulopsis*, we identified new populations of *P. thompsoni* in the Huachuca Mountains and *P. glandulosa* along the Verde Rim (Fig. 2; Table 1). Additionally, we confirmed the current presence of *P. simplex* along Fossil Creek, as well as *P. conica* at an unnamed spring near Union Pass (UNION) in the Black Mountains. In fact, our confirmation of the presence of *P. conica* at the Cool Spring (COOL) sampling site in the Black Mountains contrasts with previous work that suggested this species was extirpated from this site (Hurt, 2004). In general, we successfully demarcated several species of *Pyrgulopsis* at Arizona's peripheries (Table 1; Fig. 2). However, identifying the true geographical extent of the different species, including transitional and overlapping zones between individual Arizona springsnails, will require more fine-scale, transect-based sampling across the state, with a focus on high-grade perennial springs and seeps that have not been searched before.

Geographic proximity generally explains estimated genetic differentiation and changing *Pyrgulopsis* taxonomy among sampling sites (Fig. 2) and that is consistent with the observation that gastropods are in general poor active dispersers (Strong *et al.*, 2007). Despite the tendency of gastropods to display patterns of genetic differentiation that are closely tied with biogeographical patterns, we recovered the same springsnail species in sites that were geographically widely separated. Among the various springsnail populations found in central Arizona, the populations of *P. conica* recovered in the Cool (COOL) and Union Pass (UNION) springs of the Black Mountains were separated by a relatively long distance (24.6 km). Similarly, previous studies have identified *P. thompsoni* populations in two different watersheds on either side of the Huachuca Mountains (Hurt, 2004), and populations of *P. bacchus* are known to occur in Nevada's Spring Mountains and Arizona's Grand Wash, areas that are nearly 130 km apart (McKelvey *et al.*, 2020). Though direct dispersal is possible, the large geographical distances involved and the presence of significant topographical barriers to such dispersal suggest that the movement of these snails most likely is made possible by secondary actor(s). Clearly, anthropogenic dispersal is a common mechanism among molluscs, and this includes dispersal in or on boats (Shaw, Hogan & McIntosh, 1986; Griffiths *et al.*, 1991; Leung, Bossenbroek & Lodge, 2006), as well as due to the direct manipulation of streams to increase headwater transfer (Liu, Hovingh & Hershler, 2015). Additionally, waterbirds are known to be dispersers of various aquatic

organisms (Figuerola & Green, 2002; Green & Figuerola, 2005), including molluscs (Gittenberger *et al.*, 2006; Kawakami, Wada & Chiba, 2008; van Leeuwen *et al.*, 2012; Wada, Kawakami & Chiba, 2012; Simonová *et al.*, 2016). With their small size and operculum to reduce desiccation, springsnails are likely able to disperse over long distances between suitable habitats in the feathers or undigested in the faeces of waterfowl (e.g. ducks). Studying the evolutionary relationship between springsnails and birds may help illuminate how these small molluscs are able to colonize and spread to new or uninhabited wetlands. Alternatively, rather than the current apparently disjunct springsnail distributions recovered in Arizona and elsewhere (e.g. *P. kolobensis* populations found across the Wasatch Mountain divide in north-eastern Utah; Liu *et al.*, 2015) being evidence of unique dispersal events, they may be the result of slow extirpation events through time of once widespread and interconnected populations. More complete sampling of *Pyrgulopsis* across Arizona will be required to investigate these two competing hypotheses.

Conservation of Arizona's springsnails

For the effective management of rare or at-risk molluscs, state and federal wildlife agencies are increasingly relying on genetic analysis to better understand the identity and haplotypes of newly discovered populations so as to place them in context with known populations (Lysne *et al.*, 2008; Hershler *et al.*, 2014). The identity and relatedness of these molluscs with others across the landscape have set the foundation for federal Endangered Species Act listing decisions and subsequent recovery actions, or partner-based management commitments through conservation agreements. In our study, we describe the COI diversity of extant *Pyrgulopsis* from across Arizona, comprising five described and three undescribed species (Table 1; Fig. 2). Not only are such efforts essential to facilitate the conservation of extant diversity, they are vital for informed decision-making during reintroductions. We confirmed the current status of haplotypes of the Three Forks springsnail *P. trivialis*, which occurs in the Boneyard Bog site of the eastern Gila watershed (Hurt, 2004) and is listed as critically endangered in the IUCN Red List (https://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T18990A1939580.en). A total of three unique haplotypes were recovered across the three Boneyard Bog sites, with the haplotype that was previously identified by Hurt (2004) being present at all three sites and the remaining two haplotypes being specific to one site (i.e. BYB4; Supplementary Material Table S2). This information will aid state and federal wildlife managers in deciding which source populations to use in reintroducing this species to extirpated springs further downstream in the watershed. We suggest using individuals from the BYB4 site (Figs 1, 2) due to recovery of the most genetic diversity at this site (Table 1). With freshwater habitats projected to be one of the most impacted systems by climate change (Opdam & Wascher, 2004; Kundzewicz *et al.*, 2008), establishing baseline and geographical distributions, including tracking changes of genetic diversity through time, is increasingly important for future conservation efforts of these springsnails and, in general, for other freshwater species.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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