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SHORT COMMUNICATION

Molecular evidence for cryptic species in a narrowly endemic western North American springsnail (*Pyrgulopsis gilae*)

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Abstract *Pyrgulopsis gilae* is a small springsnail that is narrowly distributed along the forks of the upper Gila River and currently being managed as a threatened and sensitive species by the State of New Mexico and United States Forest Service, respectively. A previous phylogeographic study of this species based on mitochondrial COI sequences delineated substantial divergence between several populations along the lower and upper reaches of the East Fork Gila River. The present study surveyed COI variation among a larger number of populations across the entire geographic range of *P. gilae*. Three haplotype groupings were delineated that were congruently resolved as clades by a Bayesian analysis. One of the clades was composed of populations along the lower East Fork and mainstem Gila River and corresponds to *P. gilae* as originally circumscribed. The other two clades were composed of populations along the Middle Fork and upper East Fork Gila River that were recently referred to *P. gilae*. These three geographically isolated clades do not share any haplotypes, have significant F_{ST} values, and are differentiated from each other by 3.9–6.3 % sequence divergence. Based on this evidence we suggest that the clades represent distinct species and should be managed as separate conservation units pending taxonomic revision of *P. gilae*. This study provides additional evidence that geographically disjunct subunits of *Pyrgulopsis* species often represent distinct monophyletic lineages that may warrant formal taxonomic recognition, and thus underscores the importance of fine-scale conservation genetics studies of these imperiled organisms.

Keywords Phylogeography · Gastropoda · Springs · Conservation · Cryptic species

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Introduction

The hydrobiid gastropod genus *Pyrgulopsis* (134 species), which originated (minimally) in the late Miocene (Liu and Hershler 2005), is one of the most diverse elements of the western North American aquatic biota. Most of the tiny species (1–8 mm shell height) in this genus (commonly known as springsnails) have very small geographic ranges, which often consist of a single spring or single spring complex. Congeners are scattered throughout much of the West, sometimes forming local species flocks. The high diversity and local endemism that characterizes this species radiation appears to be a product of limited vagility and high fidelity to insular spring-fed habitats (Hershler and Sada 2002). These natural history attributes further suggest that *Pyrgulopsis* species may be highly differentiated within their typically narrow geographic ranges. Genetic studies have documented strong structuring in various congeners consistent with this hypothesis (Liu et al. 2003; Hurt 2004; Moline et al. 2004; Hershler et al. 2007; Liu and Hershler 2012), while only a few members of the genus have instead been shown to be weakly subdivided (Hershler and Liu 2004; Liu and Hershler 2007). Such

phylogeographic investigations are not only of interest from the biological perspective, but also are needed to help guide the conservation and management of these biodiversity jewels, which are threatened by various anthropogenic stressors and increasingly are becoming a focus of attention of land managers (e.g., USFWS 2012a, b, c).

Pyrgulopsis gilae, commonly known as the Gila springsnail, is distributed in several dozen springs and seeps in the upper Gila River basin of southwestern New Mexico (NMDGF 2010; USFWS 2011a, b). The geographic range of this species is markedly disjunct, consisting of several short reaches of the East Fork and Middle Fork (Gila River) watersheds and one locality along the mainstem Gila River (USFWS 2011a; Fig. 1). The genetic connectivity of the geographically isolated groups of populations of *P. gilae* has not been well established. Hurt (2004) studied mitochondrial cytochrome *c* oxidase subunit I (COI) variation of this species along the East Fork Gila River and delineated considerable divergence between several populations in the lower reach of this drainage (the type locality area) and a population about 20 km to the northeast along Taylor Creek. She suggested that the latter may merit recognition as a distinct subspecific taxonomic unit pending additional study. Here we further evaluate the genetic diversity and population structure of *P. gilae* using mitochondrial DNA sequences from a larger suite of populations that spans the entire geographic range of this species. We also discuss the implications of our findings for the taxonomy and conservation of *P. gilae*, a former candidate for federal listing (USFWS 2011b) which is vulnerable to several possible stressors (natural stochastic events, wetland habitat degradation, water contamination; NMDGF 2010) and which is currently being managed as a threatened and sensitive species by the State of New Mexico (NMDGF 2010) and United States Forest Service (USFS 2007), respectively.

Materials and methods

Samples were collected by RH, BL and Marilyn Myers from 12 sites across the entire geographic range of *P. gilae* (Fig. 1). Specimens were preserved in 90–95 % ethanol. Locality details and other pertinent information for these samples are in Table 1. Partial sequences of the COI and NADH dehydrogenase subunit I (NDI) genes were obtained for 2–10 specimens from each sample, totaling 84 new sequences for COI (see explanation below for NDI). Six previously published COI sequences of *P. gilae* (Hurt 2004; Liu and Hershler 2005) and representative sequences from 11 other congeners were also included in the phylogenetic analysis. Trees were rooted with sequences of *Floridobia floridana* following Hershler et al. (2003).

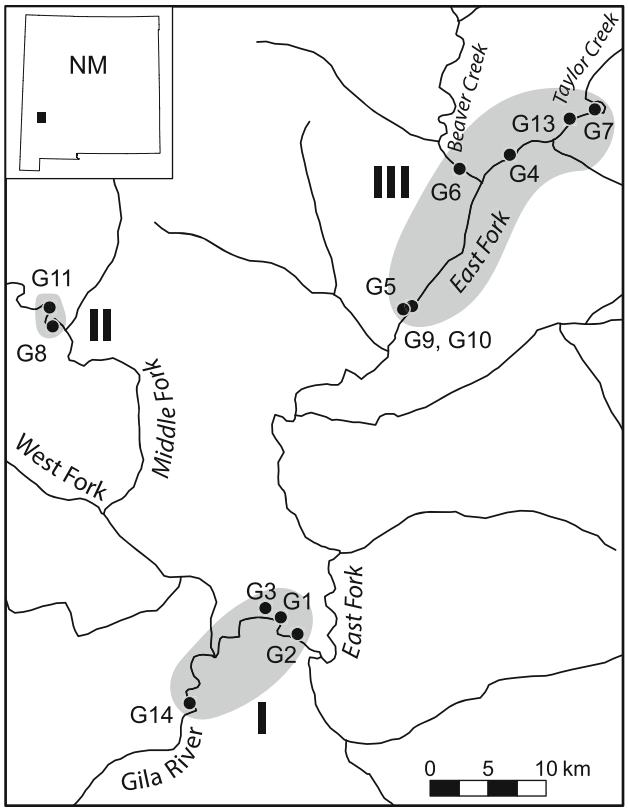

Fig. 1 Map showing the locations of *P. gilae* collection sites. Sample codes are from Table 1. The geographic locations of clades delineated by molecular evidence (*I–III*) are shaded *grey*

Genomic DNA was extracted from entire snails using a CTAB protocol (Bucklin 1992). LCO1490 and HCO2198 (Folmer et al. 1994) were used to amplify a 710 base pair (bp) fragment of COI and ND43F and RND592F (Liu et al. 2003) were used to amplify a 550 bp fragment of NDI. The amplification conditions described in Liu et al. (2003) were used. We could not amplify NDI from samples 8 and 11 despite experimenting with various conditions and redesigned primers. The amplified polymerase chain reaction product was sequenced following Liu et al. (2003). Sequences were determined for both strands and then edited and aligned using Sequencher™ version 5.0.1.

Since we could not amplify the NDI gene from two samples and there are no previously published sequences to fill this data gap, we are only reporting our mtCOI results in detail herein. MrModeltest 2.3 (Nylander 2004) was used to obtain an appropriate substitution model (using the Akaike Information Criterion) and parameter values for the Bayesian analysis, which was performed using MrBayes 3.2.1 (Ronquist et al. 2012). Metropolis-coupled Markov chain Monte Carlo simulations were performed with four chains for 2,000,000 generations and Markov chains were sampled at intervals of 10 generations to obtain 200,000 sample points. We used the default settings for the priors on topologies and the

Table 1 Samples of *P. gilae* used for molecular analysis, with sample codes (referred to in the text and figures) and locality details

Code	Locality (all in New Mexico)	Voucher	Latitude	Longitude
G1	Unnamed spring along East Fork Gila River, 1.53 km north and 2.9 km east of State Route 527 bridge crossing, Grant County	1135043	33.1917	−108.1742
G2	Unnamed spring along East Fork Gila River, 1.29 km north and 0.56 km west of Black Canyon confluence, Grant County	1135052	33.1864	−108.1675
G3	Unnamed spring along East Fork Gila River, 1.53 km north and 2.38 km east of State Route 527 bridge crossing, Grant County	1135055	33.1946	−108.1804
G4	Unnamed hillside seep along Taylor Creek, ca. 0.32 km south and 0.93 km west of Wall Lake dam (below Wall Lake), Catron County	1135060	33.3457	−108.0904
G5	Unnamed hillside seep, 1.61 km north, 0.97 km east of Burnt Corral Canyon, Catron County	1123590	33.2951	−108.1268
G6	Unnamed spring along Beaver Creek, 0.29 km north and 0.40 km west of Taylor Creek confluence, Catron County	1135064	33.3405	−108.1097
G7	Unnamed spring along Taylor Creek, Whitewater Canyon, 50 m west of Whitetail Canyon, Catron County	1123593	33.3613	−108.0576
G8	Unnamed spring along Middle Fork Gila River, ca. 0.97 km north and 0.64 km west of Jordan Canyon, Grant County	1123432	33.2848	−108.2667
G9	Fall Spring along East Fork Gila River, 1.61 km north and 0.56 km east of Burnt Corral Canyon, Catron County	1135057	33.2940	−108.1302
[a]G10	Fall Spring along East Fork Gila River, 1.61 km north, 0.56 km east of Burnt Corral Canyon, Catron County	1135058	33.2940	−108.1302
G11	Unnamed spring along Middle Fork Gila River, ca. 0.48 km north and 0.48 km west of Jordan Canyon, Grant County	1135068	33.2909	−108.2681
G13	Unnamed spring along Taylor Creek, 0.80 km north and 1.13 km east of Wall Lake Dam (above Wall Lake), Catron County	1135062	33.3581	−108.0673
G14	"Alum Spring" along Gila River, ca. 1.93 km south and 0.16 km west of State Route 527 bridge crossing, Grant County	1068942	33.1618	−108.2081

[a] Very small (juvenile) specimens initially thought to be distinct from *P. gilae*

HKY + I + G model parameters selected by MrModeltest as the best fit model. At the end of the analysis, the average standard deviation of split frequencies was less than 0.01 (0.0055) and the potential scale reduction factor (PSRF) was 1, indicating that the runs had reached convergence. The sampled trees with branch lengths were used to generate a 50 % majority rule consensus tree with the first 25 % of the samples removed to ensure that the chain sampled a stationary portion.

Genetic relatedness within *P. gilae* was further assessed by a haplotype network that was generated with TCS version 1.21 using the default settings (Clement et al. 2000). Sequence divergences within and between *P. gilae* lineages were calculated (using the HKY distance selected by MrModeltest) in PAUP*4.10. (Swofford 2003) and Excel. Structuring of variation among lineages was evaluated by an AMOVA using Arlequin 3.5 (Excoffier and Lischer 2010).

Results

The alignment of 84 COI sequences of *P. gilae* that were generated for this study yielded 658 bp. The Bayesian analysis yielded a topology in which specimens of this species formed a well supported (100 % posterior probability) clade that was sister to a lineage consisting of *P. deserta* and an undescribed congener (Pmim) from the Mimbres River drainage (Fig. 2a). *P. gilae* was subdivided into three well supported (100 % posterior probability) clades (Fig. 2a, I–III) composed of specimens from "Alum Spring" (along the mainstem Gila River) and the lower reach of the East Fork watershed (I), specimens from the Middle Fork watershed (II), and specimens from the upper reach of the East Fork watershed (III) (Fig. 1). These clades differed from each other by 3.9–6.3 % sequence divergence while variation within each was minor (Table 4). A separate analysis of the NDI dataset congruently resolved clades I and III (100 % posterior probability in both cases); note that specimens from clade II could not be successfully amplified for this gene.

Pyrgulopsis gilae was structured into three groups in the COI haplotype network congruent with the Bayesian topology (Fig. 2b). Twenty-three haplotypes were detected (Table 2, GenBank accession numbers KC571284–KC571306), 21 of which were restricted to single populations. The others were

Table 2 Frequency distribution of COI haplotypes detected in *P. gilae*

Haplotype	Sample												
	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G13	G14
I	2												
II	1												
III		9											
IV		1											
V			4										2
VI			1										
VII			1										
VIII													1
IX				4									
X				1			10					8	
XI				1									
XII				1									
XIII					7								
XIV						2							
XV						6							
XVI						1							
XVII									1				
XVIII									8	4			
XIX								1					
XX								1					
XXI								3					
XXII								1					
XXIII											2		
n	3	10	6	7	7	9	10	6	9	4	2	8	3

n sample size

shared by two populations along the lower East Fork and mainstem Gila River (haplotype V), and by three populations along the upper East Fork (haplotype X). Five samples (G5, G7, G10, G11, G13) each contained a single haplotype. The TCS analysis recovered three well differentiated haplotype groups (Fig. 2a, I–III) which were composed of specimens from "Alum Spring" (along the mainstem Gila River) and the lower reach of the East Fork watershed (I); specimens from the Middle Fork watershed (II); and specimens from the upper reach of the East Fork watershed (III) (Fig. 1). The clades differed from each other by 3.9–6.3 % sequence divergence while variation within each was minor (Table 4). An AMOVA indicated that most of the detected variation (86.7 %) was partitioned among these groups; variation within populations and among populations within the groups was much smaller (11.04, 2.04 %, respectively), but nonetheless was significant (Table 3).

Discussion

Hurt (2004) previously delineated distinct COI subgroups (clades) of *P. gilae* along the lower and upper reaches of the East Fork Gila River based on a single-strand conformation polymorphism (SSCP) analysis. Our study has more fully delineated the scope and content of these clades and has also revealed a third clade along the Middle Fork Gila River, which was not sampled by Hurt (2004).[1] Clade I corresponds to *P. gilae* as originally circumscribed (Taylor 1987); the other two clades are composed of populations that were subsequently referred to this species (NMDGF 2010, USFWS 2011a). The three reciprocally monophyletic groups are geographically isolated, do not share any haplotypes, are delineated by significant F_{ST} values, and their COI sequence divergences (3.9–6.3 %)

[1] Hurt (2004) detected four haplotypes from the lower reach of the East Fork (Pgil1, Pgil2, Pgil3, Pgil4) which differed by 0.16–0.75 %. Our analysis of specimens from this area (and one site along the closely proximal mainstem Gila River) detected eight haplotypes (I–VIII) which differed from each other by 0.15–1.2 % (note that our haplotype II is identical to Pgil1). Hurt (2004) sampled one site along the upper East Fork (Wall Spring) and detected a single haplotype (Pgil6). We sampled this spring (G13 sample) and 6 additional sites along the upper East Fork and found total ten haplotypes which differed by 0.15–1.52 %. Note that our haplotype X is identical to Pgil6.

Table 3 Genetic differentiation among *P. gilae* clades

Source of variation	d.f.	Variance components	% of variation	Φ statistic
Among groups	2	14.52	86.77	0.87*
Among populations within groups	10	1.85	11.04	0.83*
Within populations	71	0.37	2.19	0.98*

Sub-groups = (G1, G2, G3, G14), (G8, G11), and (G4, G5, G6, G7, G9, G10, G13). Asterisked Φ values are highly significant ($P < 0.001$)

fall well into the range observed for other *Pyrgulopsis* species (1.1–13.1 % for COI; Liu and Hershler 2005). Based on this information and the obvious differences between the shells of these groups of populations (in preparation), we consider it likely that the three clades are distinct species. These findings are preliminary in that our samples were analyzed in detail for only a single mitochondrial gene and have not been accompanied by detailed morphologic studies. Nonetheless, based on our evidence that these three groups of populations represent independent evolutionary trajectories, we recommend that

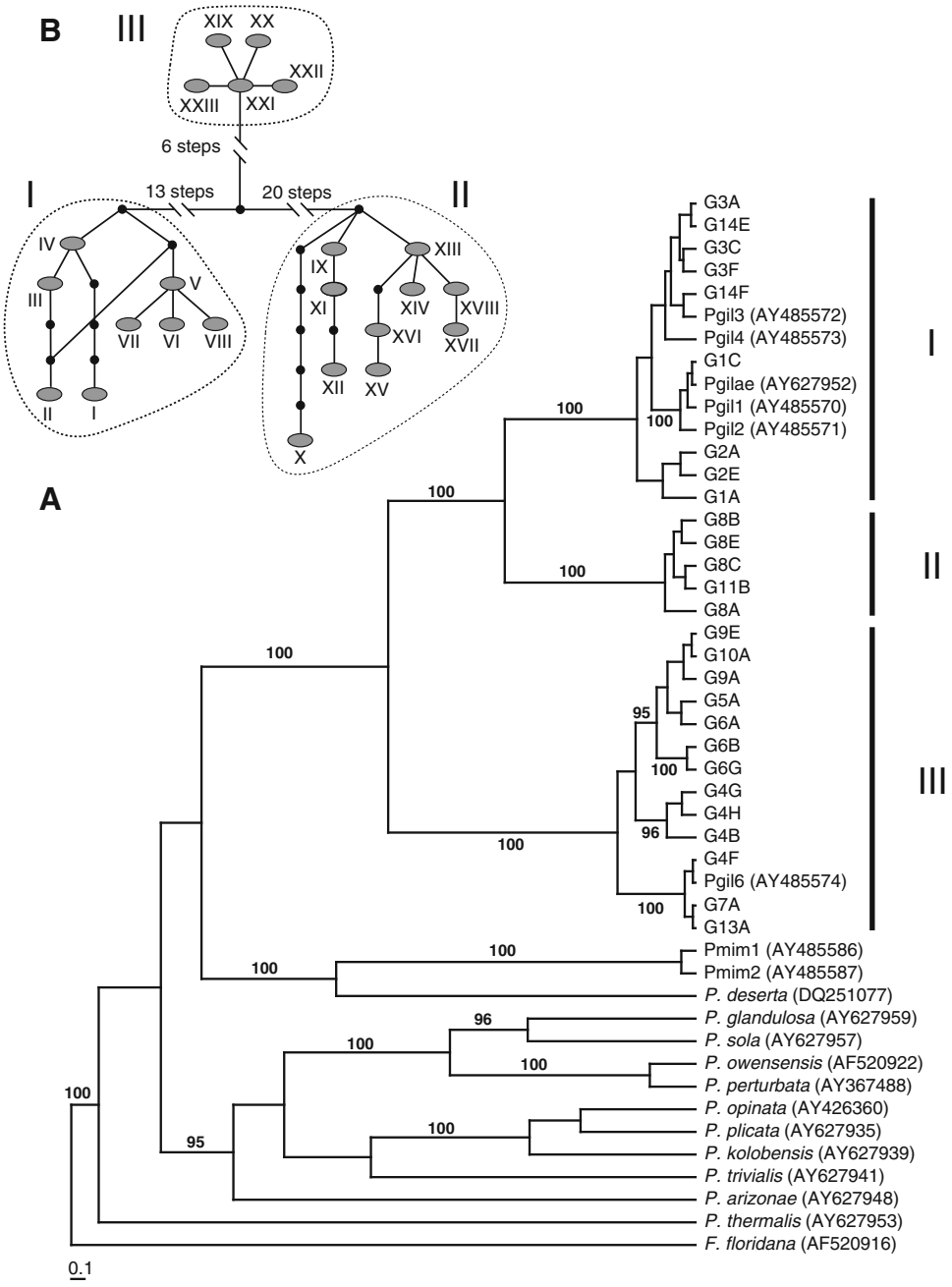

Fig. 2 **a** Bayesian tree. **b** Network of COI haplotypes (*I–XXIII*). GenBank accession numbers and sample codes (from Hurt 2004; Liu and Hershler 2005) are provided for previously published sequences. Posterior probabilities of nodes are provided when ≥95 %

Table 4 Mean COI sequence divergence within and among *P. gilae* clades

	Clade I	Clade II	Clade III
Clade I	0.006 ± 0.003		
Clade II	0.039 ± 0.002	0.003 ± 0.001	
Clade III	0.063 ± 0.004	0.046 ± 0.005	0.011 ± 0.012

I G1, G2, G3, G14; *II* G8, G11; *III* G4, G5, G6, G7, G9 G10, G13

they be managed as separate conservation units pending a taxonomic revision of *P. gilae*.

Our finding of a close (sister) relationship between lineages distributed along the Middle Fork (clade II) and along the lower reach of the East Fork and mainstem Gila River (clade I) is also of interest from the biogeographic perspective. Although the late Cenozoic history of the upper Gila River is generally one of increasing integration (Mack 2004; Connell et al. 2005) rather than vicariance, the development of the northwest trending "Gila Hot Springs graben" (Ratté et al. 1979) provides a plausible mechanism for divergence of the clade composed of these two lineages. The springs inhabited by these snails are situated along normal faults within and along the boundaries of the Gila Hot Springs graben (Witcher and Lund 2002), a down-dropped depression that is thought to be late Tertiary in age (Ratté and Gaskill 1975; Ratté et al. 1979; Elston 1981). (Note that the springs along the upper East Fork inhabited by members of clade III are associated with a different group of faults [Ratté et al. 1979: Fig. 16]). We speculate that the subsequent divergence of clades I and II is related to the history of spring activity along this fault zone.

The development of conservation plans for western springsnails traditionally has been based on the tacit assumption that these organisms are genetically homogeneous across their geographical ranges. Consequently, these plans do not include explicit strategies to maintain genetic diversity, which are considered an integral component of modern conservation biology (Allendorf et al. 2013). This study adds to a growing body of molecular evidence that, contrary to traditional interpretations, geographically disjunct subunits of *Pyrgulopsis* species are usually evolutionarily distinct, and sometimes represent monophyletic lineages that may warrant formal taxonomic recognition. The data presented herein suggest that even those congeners that are narrowly distributed within a single drainage basin can exhibit strong population structure, as might be expected given the remarkable propensity for local differentiation in *Pyrgulopsis*. These findings suggest that it would be prudent for land managers to treat disjunct populations of springsnails as potentially evolutionarily distinct (and thus a conservation priority) unless there is evidence to suggest otherwise. Additional studies are urgently needed to detail the genetic diversity of other congeners, especially the numerous narrow-range endemics which are most vulnerable to habitat perturbation (per Harvey et al. 2011).

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