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Genetic divergence, population structure and historical demography of rare springsnails (*Pyrgulopsis*) in the lower Colorado River basin

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Abstract

Springsnails of the genus *Pyrgulopsis* are the most diverse group of freshwater gastropods in North America and current estimates show that *Pyrgulopsis* contains ~120 different species, many of which are at risk of extinction. Some factors contributing to their exceptional diversity include poor dispersal ability and extreme habitat specificity based on water availability, chemistry and depth. Most taxa exhibit high degrees of endemism, with many species occurring only in a single spring or seep, making springsnails ideal for studies of speciation and population structure. Here I present data from a survey of genetic variation at the mitochondrial gene cytochrome oxidase I from 37 populations and over 1000 individuals belonging to 16 species of *Pyrgulopsis* distributed throughout the lower Colorado River basin. High levels of interspecific sequence divergence indicate that *Pyrgulopsis* may have colonized this region multiple times beginning in the late Miocene (~6 Ma); earlier than previous estimates based on fossil evidence. Estimates of nucleotide diversity differ greatly among species and may reflect differences in demographic processes. These results are used to identify factors contributing to radiation of species in this region. The implications of this evolutionary history and genetic variation are discussed in relation to future management and conservation.

Keywords: conservation, Mollusca, mtDNA, phylogeography, population structure, *Pyrgulopsis*

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Introduction

Widespread elimination of freshwater habitat has led to a recent emphasis on the conservation of freshwater invertebrates. In this regard, molluscs are of particular concern both because of their diversity, it is estimated that the western USA has ~450 native freshwater snail species, and because of their sensitivity to environmental change. Extinctions of nonmarine molluscs from human-related causes outnumber those of birds and mammals combined (Ponder *et al*. 1995). Despite their often small size and inconspicuous nature, molluscs play a key role in maintaining ecosystem health, occupying an important niche in basal food chains as major consumers of aquatic plants and as a critical food source for fish, amphibians, reptiles and birds.

The molluscan family Hydrobiidae is the most diverse group of aquatic snails in the world, encompassing almost 400 genera. Among these, the hydrobiid genus *Pyrgulopsis* is the most species-rich molluscan genus in North America, with an estimate of ~120 species (Kabat & Hershler 1993; Liu *et al*. 2003). Members of this genus, commonly called springsnails, are small (1–8 mm in length), gonochoristic snails with distributions ranging from southern Canada to northern Mexico (Hershler 1994).

Springsnails possess several life history traits that make them vulnerable to extinction (Dillon 1988). First, they have restrictive habitat requirements, typically occurring in pristine coldwater or thermal springs within a few metres of the spring source, where dissolved carbon dioxide and calcium concentrations are high (O'Brien & Blinn 1999; Mladenka & Minshall 2001). Slight changes in water chemistry or temperature, particularly contamination and trampling of vegetation resulting from livestock use, can quickly eliminate a population. Second, springsnails are poor dispersers, rarely moving more than a few metres per generation, while adequate habitats are generally isolated from each other by kilometres of inhospitable water or arid

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landscape. Therefore, once a springsnail population has been extirpated, there is a low probability of recolonization (Ponder & Colgan 2002).

Habitat specificity and low dispersal capabilities have contributed to a high degree of endemism in this genus, with many species occurring only within a single spring or seep. Therefore, dewatering of a single springhead for human related activities can lead to the loss of an entire species (Landye 1981; Ponder & Colgan 2002). Currently, over one third of the recognized species of *Pyrgulopsis* are candidates for listing under the Endangered Species Act (Hershler 1994). Three described species of *Pyrgulopsis* have gone extinct since their description in the early 1900s and it is likely that other species have been lost before they have been discovered.

Effective management of endangered species requires reliable information on occupied habitat, population structure and taxonomic relationships. Early studies of springsnail taxonomy relied on shell morphology (Taylor 1970). Species in the genus *Pyrgulopsis* are distinguished by a combination of small size, ovate to ovate–conic shell and a penis with relatively few glands (Hershler 1994). However, shell characteristics of freshwater gastropods are simple in comparison with marine species and have been shown to vary widely in response to environmental conditions (Boeters 1982). Hershler & Thompson (1986) redefined the genus based on penial morphology. While this method has had greater success in distinguishing between closely related taxa, relationships between species have been difficult to resolve due to repeated instances of morphological homoplasy (Hershler 1994; Liu *et al*. 2003). Furthermore, the localized nature of these fauna could contribute to cryptic species that may not be readily detected by morphological criteria. Therefore, detailed genetic studies are needed to: (i) reliably document the extent and patterns of divergence among morphologically defined springsnail species, (ii) identify genetically unique populations that qualify for special protection, and (iii) to further investigate regions of endemism in this genus.

In addition to contributing vital information for invertebrate conservation, information regarding the geographical distribution of genetic variation in springsnails could improve our understanding of speciation processes and the aquatic phylogeography of western North America. Hydrobiids have been present in the southwestern USA since the mid-Tertiary (Hershler 2000), therefore their distribution may reflect geological events that have occurred throughout the late Cenozoic. Their limited dispersal ability has contributed to a rapid radiation of species tightly linked to drainage history. Previous genetic studies of related hydrobiid taxa have uncovered extensive variation on a microgeographical scale within drainage systems (Colgan & Ponder 1994). The Colorado River and its tributaries have been the subject of numerous biogeography studies because of the dynamic geological history of the region. Thus far, such studies have focused almost exclusively on fish taxa (Minckley *et al*. 1986; Douglas *et al*. 1999). Information about genetic variation of codistributed invertebrate species with limited vagility will add insight to our understanding of the history of waterways in the Basin and Range Province.

There are two specific aims of this study. First, I examined the geographical distribution of genetic variation in the mitochondrial gene cytochrome oxidase I (COI) within and between springsnail populations belonging to the 16 described *Pyrgulopsis* species that occur in the lower Colorado River drainage. These data were used to: (i) test the validity of morphologically defined species, (ii) identify genetically unique populations in need of protection, and (iii) assess levels of genetic diversity within populations. Information regarding the genetic structure of springsnail populations will be valuable for management agencies in designing conservation strategies for these taxa. Second, I used a phylogeographical approach to identify evolutionary processes that could have contributed to the radiation of *Pyrgulopsis* species throughout this region.

Materials and methods

Collections

From July 2001 to October 2002, ~30 individuals were collected from each of 37 springsnail populations occurring in Utah, Arizona and New Mexico (Fig. 1, Table 1). Populations were defined as the group of individual snails existing at a single spring. Snails were typically found attached to hard substrates within several metres of the springhead. All but two of the sampled locations occur in the Colorado River drainage. San Bernardino Spring and Mimbres Spring are located just beyond the Gila River drainage, in the Rio Yaqui and Mimbres River drainages, respectively. Samples were obtained from all of the known *Pyrgulopsis* species in the lower Colorado River drainage system and nearly all known populations (see Hershler 1994 and Hershler & Landye 1988 for mapping locations). Snails were identified on the basis of general form, shell morphology and ecological habitat. All but two of the sampled taxa were distributed allopatrically. The exceptions were *P. gilae* and *P. thermalis*, which co-occur in several springs along the upper Gila River in New Mexico. Specimens were stored in absolute ethanol until DNA processing. Voucher specimens for each sampled population were reposited in the Melville Malacological Collection, Arizona State University.

Laboratory methods

For most samples, total genomic DNA was isolated using the Chelex protocol described by Walsh *et al*. (1991). DNA

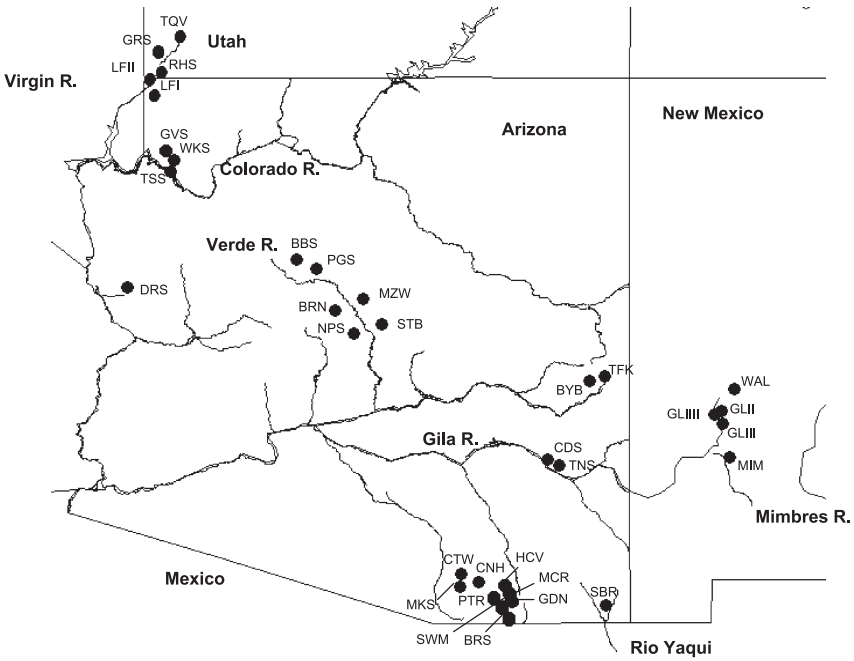

Fig. 1 Map showing collection sites for *Pyrgulopsis* specimens. Population abbreviations and information given in Table 1.

from *P. thermalis* was extracted using a modification of the CTAB protocol described by Bucklin (1992). A 658 bp segment of the COI gene was amplified using the universal primers LCO-1490 and HCO-2198 (Folmer *et al*. 1994) at a final concentration of 0.5 μM each with an annealing temperature of 50 °C. Polymerase chain reactions (PCR; 50 μL total volume) contained ~50 ng template DNA, 3.0 mM $MgCL_2$, 0.2 mM each of dNTPs, *Taq* DNA polymerase and 1 × *Taq* DNA polymerase buffer (Promega). Alleles were sequenced directly from PCR products on an Applied Biosystems 377 automated sequencer. This sequence was used to make two internal primer sets specific for *Pyrgulopsis*. The primer set LCO-1728 (5′-TCGGTGCTCCAGATATAGCAT-3′) and HCO-2070 (5′-GTAATAGCACCGGCCAGAAC-3′) amplifies a 342 bp region used for single strand conformational polymorphism (SSCP) analysis of most taxa examined in this study. The second primer set, LCO-1824 (5′-TCAGGCTTAGTTGGTACAGCTC-3′) and HCO-2121 (5′-CCAGCTGGATCAAAGAAAGC-3′), was used to amplify a 297 bp fragment used for SSCP analysis of all *P. thermalis* samples. SSCP analysis was performed using the same PCR conditions but was scaled down to a total volume of 5 μL and included 1 μCi of ^{32}P-dATP in each reaction. Alleles were separated by electrophoresis at 4 °C on a 6% polyacrylamide gel. The gel was then transferred to Whatman paper, dried and exposed to X-ray film (Kodak XOMAT AR) overnight. All unique alleles were then sequenced using the primer set LCO-1490 and HCO-2198. SSCP is a highly efficient technique for rapid screening of DNA polymorphisms from PCR products. Under optimal conditions, this method has been shown to identify single point mutations with up to 100% sensitivity (Glavac & Dean 1993). However, it is likely that some SSCP haplotypes represent multiple sequence classes.

Several studies have reported the presence of mitochondrial pseudogene copies in nuclear DNA (Zhang & Hewitt 1996; Williams & Knowlton 2001). Amplification of nuclear pseudogenes could lead to confounding results in phylogenetic analysis because compared sequences may not be homologous. In this study, I obtained both forward and reverse sequence products, and none of the PCR products appeared to be contaminated by a second product. Furthermore, all PCR products were of the same size and there were no insertions, deletions or stop codons as would be expected for a pseudogene. Therefore, I feel confident that the sequences presented here are mitochondrial in origin.

Statistics

Sequences were aligned manually using BIOEDIT v5.0.9 (Hall 1999) and are deposited in GenBank under Accession nos AY485532–AY485597 (Table 1). A neighbour-joining (NJ) tree was constructed using Kimura 2-parameter (K2P) genetic distances between all uniquely identified sequences of COI. Bootstrap confidence estimates for each node were based on 1000 replicates (Felsenstein 1985). A maximum-parsimony (MP) tree was also constructed using the close-neighbour-interchange algorithm to search through possible trees; bootstrap confidence intervals (1000 replicates) were performed to assess confidence at each node. COI sequence from *Graziana alpestris* was used to root the tree (Wilke *et al*. 2001); this sequence was identified by BLAST search and

Table 1 Systematic list of the *Pyrgulopsis* taxa and populations used in the study and the sample size (*N*) in each. Site abbreviations correspond to those given in Figs 1 and 4

Drainage	Spring	*N*	Map symbol	GenBank Accession nos
Virgin River				
P. kolobensis	Toquerville	33	TQV	AY485532–AY485533
P. deserta	Green	28	GRS	AY485534–AY485539
	Littlefield I	31	LFI	
	Littlefield II	33	LFII	
	Red Hill	31	RHS	
Colorado River				
P. bacchus	Grapevine	30	GVS	AY485540–AY485545
	Whiskey	33	WKS	
	Tassi.	34	TSS	
P. conicus	Dripping	30	DRS	AY485546
Verde River				
P. morrisoni	Bubbling	26	BBS	AY485546–AY485551
	Page	30	PGS	
P. montezumensis	Montezuma Well	34	MZW	AY485552–AY485553
P. solus	Brown	26	BRN	AY485554–AY485556
P. glandulosus	Nelson Place	25	NPS	AY485557
P. simplex	Strawberry	31	STB	AY485558
Gila River				
P. trivialis	Boneyard Bog	31	BYB	AY485559–AY485560
	Three Forks	31	TFK	
P. sancarlosensis	Cold	30	CDS	AY485561–AY485569
	Tom Niece	34	TNS	
P. gilae	Gila I	32	GLI	AY485570–AY485574
	Gila II	29	GLII	
	Gila III	29	GLIII	
	Wall	14	WAL	
P. thermalis	Gila I	31	GLII	AY485575–AY485585
	Gila II	5	GLII	
	Gila III	31	GLIII	
P. mimbres	Mimbres	34	MIM	AY485586–AY485587
P. thompsoni	Bear	32	BRS	AY485588–AY485594
	Canelo Hills	30	CNH	
	Cottonwood	30	CTW	
	McClure	32	MCR	
	Garden	36	GDN	
	Cave	30	HCV	
	Monkey	30	MKS	
	Peterson Ranch	36	PTR	
	Sawmill	32	SWM	
Rio Yaqui				
P. cochisi	San Bernardino	31	SBR	AY485595–AY485597

was shown to form an outgroup to all *Pyrgulopsis* taxa in both NJ and MP trees. Phylogenetic analysis, transition/transversion and d*N*/d*S* ratios were estimated using MEGA v2.1 (Kumar *et al*. 2001).

Relative rate tests for constancy of substitution rates and branch length estimates were obtained using PHYLTEST v2.0 (Kumar 1996). A molecular clock rate of $1.83 \pm 0.21 \times 10^{-8}$ ($1.83 \pm 0.21\%$ population divergence per million years) was used to estimate species divergence times and in calculating effective population sizes. This calibration was based on the split of the Hydrobia/Peringi lineage and an unnamed hydrobiine taxon that evolved on a Mediterranean island 5.33 Ma (Wilke & Pfenninger 2002; Liu *et al*. 2003; Wilke 2003) and is consistent with estimated COI divergence rates for several invertebrate transisthmian species pairs (Bermingham & Lessios 1993; Knowlton & Weigt 1998).

Hierarchical partitioning of genetic variation within and between populations and between morphologically defined species was examined as described by Weir & Cockerham

(1984) and implemented in analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992; Schneider *et al*. 2000). In a separate AMOVA, I also examined the partitioning of genetic variation among species belonging to different subdrainages. A minimum spanning network (MSN), illustrating the minimum number of mutational steps between haplotypes, was constructed using the algorithm of Rohlf (1973). Population and species pairwise genetic distances were estimated using a modification of the net D_A distance of Nei & Li (1979), where K2P genetic distances were substituted for the raw number of nucleotide differences between alleles. AMOVA, MSN and population pairwise genetic distances estimates were computed using ARLEQUIN version 2.0 (Schneider *et al*. 2000).

Standard descriptive polymorphism statistics including nucleotide diversity (π), haplotype frequencies, and Tajima's *D*-statistic were calculated with ARLEQUIN version 2.0. Statistical significance of Tajima's *D*-statistic was adjusted for multiple tests using a sequential Bonferroni correction (Rice 1985). These analyses were confined to a 524 bp subset of PCR product after removing all nucleotide sites that were missing in > 5% of sequences. Effective female population sizes (N_{ef}) for all polymorphic populations were calculated in two different ways. The first method used the net nucleotide diversity (π) for estimation of θ_f. For the second estimate, I used the computer application FLUCTUATE (Kuhner *et al*. 1998) to estimate the long-term historical effective population size. This program uses a maximum likelihood coalescent approach to simultaneously estimate the exponential growth parameter (g) and θ_f from population sequence data, where $\theta_f = 2N_{ef}\mu$, and N_{ef} is the female effective size for mitochondrial genes. The search strategy used was as follows: ten short chains of 1000 steps each, sampling every 20th step, followed by two long chains of 15 000 steps each, sampling every 20th step.

To test for isolation by distance, a Mantel test was used to measure the correlation between species pairwise stream and Euclidean distances with the species pairwise K2P genetic distances. In addition, I performed a partial Mantel test relating geographical and genetic distances while controlling for species in a third matrix (Smouse *et al*. 1986). Both tests were performed using PASSAGE version 1.0.3.8 (Rosenberg 2003).

Results

Sequence data

SSCP analysis identified 66 unique alleles among the 1105 surveyed individuals. Distribution of haplotypes among populations is summarized in Table 2. In total, 210 of 658 nucleotide sites (31.9%) were polymorphic and 177 sites were parsimony informative. The empirical nucleotide composition was A–T biased. Average base pair frequencies were 37.4% T, 25.0% A, 19.0% C and 18.6% G, similar to that found for the hydrobiid genus *Tryonia* (Hershler 2000). Transitions outnumbered transversions, with an average transition/transversion ratio of 11.38. The overall average d*N*/d*S* ratio was 0.005, consistent with strong purifying selection commonly found in mitochondrial coding genes.

Tajima's *D*-statistic was significantly greater than zero in two populations after adjusting for multiple tests (Table 2). Positive *D*-statistics result when relatively divergent haplotypes in a population are found in too-even frequencies. This may result from balancing selection or following a population bottleneck where rare haplotypes that are phylogenetically intermediate to common haplotypes are lost from a population. The highest observed *D*-statistics were found in the Red Hill and Green Spring populations of *P. deserta* ($D = 3.496$, $P < 0.0001$; $D = 3.539$, $P < 0.0001$, respectively). These two populations are found about 10 km apart in the Virgin River drainage in St. George, Utah and are each characterized by two very divergent alleles at nearly equal frequencies. Alleles *Pdes 1* and *Pdes 2* (Table 3), found in the Green Spring population, are separated by a minimum of 19 mutational steps and are at frequencies of 54% and 46%, respectively. Similarly, alleles *Pdes 6* and *Pdes 7*, found in the Red Hill Spring population, are separated by a minimum of 15 mutational steps and are at frequencies of 55 and 45%, respectively. No haplotypes are shared between these two populations.

Diversity within populations and effective population size

Nucleotide diversity per site (π) within populations ranged from 0 to 1.87% with an average of 0.36%. The highest value of nucleotide diversity was found in Green Spring and San Bernardino Spring, $\pi = 1.87\%$ for both populations. The number of haplotypes per population ranged from 1 to 5, with an average of 2.02. *Pyrgulopsis sancarlosensis* had the largest number of haplotypes per population; Cold Spring and Tom Niece Spring had five and four haplotypes, respectively. Fourteen populations were fixed for a single haplotype.

Under the assumption of the infinite-sites Wright–Fisher model, nucleotide diversity of mitochondrial DNA (mtDNA) can be used to give unbiased estimates of the long-term historical effective female population size. Assuming a molecular clock of 1.83% sequence divergence per million years, the effective female population size across all polymorphic populations ranged from 5464 found in Gila II population of *P. gilae* and Canelo Hills populations to 355 191 found in the Bubbling Spring population, with an average of ~77 000 per population, across all populations. N_{ef} for populations that showed a significant Tajima's *D*-statistic were not evaluated because they violated the assumptions of the Wright–Fisher model.

Table 2 Estimates of the number of different mitochondrial haplotypes, nucleotide diversity (π), Tajima's statistic D, nucleotide diversity estimate of N_{ef}, and the joint ML estimates of N_{ef} and the growth parameter (g) for each of the studied populations. *$P < 0.05$, **Significance after Bonferroni adjustment for multiple tests. ***Estimates not calculated due to small sample size

Population	No.	π	D	N_{ef} (π)	ML N_{ef} (± 95% CI)	g (± 95% CI)
P. kolobensis	2					
Toquerville	2	0.0004	−0.164	21 857	88 743 (± 21 742)	2 288 (± 1 454)
P. deserta	6					
Green	2	0.0187	3.539**	—	123 387 (± 50 338)	−207 (± 105)
Littlefield I	2	0.0005	0.445	27 322	6 120 (± 1 392)	−2 024 (± 9 173)
Littlefield II	1	—	—	—		—
Red Hill	2	0.0140	3.496**	—	61 366 (± 23 241)	−312 (± 69)
P. bacchus	6					
Grapevine	2	0.0008	1.578*	—	49 945 (± 17 350)	1 197 (± 1 892)
Whiskey	3	0.0018	−0.117	98 360	184 808 (± 48 732)	483 (± 483)
Tassi	3	0.0005	−1.607*	—	73 224 (± 20 135)	16 (± 833)
P. conicus	1					
Dripping	1	—	—	—	—	—
P. morrisoni	5					
Bubbling	4	0.0065	1.020	355 191	85 683 (± 23 991)	−330 (± 260)
Page	1	—	—	—		
P. montezumensis	2					
Montezuma	2	0.0001	−1.138	5 464	61 366 (± 23 241)	10 000 (± 7 842)
P. solus	3					
Brown	3	0.0009	−0.190	49 180	154 153 (± 33 095)	5 095 (± 690)
P. glandulosus	1					
Nelson Place	1	—	—	—	—	—
P. simplex	1					
Strawberry	1	—	—	—	—	—
P. trivialis	2					
Boneyard Bog	1	—	—	—	—	—
Three Forks	1	—	—	—	—	—
P. sancarlosensis	9					
Cold	5	0.0014	−1.125	76 502	173 333 (± 52 159)	787 (± 617)
Tom Niece	4	0.0025	−0.754	136 612	198 360 (± 44 233)	−42 (± 213)
P. gilae	5					
Gila I	2	0.0008	1.597*	—	39 890 (± 29 346)	4 028 (± 3 566)
Gila II	2	0.0002	−1.507*	—	39 890 (± 29 346)	−2 (± 1 135)
Gila III	1	—	—	—	—	—
Wall	1	—	—	—	—	—
P. thermalis	11					
Gila I	2	0.0002	−1.506	5 738	22 896 (± 5 622)	4 039 (± 2 646)
Gila II	5	***	***	***	***	***
Gila III	4	0.0020	−0.432	109 289	104 316 (± 24 633)	777 (± 716)
P. mimbres	2					
Mimbres	2	0.0005	−0.636	27 322	117 704 (± 31 274)	4 645 (± 1 624)
P. thompsoni	7					
Bear	1	—	—	—	—	—
Canelo Hills	2	0.0001	−1.147	5 464	49 289 (± 65 761)	Nan
Cottonwood	2	0.0024	−1.552	131 147	95 573 (± 34 701)	−290 (± 197)
McClure	1	—	—	—	—	—
Garden	1	—	—	—	—	—
Cave	1	—	—	—	—	—
Monkey	2	0.0004	−1.004	21 857	93 715 (± 29 132)	3 757 (± 2 067)
Peterson Ranch	2	0.0031	−1.850*	—	61 366 (± 23 241)	−392 (± 153)
Sawmill	1	—	—	—	—	—
P. cochisi	3					
San Bernardino	3	0.0187	1.632*	—	178 797 (± 58 907)	−149 (± 76)

Table 3 Haplotype frequencies for all populations where haplotypes are named starting with P (for genus name), followed by three letters indicating the species name and a number indicating the haplotype within species. Sample sizes are given in Table 1

Population	Haplotype frequencies										
P. kolobensis	*Pkol1*	*Pkol2*									
Toquerville	0.879	0.121	—	—	—	—	—	—	—	—	—
P. deserta	*Pdes1*	*Pdes2*	*Pdes4*	*Pdes5*	*Pdes6*	*Pdes7*					
Green	0.536	0.464	—	—	—	—	—	—	—	—	—
Littlefield I	—	—	0.194	0.806	—	—	—	—	—	—	—
Littlefield II	—	—	—	1.000	1.000	—	—	—	—	—	—
Red Hill	—	—	—	—	0.548	0.452	—	—	—	—	—
P. bacchus	*Pbac1*	*Pbac2*	*Pbac3*	*Pbac5*	*Pbac6*	*Pbac8*					
Grapevine	0.597	0.433	—	—	—	—	—	—	—	—	—
Whiskey	0.151	—	—	—	0.818	0.030	—	—	—	—	—
Tassi	—	0.030	0.941	0.030	—	—	—	—	—	—	—
P. conicus	*Pcon1*										
Dripping	1.000	—	—	—	—	—	—	—	—	—	—
P. morrisoni	*Pmor1*	*Pmor2*	*Pmor3*	*Pmor4*	*Pmor5*						
Bubbling	0.461	0.154	0.115	0.231	—	—	—	—	—	—	—
Page	—	—	—		1.000	—	—	—	—	—	—
P. montezumensis	*Pmon1*	*Pmon2*									
Montezuma	0.971	0.029	—	—	—	—	—	—	—	—	—
P. solus	*Psol1*	*Psol2*	*Psol3*								
Brown	0.719	0.250	0.031	—	—	—	—	—	—	—	—
P. glandulosus	*Pgla1*										
Nelson Place	1.000	—	—	—	—	—	—	—	—	—	—
P. simplex	*Psim1*										
Strawberry	1.000	—	—	—	—	—	—	—	—	—	—
P. trivialis	*Ptri1*	*Ptri2*									
Boneyard Bog	1.000	—	—	—	—	—	—	—	—	—	—
Three Forks	—	1.000	—	—	—	—	—	—	—	—	—
P. sancarlosensis	*Psan1*	*Psan2*	*Psan3*	*Psan4*	*Psan5*	*Psan6*	*Psan7*	*Psan8*	*Psan9*		
Cold	0.867	0.038	0.038	0.038	0.038	—	—	—	—	—	—
Tom Niece	—	—	—	—	—	0.875	0.031	0.031	0.031	—	—
P. gilae	*Pgil1*	*Pgil2*	*Pgil3*	*Pgil4*	*Pgil6*						
Gila I	0.563	0.437	—	—	—	—	—	—	—	—	—
Gila II	—	—	0.967	0.033	—	—	—	—	—	—	—
Gila III	—	—	1.000	—	—	—	—	—	—	—	—
Wall Spring	—	—	—	—	1.000						
P. thermalis	*Pthe1*	*Pthe2*	*Pthe3*	*Pthe4*	*Pthe5*	*Pthe6*	*Pthe7*	*Pthe8*	*Pthe9*	*Pthe10*	*Pthe11*
Gila I	0.968	0.032	—	—	—	—	—	—	—	—	—
Gila II	—	—	0.200	0.200	0.200	0.200	0.200	—	—	—	—
Gila III	—	—	—	—	—	—	—	0.613	0.323	0.323	0.323
P. mimbres	*Pmim1*	*Pmim2*									
Mimbres	0.912	0.088	—	—	—	—	—	—	—	—	—
P. thompsoni	*Ptho1*	*Ptho2*	*Ptho3*	*Ptho4*	*Ptho5*	*Ptho8*	*Ptho9*				
Bear	1.000	—	—	—	—	—	—	—	—	—	—
Canelo Hills	0.967	0.033	—	—	—	—	—	—	—	—	—
Cottonwood	—	—	0.933	0.067	—	—	—	—	—	—	—
McClure	—	—	—	—	1.000	—	—	—	—	—	—
Garden	—	—	—	—	1.000	—	—	—	—	—	—
Cave	—	—	—	—	1.000	—	—	—	—	—	—
Monkey	—	—	—	—	—	0.933	0.067	—	—	—	—
Peterson	0.944	—	—	—	0.056	—	—	—	—	—	—
Sawmill	1.000	—	—	—	—	—	—	—	—	—	—
P. cochisi	*Pber1*	*Pber2*	*Pber3*					—	—	—	—
San Bernardino	0.750	0.219	0.031	—	—	—	—	—	—	—	—

A second approach was used for estimating effective population size developed by Kuhner *et al.* (1998), which utilizes a maximum likelihood coalescent approach to simultaneously estimate theta (τ) and the exponential growth parameter (g). This method gave point estimates of N_{ef} ranging from 6120 for the Littlefield I population to 198 360 in the Tom Niece Spring population. The exponential growth rate was positive for nine populations, six populations showed patterns consistent with population decline and six populations showed no significant growth rate. Eight of nine populations with positive growth rates also had negative values of Tajima's *D*-statistic, as expected after a population expansion. The *P. gilae* I population had a significantly positive *D*-statistic and a significantly positive exponential growth rates. Four of the six populations with negative growth rates also had significantly positive *D*-statistics, consistent with the loss of rare, intermediate alleles following a population bottleneck.

Population structure and phylogenetic analysis

In the NJ tree (Fig. 2), all but one morphologically defined species form monophyletic groups that have high bootstrap support (see also the MSN tree in Fig. 3). The exception is the species pair, *P. thompsoni* and *P. conicus*. *P. thompsoni* is paraphyletic with respect to *P. conicus*, haplotype Ptho 5 groups with Pcon 1 with significant bootstrap support (bootstrap NJ = 51%). In general, species groups appear to form a nested hierarchy of clades. There is a large, well-supported clade that includes all taxa from the Verde, Colorado and Santa Cruz watersheds (bootstrap NJ = 97%). Within this group all haplotypes from taxa in the Verde drainage form a cluster that also includes all *P. thompsoni* and *P. conicus* haplotypes (bootstrap NJ = 68%). *P. bacchus* forms a sister taxa to this grouping. Interestingly, the geographically distant taxa *P. deserta* and *P. mimbres* haplotypes, separated by 717 km Euclidean distance and 1721 km of stream distances, form a sister group with significant bootstrap support (bootstrap NJ = 72%); however, the average genetic distance between the two species is high (= 7.86%). *P. thermalis* is the most genetically distinct of all the examined species with an average net genetic distance of 10.2% from all other species, forming an outgroup to all other taxa with high bootstrap support (bootstrap NJ = 99%).

Maximum-parsimony (MP) analysis yielded 57 candidate trees and branch support for internal nodes was less than that found for the NJ tree. Topology of the two trees differed slightly. Again, all but one of the morphologically defined species formed monophyletic clades. In the MP analysis *P. bernadensis* was paraphyletic; the divergent haplotype, *Pber1*, did not cluster with the other two haplotypes, *Pber2* and *Pber3*. *P. thompsoni* was monophyletic with low support (bootstrap MP = 47%). *P. thermalis* formed an outgroup to all other species (bootstrap NJ = 74%). Bootstrap values for all other internal nodes were < 50%.

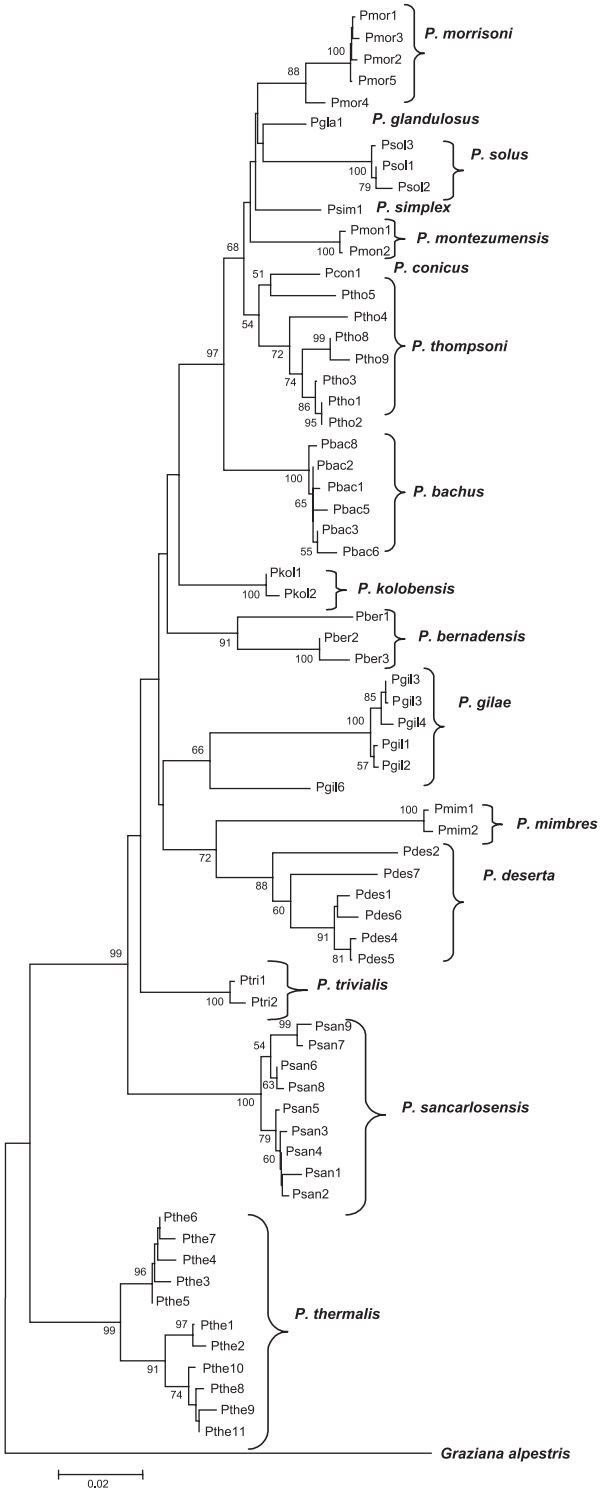

Fig. 2 Neighbor-joining tree of mtDNA sequence data. Numbers are bootstrap percentages for well-supported clades.

Results from hierarchical AMOVA (Table 4), which partitioned individuals by population and then by morphologically

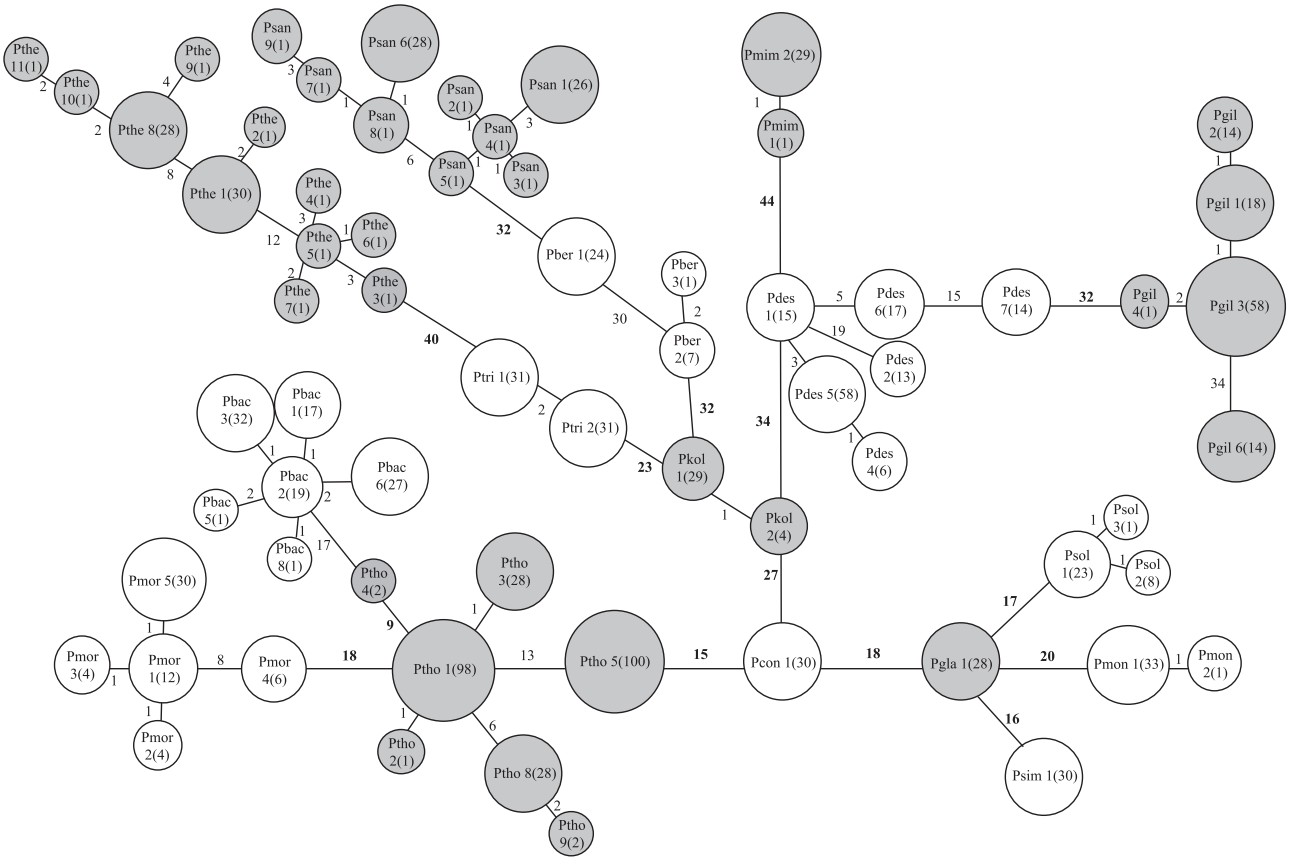

Fig. 3 Minimum spanning network showing all COI haplotypes. Haplotype IDs are indicated in circles. Numbers in parentheses correspond to the number of individuals with a given haplotype. Numbers on lines indicate the number of mutational steps between haplotypes.

Table 4 Hierarchical analysis of molecular variation (AMOVA) for 35 populations belonging to 16 morphologically defined species within the springsnail genus *Pyrgulopsis*

Source of variation	Variance components	Per cent of total	F_{CT}	F_{SC}	F_{ST}
Among species	17.38	80.57	0.806		
Among populations within species	3.39	15.73		0.809	
Within populations	0.80	3.70			0.963
Total	21.57	100.00			
Among drainages	0.24	1.09	0.011		
Among species within drainages	18.78	86.44		0.874	
Within species	2.71	12.47			0.875
Total	21.73	100.00			

defined species, showed that the majority of the genetic variance (80.57%) can be explained by differences among species. A smaller but significant portion of the genetic variance (15.73%) was attributed to multiple populations within species. The remaining 3.70% of the total genetic variation was due to within population variation. The second AMOVA testing for the partitioning of genetic variation among species within the five subdrainages of the lower Colorado showed that none of the genetic variation could be explained by drainage systems, suggesting that watersheds are not biologically relevant groupings.

The overall genetic pattern showed higher among-population variation than within-population variation. All geographically structured *Pyrgulopsis* taxa (i.e. taxa found at multiple springs) were found to contain significant genetic structure, with many conspecific populations composed entirely of private alleles. K2P genetic distances between individuals, within populations, were all < 2.10% and

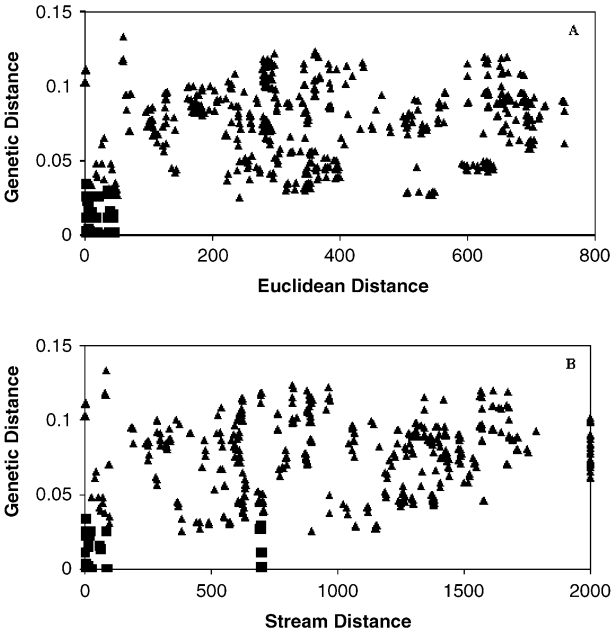

Fig. 4 Population pairwise Kimura two-parameter genetic distances plotted against (A) Euclidean and (B) stream distances. Squares indicate comparisons between conspecific populations and triangles indicate comparisons between populations belonging to different species.

averaged 0.198%, while genetic distances between conspecific populations ranged from 0 to 3.44% and averaged 1.38% (Table 5). Net genetic distances between species ranged from 2.19 to 12.46% and averaged 7.28%.

Relationship between genetic distance and geographical distance

Results from the first Mantel test comparing all pairwise population K2P distances with geographical Euclidean and stream distances (Fig. 4) were highly significant ($r = 0.223$, $P = 0.003$ and $r = 0.225$, $P < 0.001$ for Euclidean and stream distances, respectively). However, a partial Mantel test showed no significant correlation between genetic and geographical distances when comparisons between conspecific populations were removed ($P > 0.10$ for both tests), indicating that the overall relationship between geographical and genetic distances is due to taxonomic structure and does not necessarily indicate a pattern of isolation-by-distance.

Discussion

This study provides the first comprehensive examination of the structuring genetic variation within and between species of the springsnail *Pyrgulopsis* in the lower Colorado River Basin, many of which are at risk of extinction. Hierarchical analysis of mtDNA has revealed large genetic distances between species, significant structure among conspecific populations, and substantial genetic variation within populations. These results demonstrate that springsnails make an ideal model organism for the study of population structure and speciation and may also be valuable for management and conservation planning. Prioritizing populations for protection, initiating captive populations, and performing reintroductions require information on the genetic structuring of populations. Furthermore, springsnails are excellent candidates to serve as bioindicators for managing habitats rich in invertebrate diversity. Endemic springsnail populations in the southwest are relictual; fossil evidence for species of *Pyrgulopsis* dates back to the Pliocene and early Pleistocene (Taylor 1985, 1988). It follows that their distribution reflects habitats that were historically widespread, but are now limited and are likely to contain other endemic species.

Population demography

Results from both Tajima's *D*-statistic and maximum likelihood growth parameter estimates provide evidence that several of the populations may have undergone recent or historical population bottlenecks. Given the stringent ecological parameters limiting springsnail distributions, this result is not surprising. Spring outflows can vary greatly depending on levels of precipitation and groundwater pumping; these fluctuations are likely to have a large effect on springsnail numbers (Myers & Resh 1999). The large *D*-statistics in Green Spring, Red Hill Spring and San Bernardino Spring are the result of large genetic distances between haplotypes that occur in a single population. An alternative explanation for these results is that the large genetic distances between haplotypes within a single population could be due to population admixture. This is particularly likely in Peterson Ranch, where the rare divergent haplotype, *Ptho5* is fixed in several nearby springs.

The high levels of genetic diversity found in many populations indicate that historical long-term effective sizes have been quite large. These results are encouraging for the long-term persistence of springsnail populations because they suggests that, despite recent disturbances to freshwater habitat, springsnail populations have maintained sufficiently large numbers to retain potentially adaptive genetic variation. However, continued loss of habitat could result in lowered numbers and erosion of genetic variation in the future.

Taxonomic units and units of conservation

Both phylogenetic analyses and AMOVA results generally support morphologically defined species designations. Fourteen of 16 species were monophyletic with significant bootstrap support and the majority of the genetic variation

Table 5 Average Kimura 2-parameter genetic distances (× 100) between conspecific populations and between species

	Genetic distance between popn	Genetic distance between species													
		P. kol	*P. des*	*P. bac*	*P. con*	*P. mor*	*P. mon*	*P. sol*	*P. gla*	*P. sim*	*P. tri*	*P. san*	*P. gil*	*P. the*	*P. mim*	*P. tho*
P. kolobensis	—															
P. deserta	1.22	6.24														
P. bacchus	0.14	5.91	8.12													
P. conicus	—	5.34	8.25	4.36												
P. morrisoni	0.66	5.66	8.66	4.83	3.93											
P. montezumensis		6.63	8.36	4.37	4.37	4.03										
P. solus	—	6.59	8.67	6.28	5.01	4.62	4.81									
P. glandulosus	—	5.55	7.45	4.03	2.54	2.93	3.15	3.36								
P. simplex	—	5.56	7.69	4.99	3.76	3.34	3.76	4.81	2.54							
P. trivialis	0.38	4.48	6.62	7.33	7.02	7.50	7.25	8.37	6.15	6.60						
P. sancarlosensis	1.47	7.22	8.13	7.97	8.52	8.19	8.77	10.17	6.84	8.77	6.17					
P. gilae	3.44	6.97	7.32	8.07	7.92	7.66	9.43	8.91	8.28	9.02	8.17	8.00				
P. thermalis	2.25	8.47	10.61	10.99	11.11	10.95	11.12	11.57	9.96	10.95	7.75	8.45	9.65			
P. mimbres	—	9.29	7.86	10.21	11.37	11.45	10.68	11.38	10.20	11.15	9.77	9.50	10.80	12.46		
P. thompsoni	1.44	5.54	7.72	3.97	2.19	3.73	4.11	4.70	2.46	3.08	6.67	7.66	8.43	10.15	10.92	
P. bernadensis	—	6.15	8.42	6.87	6.86	7.25	7.16	8.17	6.09	6.59	6.70	8.04	8.85	9.37	8.97	6.85

P. kol, *P. kolobensis*; *P. des*, *P. deserta*; *P. bac*, *P. bacchus*; *P. con*, *P. conicus*; *P. mor*, *P. morrisoni*; *P. mon*, *P. montezumensis*; *P. sol*, *P. solus*; *P. gla*, *P. glandulosus*; *P. sim*, *P. simplex*; *P. tri*, *P. trivialis*; *P. san*, *P. sancarlosensis*; *P. gil*, *P. gilae*; *P. the*, *P. thermalis*; *P. mim*, *P. mimbres*; *P. tho*, *P. thompsoni*.

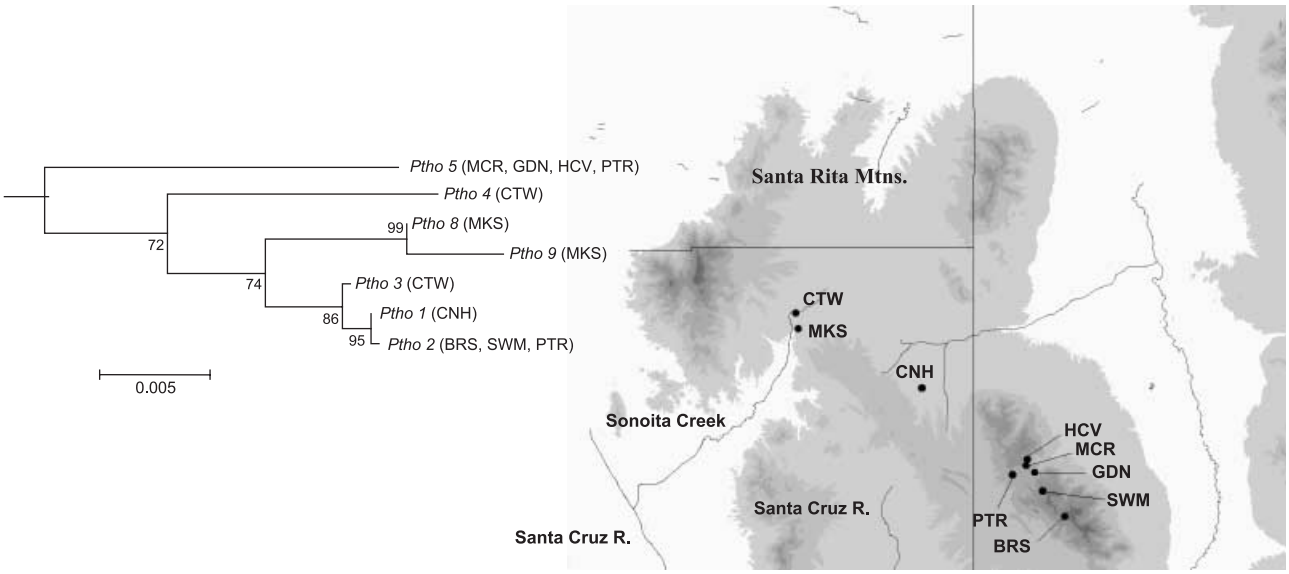

Fig. 5 Digital elevation map showing distribution of mitochondrial haplotypes in the nine surveyed populations of *P. thompsoni*.

was accounted for by current taxonomic classification. However, the paraphyletic relationship observed between *P. conicus* and *P. thompsoni* may be an indication of initial wrong identification and warrants further investigation by additional molecular markers.

In several taxa, high levels of genetic subdivision are found below the species level that suggest subspecific taxonomic units may be appropriate and further investigation with additional molecular markers is needed. Several conspecific populations have genetic distances that fall within the range of interspecific distances observed in this study as well as in surveys of COI in *Pyrgulopsis* (Liu *et al*. 2003) (COI genetic distances ranged from 1.1 to 11.6%) and in the related hydrobiid genus *Tryonia* (Hershler 2000) (COI genetic distances ranged from 1.3 to 14.8%). However, as mtDNA lacks recombination and selection acting on a single mitochondrial gene will affect the entire molecule, corroborating evidence from nuclear data is needed prior to reassigning taxonomic status.

The *P. gilae* population at Wall Spring, a small spring located near Taylor Creek in the Gila National Forest, is both geographically and genetically distinct from the other surveyed *P. gilae* populations. The Wall Spring population is fixed for a single haplotype, *Pgil 6*, that has a minimum of 34 mutational steps from other conspecific haplotypes and forms an outgroup to all other *P. gilae* haplotypes with 100% bootstrap support. This population has the largest within-species interpopulation distance observed in this study, with an average genetic distance of 6.23% from all other conspecific alleles.

P. deserta is only known from springs along the Virgin River in southwestern Utah. Analysis of four populations has shown significant genetic differences between (i) Green Spring population; (ii) Littlefield Population I and Littlefield Population II; and (iii) Red Hill Spring. These three groups possess nonoverlapping sets of haplotypes with large genetic distances between them, the average net pairwise genetic distance between these three groups is 1.50%.

Within *P. thompsoni* (Fig. 5), the three populations on the east slope of the Huachuca Mountains, including McClure Spring, Garden Spring and Huachuca Cave Spring, are fixed for the divergent haplotype *Ptho* 5, which has a large average genetic distance from all other conspecific haplotypes (average K2P genetic distance = 2.86%).

The two *P. thompsoni* populations that occur at lower elevations along Sonoita Creek and in the San Rafael Valley possess all unique alleles with large genetic distances from other conspecific haplotypes. Cottonwood Spring is located in a cold-water spring along Sonoita Creek between Sonoita and Patagonia, Arizona. This population is the most distant geographically, and is fixed for two unique alleles, *Ptho3* and *Ptho4*. Finally, the Monkey Springs population is both genetically and environmentally unique. This population is fixed for two unique haplotypes, *Ptho8* and *Ptho9*, which are substantially divergent from all other *P. thompsoni* alleles and form a monophyletic group with high bootstrap support (bootstrap NJ = 99). Monkey Spring is an isolated constant temperature thermal spring, averaging 28 °C. It once supported several unique fish taxa indicating long isolation from the surrounding watershed. These include a now extinct species of pupfish (*Cyprinodon arcuatus*), an extinct form of the chub *Gila intermedia*, and a unique population of Gila topminnow (*Poeciliopsis occidentalis*), classified as an ESU, which still exists there (Minckley 1999; Parker *et al*. 1999).

Based on male and female genitalia and shell descriptions, *P. thermalis* is the most morphologically distinct species of

the genus (Taylor 1987). The three sampled *P. thermalis* populations, which occur sympatrically with *P. gilae*, are endemic to several thermal springs along the east fork of the Gila River. No haplotypes are shared between the three populations and all form reciprocally monophyletic groups in both NJ and MP analysis. The average net interpopulation genetic distance between these populations was 2.25%. The *P. thermalis* population at Gila Spring II is the most distinct population, with an average genetic distance of 2.62% from the other conspecific populations. The *P. thermalis* II population forms an outgroup to *P. thermalis* I and III with high bootstrap support (bootstrap NJ = 91%).

Phylogenetics and biogeography

Vicariant events shaping the distribution of *Pyrgulopsis* species may reflect freshwater drainage rearrangements, tectonic shifts, or possibly migratory flyways of waterfowl (Taylor 1985; Ponder *et al*. 1994; Liu *et al*. 2003). Several lines of evidence suggest that phylogeographical patterns are not congruent with contemporary drainage patterns. Phylogenetic analysis shows that species did not usually cluster according to their respective subdrainage, with the exception of species found in the Verde rainage. All Verde taxa grouped together, however, bootstrap support for this clade was not high. Mantel test results for isolation by stream distance were nonsignificant. Finally, results from AMOVA showed that contemporary drainage systems could not account for any of the observed genetic variation. Limited biogeographical interpretations can be made from this data, however it is possible to place divergence of lineages into a general geological context.

Levels of sequence divergence in COI suggest that diversification of *Pyrgulopsis* species correlates to the late Miocene to the early Pleistocene; a time when extension and faulting throughout the Basin and Range region resulted in dramatic changes to drainage patterns. This is consistent with observed levels of COI divergence in *P. micrococcus* lineages in Death Valley, CA (Liu *et al*. 2003). Variation in climate resulting from changes in latitude, altitude and glaciation also certainly affected the distribution of freshwater taxa. The Miocene to Pliocene saw a shift from a subtropical setting towards a cooler drier climate. Increasing aridity in recent times promoted fragmentation of watersheds and isolation of resident taxa (Minckley *et al*. 1986). Phylogenetic analysis indicates that the upper Gila River drainage was likely the first area to be colonized. *P. thermalis* sequences form the most basal lineage in the tree; this species is an outgroup to all other taxa with an average divergence of 11.10%, suggesting that it has been isolated since the late Miocene (~6 Ma). This is consistent with geological evidence that thermal-spring species may have been the first to colonize the southwest (Taylor 1987). All other taxa from the upper Gila River, including *P. sancarlosensis*, *P. gilae* and *P. trivialis* have very large interspecific genetic distances and do not cluster with other species in the NJ or MP trees.

The occurrence of springsnails in the San Bernardino area is consistent with geological evidence indicating a connection between the Gila River and the Rio Yaqui by way of the San Simon Trough (Melton 1960; Minckley *et al*. 1986). This connection was severed in the early Pleistocene as lava flow in the San Bernardino region and uplift in the Chiricahuas caused Mexican drainages to turn southward towards the Rio de Bavispe. As further support of this connection, codistribution of aquatic taxa in the Gila and Yaqui watersheds is found in several fishes including the Gila topminnow (*Poeciliopsis occidentalis*) and the Sonoran Sucker (*Catostomus insignis*) (Hendrickson *et al*. 1980; Minckley *et al*. 1986).

The two species from the Virgin River drainage, *P. deserta* and *P. kolobensis*, also appear to be of considerable antiquity; both taxa have high levels of interspecific sequence divergence, and in the case of *P. deserta* there are high levels of interpopulation and intrapopulation genetic variation as well. One surprising result was the sister relationship between *P. mimbres* and *P. deserta*. Because there is no known connection between the Virgin and Mimbres Rivers, the relationship between these two taxa is not easily explained.

High bootstrap support indicates a monophyletic group comprising all haplotypes from the Lower Colorado, Verde and Santa Cruz Rivers. The maximum genetic divergence separating these taxa is < 5%. This genetic distance is consistent with a divergence time that postdates uplifts severing the connection between the Gila and Santa Cruz Rivers. The upper Gila River then flowed south into Mexico or into a closed basin near Safford, Arizona; possibly explaining why there is no connection between taxa in the Gila River and those from the rest of the lower Colorado River. Within this group, the paraphyletic relationship between *P. conicus* and *P. thompsoni* haplotypes may be due to an overland dispersal event from the Santa Cruz to the Colorado as there is no known connection between these rivers. The greater genetic diversity of *P. thompsoni* implies it is the older of the two taxa. *P. conicus* formerly occurred at three different springs along the Colorado River near Kingman, Arizona. Genetic analysis of these populations would be valuable for further analysing the relationship between these species. Unfortunately, two of the three populations have become extirpated within the last decade. Taxa within the Verde drainage show low levels of genetic divergence, indicating colonization in this region is probably relatively recent. Colorado and Verde River haplotypes are closely related, and may support an early connection between them via the Bill Williams drainage.

Conclusions

Our molecular phylogeny of Colorado springsnails demonstrates a complex relationship among taxa that cannot be

explained by drainage patterns or geographical Euclidean distances. Given the extensive tectonic and climatic changes that occurred around the time springsnails colonized the Basin and Range, these results are not surprising. Rugged landscapes, increased aridity and low vagility have led to high levels of endemism on a fine geographical scale. Molecular analysis shows high levels of genetic divergence between geographically close conspecific populations, indicating that many have been isolated for extended periods.

Despite isolation, habitat specificity and recent compromises to habitat quality, many populations have maintained haplotypic variation. This is a positive sign for the future persistence of springsnail species, many of which are candidates for listing as threatened or endangered. Research and theory both show a positive link between genetic variation and fitness traits (Saccheri *et al*. 1998; Hedrick & Kalinowski 2000). Management efforts should strive to maintain maximum genetic diversity by preserving as many populations as possible; particular emphasis should be placed on those populations with highly diverged haplotypes and unique environmental traits.

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