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SHORT COMMUNICATION

Phylogeography of an endangered Western North American springsnail

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Abstract We provide the first genetic analysis of the Bruneau Hot Springsnail (*Pyrgulopsis bruneauensis*), a federally listed (endangered) hydrobiid gastropod that is distributed in spring-fed habitats along a short reach of the Bruneau River in southwestern Idaho and threatened with extinction by groundwater withdrawal. Partial sequences of mitochondrial cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase subunit I (NDI) were obtained from 51 specimens from six sites spanning the narrow geographic range of *P. bruneauensis*. A Bayesian analysis of the combined dataset resolved this species as a well supported clade which differed from other regional congeners by 4.66–10.62% sequence divergence (COI). The 11 observed COI haplotypes in *P. bruneauensis* formed two divergent (1.42 ± 0.7%) subgroups that co-occurred at five of the six collecting sites. COI haplotype diversity was substantial (ranging up to 0.9111) in all but one sample, while nucleotide diversity was low (<0.01). AMOVA detected small but significant variation among sites, although only one sample was significantly differentiated by pairwise comparisons. Haplotype composition varied widely among the collecting localities and no obvious geographic pattern was detected. These findings suggest that translocation of snails, which was considered as a possible measure in the *P. bruneauensis* recovery plan, should be preceded by assays to ensure selection of appropriately genetically diverse source populations.

Keywords Phylogeography · Gastropoda · Endangered species · Springs · Conservation

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Introduction

The hydrobiid gastropod genus *Pyrgulopsis* (133 species) is one of the most diverse elements of the western North America aquatic biota. Most of these tiny (1–8 mm shell height), gill-breathing species are endemic to single springs, single spring systems, or local drainages (Hershler and Sada 2002). These snails are at risk of extirpation owing to their narrow geographic ranges and various anthropogenic threats to their small, fragile habitats. Six congeners have become extinct during historic times (Hershler 1994, 1998) and 70% of the extant fauna (89/127 species) is currently classified as critically imperiled (G1) by NatureServe (2010).

The Bruneau Hot Springsnail, *P. bruneauensis*, is distributed in predominantly thermal spring habitats along a short (ca. 8 km) reach of the Bruneau River in southwestern Idaho (Fig. 1). This species was ruled as endangered by the United States Fish and Wildlife Service in 1993 principally because of the threats posed by the reduction of its thermal spring habitats owing to agriculture-related groundwater withdrawal (USFWS 1993). Previous studies of this species have focused on its ecology, life history (Mladenka and Minshall 2001) and recent decline (Myler et al. 2007). The only previously published genetic data for *P. bruneauensis* are mitochondrial DNA (mtDNA) sequences from a single specimen (Hershler et al. 2003) which was delineated as a divergent (4.9–10.4% COI) terminal of uncertain

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relationships within the genus (Liu and Hershler 2005). Here we provide a baseline assessment of the genetic diversity, population structure and phylogenetic relationships of *P. bruneauensis* based on analyses of mitochondrial DNA sequences. Mitochondrial DNA polymorphisms have been frequently used in phylogeographic studies of *Pyrgulopsis* species (e.g., Liu et al. 2003; Hurt 2004; Hershler et al. 2007).

Materials and methods

Specimens

Samples were collected by Idaho (United States) Fish and Wildlife Office (IFWO) staff in 2009 from six sites, which span most of the current range of *P. bruneauensis* (Fig. 1) and include the three principal habitats (Hot Creek, thermal springs along the Bruneau River, mainstem Bruneau River) in which this species lives. Specimens were preserved in 90% ethanol. Partial sequences of cytochrome *c* oxidase subunit I (COI) were obtained from seven to ten specimens from each population sample; sample sizes were constrained by restrictions stipulated in the IFWO collecting permit. Previously published sequences of *P. bruneauensis* (obtained from a single specimen) were not included in this study because the precise location of the sampled population is unknown (Cary Myler email to RH, 5-XI-2009). Single sequences from eight other regional congeners were included in our analyses to further assess the divergence and phylogenetic relationships of *P. bruneauensis*.[1] Trees were rooted with sequences of *Floridobia floridana* following Hershler et al. (2003). Locality details and other pertinent information for all of these samples are in Table 1.

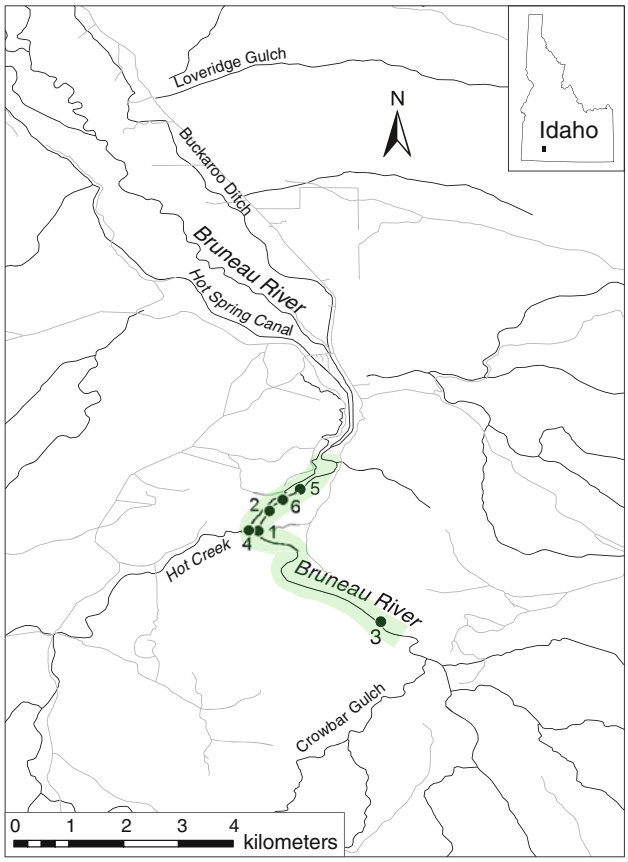

Fig. 1 Map showing the location of collection localities for *P. bruneauensis*. Drainage and roads are indicated by *black* and *grey lines*, respectively. The *shaded area* delineates the current distribution of this species (from USFWS 2007)

A parallel set of NADH dehydrogenase subunit I (NDI) sequences was also obtained and analyzed as part of this study. However, since the results from these two mitochondrial genes were entirely congruent, we only present the COI findings herein except for the phylogenetic analysis, which was based on the combined dataset.

Methods

Genomic DNA was extracted from entire snails using a CTAB protocol (Bucklin 1992). Partial sequences of COI (658 bp) were amplified and sequenced with primers LCO1490 and HCOI2198 (COI) following the protocols of Liu et al. (2003). Sequences were determined for both strands and then edited and aligned using Sequencher™ version 4.8.

Sequence divergences (GTR distance) were calculated using PAUP* (Swofford 2003). Haplotype networks were generated using TCS version 1.21 (Clement et al. 2000). The partition homogeneity/incongruence-length difference test (Farris et al. 1994; ILD) implemented in PAUP* was used to determine whether the COI and NDI datasets were consistent and could be combined for phylogenetic analysis. 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. Bayesian inference was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). In the initial analysis the burnin was set at 10% (10,000 generations) of the chain length (100,000 generations). Three runs were conducted in MrBayes using the GTR + G model selected by MrModeltest and the default random tree option to determine when the log likelihood scores reached a stable value (by plotting the log likelihood scores of sample points against generation time). The log likelihood scores started at around −8,900 and quickly converged upon a stable

[1] Preliminary analyses that included most of the currently recognized congeners did not robustly delineate the sister taxon of *P. bruneauensis*.

Table 1 Collections used for molecular analysis, with population sample numbers (used in Fig. 1), locality details (temperatures given for the *P. bruneauensis* localities), sample sizes (*n*), and GenBank accession numbers

Species	Sample	Locality	Latitude	Longitude	*n*	GenBank acc. #
P. bruneauensis	1	Pool at mouth of Hot Creek, Owyhee County, ID (27°C)	42.76236	−115.72346	10	JN184636-184645
–	2	Spring along Bruneau River below Hot Creek confluence, Owyhee County, ID (26.1°C)	42.76553	−115.72864	10	JN184646-184655
–	3	Spring along Bruneau River above Hot Creek confluence, Owyhee County, ID (29.4°C)	42.74739	−115.71028	10	JN184656-184665
–	4	Hot Creek ca. 100 m upflow from mouth, Owyhee County, ID (31°C)	42.76245	−115.7319	7	JN184666-184184672
–	5	Spring along Bruneau River below Hot Creek confluence, Owyhee County, ID (27°C)	42.7691	−115.72346	7	JN184673-184679
–	6	Bruneau River below Hot Creek confluence, Owyhee County, ID (12.7°C)	42.76737	−115.72655	7	JN184680-184686
P. fresti	–	Tudor Warm Springs, Malheur County, OR	42.5304	−118.1834	1	FJ172474[a]
P. imperialis	–	Unnamed spring, Thacker Pass, Kings River Valley, Humboldt County, NV	41.6929	−118.1263	1	AY379450[b]
P. intermedia	–	Crooked Creek, Malheur County, OR	42.8013	−117.7349	1	FJ172460[a]
P. kolobensis	–	Unnamed spring, Pine Valley, Washington County, UT	37.3665	−113.4485	1	DQ364009[a]
P. militaris	–	Spring west of Soldier Meadow Ranch, Humboldt County, NV	41.3956	−119.1663	1	EF119077[b]
P. robusta	–	Unnamed spring, tributary to Polecat Creek, Teton County, WY	44.1352	−110.7150	1	AY379426[c]
P. sp. B	–	Teton River, Buxton Bridge crossing, Teton County, ID	43.7232	−111.1893	1	AY379446[b]
F. floridana	–	Juniper Springs, Marion County, FL	29.1839	−81.7120	1	AF520916[c]

[a] Hershler and Liu (2008)

[b] Hershler and Liu (2004)

[c] Hershler et al. (2003)

value of about −4,100 after approximately 40,000 generations in all three preliminary runs. For the final run Metropolis-coupled Markov chain Monte Carlo simulations were performed with four chains for 1,000,000 generations and Markov chains were sampled at intervals of 10 generations to obtain 100,000 sample points. The sampled trees with branch lengths were used to generate a 50% majority rule consensus topology with the first 5,000 trees, equal to 50,000 generations, removed to ensure that the chain sampled a stationary portion. A molecular clock hypothesis for the Bayesian topology (52 sequences) was tested using Tajima's (1993) non-parametric relative rate test in MEGA5 (Tamura et al. in press). A molecular clock rate of 1.62% COI divergence per million years, based on the geology-calibrated divergence of a *Pyrgulopsis* sister species pair (Hershler and Liu 2008), was used to estimate divergence times. Intra-population diversity and structuring of genetic variation among populations was assessed using Arlequin 3.5 (Excoffier and Lischer 2010). We also evaluated pairwise population differentiation using Jost's D in SPADE (Chao and Shen 2009).

Results

Fifty-one specimens of *P. bruneauensis* were sequenced for COI (Table 1). The alignment of these sequences yielded 658 bp, of which 146 sites were variable (22.2%) and 82 were parsimony informative (12.5%). Overall nucleotide composition was biased towards thymine (T) (37.9%) and adenine (A) (25.5%), followed by cytosine (C) (18.5%) and guanine (G) (18.1%) as typically observed in gastropod mitochondrial genes (e.g., Hershler et al. 2003).

Eleven *P. bruneauensis* haplotypes were detected (Table 2) which differed from each other by 0.15–1.69% sequence divergence. TCS resolved these as two distinct (Fig. 2a) and broadly sympatric groups (A, B, composed of haplotypes I, X, XI and II-IX, respectively) which differed from each other by 1.42 ± 0.7%.

The ILD tests indicated no significant incongruence between COI and NDI ($P = 0.95$) and thus we performed the Bayesian analysis based on the combined dataset of 1,188 bp. Specimens of *P. bruneauensis* formed a well supported (100% posterior probability) clade (see Fig. 2b)

Table 2 COI haplotypes

Haplotype	Base pair position															Sample					
	34	50	56	58	59	109	127	181	208	235	242	287	313	349	418	425	508	526	1	2	3	4	5	6
I	C	C	T	G	C	G	T	G	G	C	T	T	T	A	T	A	A	T	10	2	6	5	4	6
II	C	C	C	A	C	G	T	A	A	T	C	C	A	A	T	A	G	T		1	1			
III	C	C	C	A	C	G	T	A	A	T	C	C	A	G	T	A	A	T		3				
IV	C	C	C	A	C	G	T	A	A	T	C	C	A	A	T	A	G	C		1				
V	C	C	C	A	C	G	T	A	A	T	C	C	A	A	T	G	G	T		1				
VI	C	T	C	A	C	G	T	A	A	T	C	C	A	G	T	A	A	T		1				
VII	C	C	C	A	T	G	T	A	A	T	C	C	A	A	T	A	A	T		1	2	2		1
VIII	C	C	C	A	C	G	C	A	A	T	C	C	G	A	T	A	A	T			1			
IX	C	C	C	A	C	A	T	A	A	T	C	C	A	A	T	A	A	T					1	
X	T	C	T	G	C	G	T	G	G	C	T	T	T	A	T	A	A	T					1	
XI	C	C	T	G	C	G	T	G	G	C	T	T	T	A	C	A	A	T					1	

Samples correspond to those in Table 1

in the Bayesian tree; the sister taxon of this lineage was not resolved. One of the haplotype groups delineated by TCS (group A) formed a well supported clade in the Bayesian tree while the other (group B) was paraphyletic. Application of the COI molecular clock for *Pyrgulopsis* suggests that these two groups diverged about 0.88 ± 0.43 mya. This may be an overestimate because we have not taken into account the effect of ancestral polymorphism (Edwards and Beerli 2000). *Pyrgulopsis bruneauensis* differed from other congeners included in the analysis by 4.66–10.62% COI divergence.

Haplotype diversity was substantial (0.2857–0.9111) except in population 1, which was invariant; nucleotide diversity was rather low (<0.01) (Table 3). The AMOVA revealed small (27.04%) but significant (Fst = 0.2707, $P = 0.00196$) structuring among populations. Pairwise differentiation among populations was small and non significant with the exception of population 2, which significantly differed from each of the others (Fst 0.18345–0.69027, $P < 0.001$; Jost's Dest 0.376–0.645) (Table 4). There was no obvious geographic pattern in the distribution of haplotypes. The most common haplotype (I) was detected in all six populations (and in 33 of 51 specimens) while none of the others was found in more than four populations (Table 2). Eight of the 11 haplotypes were restricted to single populations: II–VI (population 2), VII (population 6), and IX–XI (Sample 5). The samples differed considerably in terms of haplotype composition; for example, population 1 contained only a single haplotype (I) while population 2 contained seven (I–VII).

Discussion

Our results confirm a previous finding (Liu and Hershler 2005, Fig. 2) that *P. bruneauenis* is a substantially divergent lineage within the genus *Pyrgulopsis*. These data also indicate that *P. bruneauensis* contains a considerable amount of genetic diversity within its narrow geographic range and is partitioned into two divergent haplotype subgroups. Similar studies of other locally endemic congeners have also detected substantial genetic diversity and structure (e.g., Hurt 2004; Moline et al. 2004; Hershler et al. 2007). The local differentiation of these snails may be attributed to the limited vagility and high fidelity to headspring habitats that has, more generally, shaped the extensive evolutionary diversification of *Pyrgulopsis* (Hershler and Sada 2002).

The two relatively deep (1.42% COI sequence divergence) haplotype lineages of *P. bruneauensis* that were detected in our study co-occurred at five of the six sampled sites. Previous studies of locally endemic congeners have typically delineated deep allopatric structure (Hurt 2004;

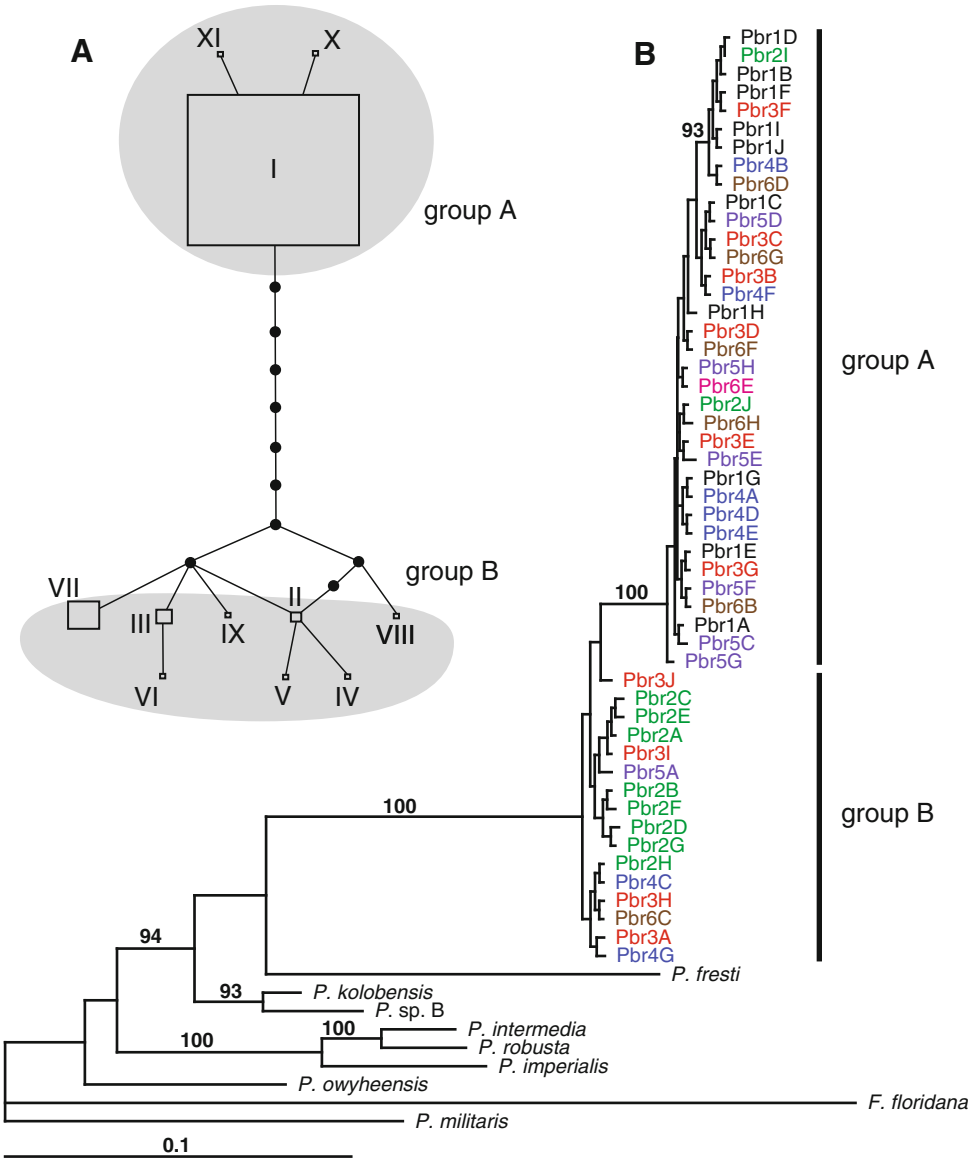

Fig. 2 **a** Unrooted haplotype network for *P. bruneauensis* based on mtCOI sequences. Haplotypes are represented by *squares* which are sized in proportion to their frequency. Branches represent mutational steps (single base pair) between haplotypes; *filled circles* represent inferred mutational steps. **b** Bayesian tree based on COI and NDI sequences. The terminals (combined COI-NDI sequences) are color coded by collection locality. Posterior probabilities of nodes are provided when >90%. The two main COI haplotype groups (A, B) are indicated in **a** by *grey ellipses* and in **b** by vertical bars

Moline et al. 2004; Hershler et al. 2007), although Hurt (2004) detected divergent sympatric haplotypes in two species (*P. bernadina*, *P. deserta*), which she attributed to population admixture. We did not discern morphological variation within our collections of *P. bruneauensis* suggesting possible cryptic species, nor do we consider incomplete lineage sorting to be a likely explanation for the sympatric occurrence of divergent lineages given that *P. bruneauensis* is highly differentiated genetically (see above) and does not closely resemble other regional congeners morphologically (Hershler 1990). Allopatric differentiation followed by secondary admixture may be a possible explanation, although we have found no evidence of a prior dispersal barrier coinciding with the estimated 0.88 mya phylogeographic break (see Jenks et al. 1998 for geologic mapping of the Hot Creek area). It is also possible that a phylogeographic break developed in this poorly dispersing species in the absence of a geographic barrier owing to stochastic factors (Irwin 2002; Kuo and Avise 2005).

Haplotype diversity appears to vary considerably among populations of *P. bruneauensis*, assuming that our population samples can be considered to represent distinct demes. This finding suggests that translocations of *P. bruneauensis*, which were discussed as a possible measure in the recovery plan for this species (USFWS 2002), should proceed with caution and be presaged by genetic surveys of the populations of concern. Based on the evidence in hand, Site 2 would appear to be the most suitable source for a translocation given its high genetic diversity (seven haplotypes) whereas Site 1, which contains a single haplotype, is least appropriate.

Table 3 Summary of COI genetic variation within samples

Sample	Haplotypes	Polymorphic sites	Haplotype diversity	Nucleotide diversity
1	1	0	0.0000 ± 0.0000	0.0000 ± 0.0000
2	7	14	0.9111 ± 0.0773	0.0070 ± 0.0043
3	4	11	0.6444 ± 0.1518	0.0077 ± 0.0046
4	2	9	0.4762 ± 0.1713	0.0065 ± 0.0042
5	4	11	0.7143 ± 0.1809	0.0048 ± 0.0032
6	2	9	0.2857 ± 0.1964	0.0039 ± 0.0027

Table 4 Pairwise genetic differentiation among samples. Jost's Dest values are given above the diagonal, Fst (AMOVA) values are given below the diagonal

	1	2	3	4	5	6
1		0.645	0.138	0.095	0.147	0.030
2	0.69037*		0.376	0.486	0.467	0.567
3	0.29321	0.18345*		−0.041	−0.014	0.034
4	0.23497	0.29505*	−0.10384		0.073	0.003
5	0.05405	0.45047*	0.02512	−0.06433		0.086
6	0.05405	0.46177*	0.00991	−0.09804	−0.12903	

* Significant ($P < 0.001$)

These findings are preliminary in that they were based on a small number of collections that were analyzed only for two mitochondrial genes. In order to more fully investigate the genetic structuring of this species and address gene flow and dispersal as they relate to planned recovery actions (USFWS 2007), additional studies should be done using microsatellites, which are especially suitable for fine-scale investigations relating to recent population history (Selkoe and Toonen 2006). These markers have been successfully used in conservation-related studies of other hydrobiid snails (Worthington Wilmer and Wilcox 2007; Liu and Hershler 2009).

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