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ORIGIN AND DIVERSIFICATION OF THE SOLDIER MEADOW SPRINGSNAILS (HYDROBIIDAE: *PYRGULOPSIS*), A SPECIES FLOCK IN THE NORTHWESTERN GREAT BASIN, UNITED STATES

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ABSTRACT

The large, western North American hydrobiid gastropod genus *Pyrgulopsis* (commonly known as springsnails) includes a series of locally endemic faunas that are thought to be species flocks. Although these assemblages are of interest from the evolutionary, biogeographic and conservation perspectives, their monophyly and phylogenetic relationships have yet to be rigorously evaluated. Here we present a molecular phylogeny (based on mitochondrial sequence data) of a putative flock of four thermal spring-dwelling springsnails that is distributed in many sites in Soldier Meadow and a single locality in Bog Hot Valley (northwestern Nevada). Our analyses support monophyly of this assemblage ('Soldier Meadow clade') and a close relationship with other regional species and suggest that the invasion of thermal habitats by these springsnails occurred independently of other such radiations within the genus. The divergence of the Soldier Meadow clade relative to its sister group is substantial (6.79–10.36% for COI, 10.35–15.88% for NDI), suggesting a split in the early Pliocene, based on the application of a COI clock for *Pyrgulopsis*. The splits within the Soldier Meadow clade into three main subunits also appear to be old events, based on their 5.78–8.54% COI divergence relative to each other. These findings are consistent with a long history of springsnail evolution in Soldier Meadow, which is intriguing given that this basin was flooded by Lake Lahontan during periods of the early and middle Pleistocene. We suggest that progenitors of the contemporary fauna survived in high elevation springs that may have been present in the basin during these pluvial periods and subsequently colonized contemporary habitats following the termination of the extreme Lake Lahontan highstands. We speculate that the broadly disjunct population (of *P. militaris*) in Bog Hot Valley, which is consistently nested within the Soldier Meadow clade in our phylogenetic analyses, is either a vicariant relict of a spring zone that may have once extended between these two areas; or was founded by a past 'jump' dispersal event from Soldier Meadow. Phylogeographic structure of springsnail populations in Soldier Meadow bears the strong stamp of geologically recent, allopatric diversification, perhaps reflecting the short time that basin floor habitats have been occupied. We describe a new species (*P. varneri*) for a series of recently discovered populations that are monophyletic, substantially divergent and morphologically distinctive. Additional studies will be necessary to confidently assess the taxonomic status of morphologically distinctive *P. limaria* and *P. umbilicata*, which are shown herein to be little divergent genetically; and a recently discovered minute springsnail that is morphologically divergent yet closely similar genetically to *P. notidicola*.

INTRODUCTION

The western North American hydrobiid gastropod *Pyrgulopsis* is the most species-rich genus of freshwater molluscs on the continent, with 126 congeners currently recognized (Liu & Hershler, 2005). These tiny, gill-breathing animals are distributed throughout much of the West and typically live in small, spring-fed habitats (hence their common name, springsnails). One of the most interesting facets of the *Pyrgulopsis* radiation is the scattered occurrence of endemic assemblages of morphologically similar congeners that are thought to be species flocks (Hershler, 1998; Hershler & Sada, 2002). The evolution of species flocks has long been a focus of intensive study (Echelle & Kornfield, 1984), yet has been relatively little investigated in non lacustrine aquatic settings (but see Echelle & Dowling, 1992; Duvernell & Turner, 1998; Sullivan, Lavoué & Hopkins, 2002; Glaubrecht & Kohler, 2004). The putative springsnail species flocks are also of interest from a biogeographic perspective because they co-occur with other endemic aquatic biota such as fishes (e.g. Williams *et al.*, 1985) and their evolutionary development is tied to the fascinating history of regional landscape and drainage (Taylor, 1985; Hershler & Sada, 2002). The endemic springsnail faunas are also a priority for conservation because their small, fragile habitats are imperilled by water development and other human mediated activities in the arid West (Melhop, 1996; Sada & Vinyard, 2002).

Despite the compelling features of these endemic assemblages, they have been little studied and rigorous investigations of their origin and diversification are precluded by the poorly known phylogenetic relationships within taxonomically difficult *Pyrgulopsis*. Classification of these snails has traditionally been based on morphological characters which are useful in diagnosing discrete units considered to be species, but provide little phylogenetic resolution (Hershler, 1994). A recent molecular

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study of about half of the species of *Pyrgulopsis* provided the first robust phylogenetic hypothesis for the genus and recovered five well supported clades composed of putative species flocks (Liu & Hershler, 2005), although sampling was not sufficient to well delineate their evolutionary development. The utility of molecular evidence in helping to resolve the relationships of endemic springsnail faunas was also underscored by another recent study which showed that geographically proximal and morphologically similar populations assigned to a single species belong to multiple, evolutionarily diverse lineages (Liu, Hershler & Clift, 2003). Here we build from these studies by generating the first in-depth molecular phylogenetic analysis of one of the putative springsnail species flocks.

Soldier Meadow is a small valley in northwest Nevada *ca* 120 km north of Gerlach that forms the northern portion of the western arm of the Black Rock Desert (Fig. 1) and was part of the vast drainage basin of Lake Lahontan (Reheis, 1999) during the Quaternary. Soldier Meadow contains extensive wetlands that are fed by numerous thermal springs (Grose & Keller, 1975; Shevenell & Garside, 2005) which are arranged in clusters or complexes along or in close proximity to several geologic faults (Nyquist, 1963). [We follow Garside & Schilling's (1979) definition of Nevada thermal waters as those having a temperature $\geq$70°F (21°C). This also follows the traditional definition of thermal springs as those that are >10°C warmer than the local mean annual air temperature. The mean annual temperature in the Soldier Meadow area is 48°F (8.9°C) (Houghton, Sakamoto & Gifford, 1975, Fig. 17).] Hershler, 1998 described four species of springsnails (Fig. 2) based on collections from 10 of these springs. Three of these are endemic to Soldier Meadow (*P. limaria* Hershler, 1998; *P. notidicola* Hershler, 1998; *P. umbilicata* Hershler, 1998) and the fourth (*P. militaris* Hershler, 1998) is distributed in this area and a spring in Bog Hot Valley, *ca* 40 km to the north-northwest (Fig. 1). The Soldier Meadow springsnails differ principally in shell shape and penial morphology (Hershler, 1998) and also may have distinct habitat requirements (Sada & Powell, 2001). One of these snails (*P. notidicola*) is a candidate for federal listing owing to its extremely narrow range and threats posed by recreational use of its habitat (USFWS, 2002) and the entire fauna is being managed under a federal recovery plan for the 'rare species of Soldier Meadows' (USFWS, 1997).

Hershler (1998:80) considered the Soldier Meadow springsnails to be a 'well defined group,' united by distinctive (but not unique) shell and anatomical morphological features, that closely resembles another congener (*P. vinyardi* Hershler, 1998) that lives in northern Nevada (Hershler, 1998:86). Hershler & Sada (2002:267) subsequently referred to the Soldier Meadow springsnails as a 'presumed [species] flock.' There have been no subsequently published investigations of this fauna aside from the inclusion of one species (*P. militaris*) in several molecular phylogenetic studies (Hershler *et al.*, 2003; Hershler & Liu, 2004a, b; Liu & Hershler, 2005). Recently, one of us (DWS) conducted a field survey of most of the Soldier Meadow springs and sampled many previously unstudied springsnail populations. In this paper, which is based on the extensive collections made during this survey, we infer the phylogenetic relationships and test the monophyly of the Soldier Meadow springsnails using DNA sequences from two mitochondrial genes, cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase subunit I (NDI). We examine the origin, age and biogeographic history of this fauna in relation to regional hydrographic history. We also utilize molecular and morphological evidence to re-evaluate the current taxonomy of the Soldier Meadow springsnails and describe a new species for a series of distinctive, recently discovered populations.

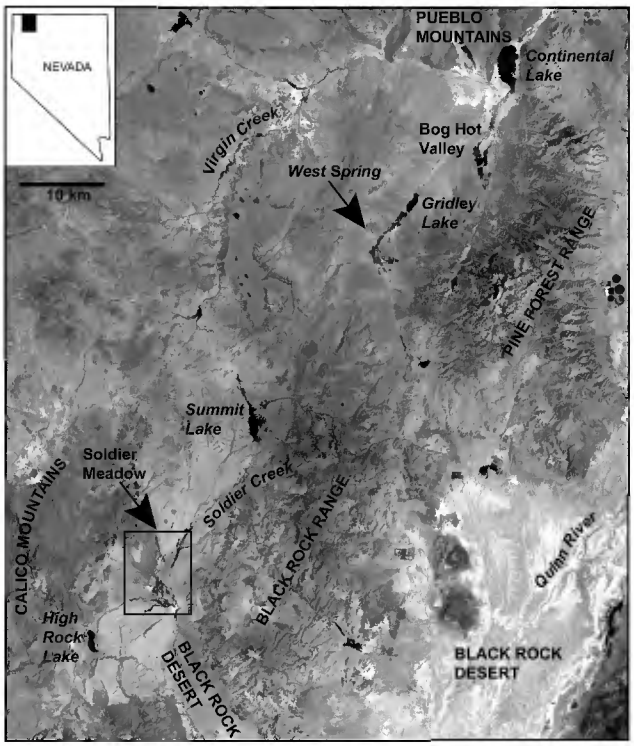

Figure 1. Landsat (ETM+, band 7) image of a portion of northwestern Nevada, showing locations of Soldier Meadow, West Spring in Bog Hot Valley (which contains a disjunct population of *P. militaris*) and other physical features mentioned in the text.

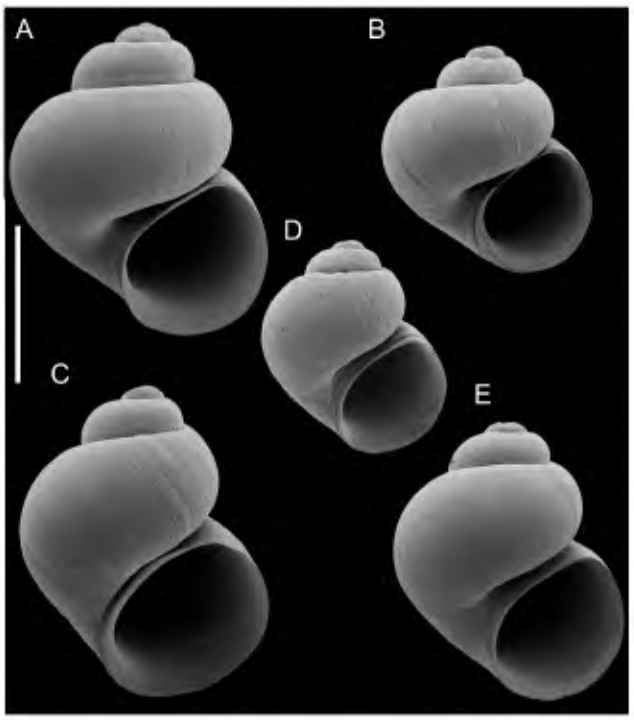

Figure 2. Scanning electron micrographs of shells of Soldier Meadow springsnails. **A**. *P. umbilicata*, paratype, USNM 860705. **B**. *P. limaria*, paratype, USNM 860706. **C**. *P. notidicola*, paratype, USNM 860707. **D**. *P. militaris*, paratype, USNM 860704. **E**. *P. militaris*, West Spring, Bog Hot Valley, USNM 883921. Scale bar = 1.0 mm.

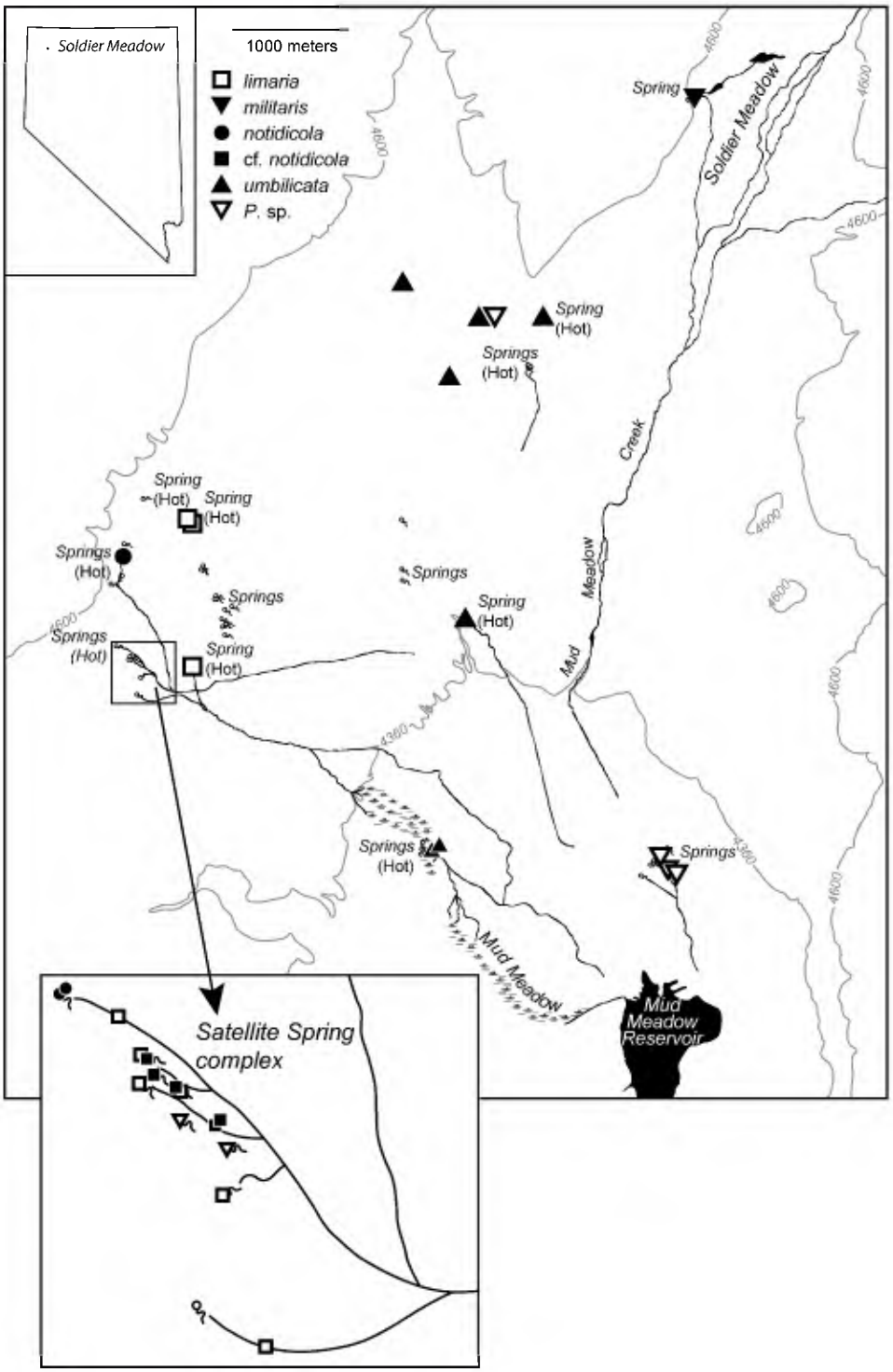

Figure 3. Map showing springsnail collection localities in Soldier Meadow, with insert showing closely proximal sites in the Satellite Spring complex. Several symbols represent two or more closely proximal localities. Topographic contour lines (grey) indicate approximate extent of flooding within Soldier Meadow during early to middle (4,600 ft) and late (4,360 ft) Pleistocene Lake Lahontan highstands. Map from United States Geological Survey 7.5 min topographic sheets (Mud Meadow, Soldier Meadow quadrangles).

MATERIAL AND METHODS

Specimens

Thirty-two samples of Soldier Meadow springsnails were included in the molecular phylogenetic study, together with a sample of the Bog Hot Valley population of *P. militaris*. Eighteen samples were from the large Satellite Spring complex, while one–five samples were taken from each of the other spring complexes in Soldier Meadow (Fig. 3). Each sample was initially divided into subsets of specimens that were relaxed with menthol crystals, fixed in formalin and preserved in 70% ETOH for morphological study; and directly preserved in

90% ETOH for molecular analysis. Voucher material from each sample was catalogued and deposited into the National Museum of Natural History (USNM) collection.

Morphology

Samples were sorted and identified based on the diagnostic shell and penial features of the Soldier Meadow species (Hershler, 1998). Identifications were facilitated by comparison with types and other original material of the Soldier Meadow species housed in the USNM. The description of the new species follows previous taxonomic investigations of springsnails (Hershler, 1998; Hershler *et al.*, 2003; also see Hershler *et al.*, 2007). Shell data consisted of counts of total number of whorls; and measurements of shell height, shell width, height of body whorl, width of body whorl, aperture height, aperture width (Hershler, 1989); and were analysed using Systat for Windows 11.00.01 (SSI, 2004).

Genetics

Two–ten specimens were typically sequenced from each population for COI and NDI. Recent studies have shown these two genes provide useful phylogenetic resolution of springsnails at the species level (Liu *et al.*, 2003; Hershler & Liu, 2004a; Liu & Hershler, 2005). Outgroups consisted of two species from Railroad Valley (*P. lockensis* Hershler, 1998; *P. villacampae* Hershler, 1998) that were depicted as most closely related to *P. militaris* in a molecular phylogenetic analysis of 62 congeners (Liu & Hershler, 2005); and three other northern Great Basin springsnails (*P. gibba* Hershler, 1995; *P. imperialis* Hershler, 1998; *P. longiglans* Hershler, 1998) that were shown to be close relatives of the Soldier Meadow fauna in more comprehensive, unpublished analyses of the genus. *Pyrgulopsis vinyardi*, which is most closely similar morphologically to the Soldier Meadow fauna (Hershler, 1998), was not included in the analysis because we do not have NDI sequences for this species. However, COI sequences of this snail differ little (0.5%) from those of *P. gibba*, which is included in our study. Trees were rooted with *Floridobia winkleyi* (Pilsbry, 1912) (*fide* Liu & Hershler, 2005). Collecting localities, sample sizes, voucher information and GenBank accession numbers are detailed in Table 1.

Genomic DNA was isolated from individual snails using a CTAB protocol (Bucklin, 1992). The DNA was visually inspected for quality and quantity by comparison with a DNA Size Standard High Molecular Weight Marker (BioRad) via electrophoresis in 1% agarose gel stained with ethidium bromide. Although the same specimens were used to survey sequence variation in both mitochondrial COI and NDI regions, some individuals amplified at only one of the two regions even after multiple attempts and therefore sample sizes for the two regions differed in some cases (Table 1).

For the COI gene, COIL1490 and COIH2198 (Folmer *et al.*, 1994; COIL1490 5′GGTCAACAAATCATAAAGATATTGG3′ and COIH2198 5′TAAACTTCAGGGTGACCAAAAAATCA3′) were used to amplify a 710 base pair (bp) fragment via polymerase chain reaction (PCR). Amplifications were conducted in a 25 μl total volume, containing 5 μl of Invitrogen optimizer buffer F (10 mM $MgCl_2$, pH 9.0) (Invitrogen, Inc.), 2.5 μl of dNTPs (2.5 mM each), 1.25 μl of each primer (10 μM), 1 unit *Taq* polymerase, 1μl of template (ca. 100 ng double-stranded DNA) and 13.8 μl of sterile water. The thermal profile for the PCR reaction consisted of an initial 2 min denaturation step at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 45°C, 2 min at 72°C and a final extension step at 72°C for 7 min. For the NDI region, ND43F and RND592F (Liu *et al.* 2003; ND43F 5′GCA TCY TAY TAG CAG TCG CTT3′and RND592F 5′TCT GCA AAR TCR AAA GGT GC3′) or 16Sar-L and RND592F (Palumbi *et al.*, 1991; 16Sar-L 5′CGC CTG TTT ATC AAA AAC AT3′) were used to amplify a *ca* 570 bp or 1500 bp fragment. Amplifications were conducted in a 25 μl total volume, containing 5 μl of Invitrogen optimizer buffer D (17.5 mM $MgCl_2$, pH 8.5) (Invitrogen, Inc.), 2.5 μl of dNTPs (2.5 mM each), 1.25 μl of each primer (10 μM), 1 unit *Taq* polymerase, 1μl of template (ca. 100 ng double-stranded DNA) and 13.8 μl of sterile water. The PCR temperature profile began with a preheat step at 94°C for 2 min. The following 35 cycles began with a denature step at 94°C for 1 min, an annealing step at 45°C for primers ND43F and RND592F or 57°C for primers 16SL and RND592F for 1 min and an extension step at 72°C for 2 min. The final extension step was extended for another 7 min.

The amplified PCR product was cleaned for sequencing using Shrimp Alkaline Phosphatase (SAP, Amersham) and Exonuclease I (ExoI, Amersham). PCR products were incubated at 37°C for 30 min and then at 85°C for another 15 min with 5 units of ExoI and 0.5 units SAP. Dye terminator cycle sequencing reactions were performed with the Beckman-Coulter Quick Start Kit according to the manufacturer's protocol but with a reaction volume of 10 μl. The cycle sequencing reactions contained 4μl Quick Start mix, 5 μl cleaned PCR product (~10–30 ng DNA) and 1 μl primer (10 μM). The COI fragment was sequenced using forward primers COIL1490 and reversed primer COIH2198. The NDI fragment was sequenced using forward primers ND43F and reverse primer RND592F. The following cycling conditions were used: 96°C for two min, then 30 cycles of 96°C for 20 s, 45°C for 20 s and 60°C for 4 min. The cycle-sequenced product was cleaned using the Beckman Coulter protocol. Fluorescent dye-labelled DNA was combined with 4 μl stop solution (equal volume of 100 mM EDTA and 3 M NaOAc pH 5.2), 1 μl glycogen (20 mg/ml) and 10 μl milli-Q H_2O, mixed well and precipitated with 60 μl cold 95% (v/v) ethanol/water. Fluorescent dye-labelled DNA was recovered by centrifuging at 13,000 rpm for 20 min at 4°C. Pellets were washed with 100 μl 70% (v/v) ethanol/water, air dried and resuspended in 30 μl of dimethylformamide. Resuspended samples were run on the Beckman Coulter CEQ8000 using method LFR-1.

Molecular data analysis

Forward and reverse sequences for each individual were assembled, edited and aligned using Sequencher™ 3.1.1. Incongruence length differences were calculated in PAUP*4.0b10 (Swofford, 2002), using the partition-homogeneity test (Farris *et al.*, 1994; ILD), to assess whether the two mitochondrial datasets differ in phylogenetic signal. We conducted 500 replicates of the ILD test using only parsimony-informative sites. Base compositional differences were evaluated with the χ^2 test. Sequence divergences (uncorrected *p* distance) within and among lineages were calculated using MEGA3 (Kumar, Tamura & Nei, 2004), with standard errors estimated by 1,000 bootstrap replications with pairwise deletion of missing data.

Phylogenetic analyses based on distance, parsimony and maximum-likelihood methods were generated using PAUP* 4.0b10. Bayesian inference was performed using MrBayes 3.04 (Huelsenbeck & Ronquist, 2001). Modeltest 3.7 (Posada & Crandall, 1998) was used to obtain an appropriate substitution model (using the Akaike Information Criterion) and parameter values for the distance, maximum-likelihood and Bayesian analyses. Appropriate genetic distance was used to generate a neighbour-joining (NJ) tree (Saitou & Nei, 1987). Maximum-parsimony (MP) analyses were conducted with equal weighting, using the heuristic search option with tree bisection reconnection branch-swapping and 100 random additions. The appropriate

Table 1. Springsnail material used in the molecular study, with specimen codes, locality details, voucher information, sample sizes for the two genes and GenBank accession numbers.

Code	Species	Locality (all Humboldt Co., NV except outgroups)	UTM (NAD 27, zone 11)		USNM voucher	Sample size		GenBank accession number	
			Northing	Easting		COI	NDI	COI	NDI
SM1	*militaris*	West Spring, Bog Hot Valley	4621221	340307	1023455	5	5	EF119076	EF119142
SM2	*limaria*	Spring brook, western Soldier Meadow	4580059	314612	1023462	6	3	EF119088–092	EF119152–153
SM3	*umbilicata*	Spring, northern Soldier Meadow	4583028	317617	1023463	4	4	EF119093–094	EF119154–156
SM6	*notidicola*	Satellite Spring, western Soldier Meadow	4580238	313978	1072233	4	4	EF119128	EF119191–192
SM7	*limaria*	Spring 150 m W of Satellite Spring	4580123	314086	1072236	5	5	EF119095–097	EF119157–161
SM8	*P.* sp.	Spring, southeast Soldier Meadow, 150 m downflow from source	4578295	318719	1072238	3	3	EF119080	EF119143
SM9	cf. *notidicola*	Satellite Spring complex, spring brook	4580078	314154	1072370	5	5	EF119132–133	EF119197–198
SM10	*limaria*	'Tole Spring,' western Soldier Meadow	4581300	314611	1072372	5	4	EF119098–100	EF119162
SM11	*P.* sp.	Spring complex (two springs), southeast Soldier Meadow	4578405	318616	1083194	8	10	EF119081	EF119144–145
SM13	*P.* sp.	Spring brook downflow from SM11	4578270	318756	1096917	4	5	EF119082–084	EF119146
SM14	*umbilicata*	Spring, northern Soldier Meadow	4583040	317091	1083247	5	5	EF119101	EF119163
SM15	*P.* sp.	Spring, northern Soldier Meadow	4583071	317182	1083249	5	4	EF119085	EF119147
SM16	*umbilicata*	Spring ca. 40 m downflow from source, northern Soldier Meadow	4582534	316836	1083250	5	5	EF119102	EF119164–165
SM19	*umbilicata*	Spring, northern Soldier Meadow	4583357	316417	1083373	5	4	EF119103	EF119166
SM20	*umbilicata*	Spring, southern Soldier Meadow	4578484	316662	1083374	4	4	EF119104	EF119167–169
SM21	*umbilicata*	Spring 5 m downflow from SM20, southern Soldier Meadow	4578479	316669	1083384	4	3	EF119105	EF119170
SM22	*limaria*	'Tole Spring' 30 m downflow from source, western Soldier Meadow	4581296	314608	1083385	5	4	EF119106–110	EF119171–174
SM23	*limaria*	Satellite Spring complex, spring 'A', 60+ m downflow from source	4579773	314247	1083387	4	4	EF119111–114	EF119175–177
SM24	*limaria*	Satellite Spring complex, spring 'B'	4579993	314200	1085669	4	3	EF119115–116	EF119178–179
SM25	*P.* sp.	Satellite Spring complex, spring 'E'	4580113	314098	1085670	5	5	EF119086	EF119148–149
SM26	*limaria*	Satellite Spring complex, spring 'F'	4580128	314094	1083389	4	3	EF119117–120	EF119180–182

Continued

Table 1. *Continued*

Code	Species	Locality (all Humboldt Co., NV except outgroups)	UTM (NAD 27, zone 11)		USNM voucher	Sample size		GenBank accession number	
			Northing	Easting		COI	NDI	COI	NDI
SM27	cf. *notidicola*	Satellite Spring complex, spring brook	4580074	314171	1083391	4	4	EF119134–135	EF119199–202
SM28	*P.* sp.	Satellite Spring complex, spring 'G'	4580034	314212	1083392	4	4	EF119087	EF119150–151
SM29, SM31	*limaria*	Satellite Spring complex, spring 'H'	4580116	314121	1085671	7	7	EF119121–123	EF119183–185
SM30	cf. *notidicola*	Satellite Spring complex, spring 'H'	4580116	314121	1093586	3	4	EF119136	EF119203–204
SM32	cf. *notidicola*	Satellite Spring complex, spring 'I'	4580119	314122	1085672	3	4	EF119137–138	EF119205–207
SM33	cf. *notidicola*	Satellite Spring complex, spring 'J'	4580151	314091	1083398	5	4	EF119139	EF119208
SM34	*limaria*	Satellite Spring complex, spring 'J'	4580151	314091	1093585	2	1	EF119124	EF119186
SM35	*notidicola*	Satellite Spring, western Soldier Meadow	4580239	313978	1083399	5	5	EF119129–130	EF119193–195
								EF119140	EF119209
SM36	*limaria*	Satellite Spring 200 m downflow from source	4580187	314112	1083400	5	3	EF119125–126	EF119187–189
SM37	*umbilicata*	Spring, central Soldier Meadow	4580460	316960	1083401	2	1	EF119127	EF119190
P146	*notidicola*	N-most spring of large complex, western Soldier Meadow	4581009	314008	1002360	1	1	EF119131	EF119196
P147	*militaris*	Spring W of Soldier Meadow Ranch	4584938	318900	1002361	5	2	AY197596*	AY426417†
								AY426362†	AY426428†
								EF119077–079	
Outgroups									
–	*gibba*	Springs W of Fee Reservoir, Surprise Valley, Lassen Co., CA	–	–	–	1	1	AY197603*	AY426413†
–	*imperialis*	Spring near Thacker Pass, Kings River Valley, Humboldt Co., NV	–	–	–	1	1	AY379450‡	AY426383†
–	*lockensis*	Big Spring, Locke's Ranch, Railroad Valley, Nye Co., NV	–	–	–	1	1	AY627932§	AY628055§
–	*longiglans*	Spring NNW of Holbrook Junction, Antelope Valley, Douglas Co., NV	–	–	–	1	1	EF119141	EF119210
–	*villacampae*	Little Warm Spring, Railroad Valley, Nye Co., NV	–	–	–	1	1	AY627933§	AY628056§
–	*F. winkleyi*	Salt marsh, Scarborough, Saco River drainage, Cumberland Co., ME	–	–	–	1	1	AF520917¶	AY628036‡

*Hershler *et al.* (2003)
†Hershler & Liu (2004a)
‡Hershler & Liu (2004b)
§Liu & Hershler (2005)
¶Hershler, Liu & Thompson (2003)

model was applied for the maximum likelihood (ML) analyses. A NJ tree with appropriate genetic distance model was used as the initial topology for branch-swapping. Node support was evaluated by 10,000 bootstrap pseudo-replicates in all but the ML analysis, in which support was based on 100 replications. In the Bayesian approaches, three short runs were first conducted using the default random tree option to determine when the log-likelihood sum reached a stable value (by plotting the log-likelihood scores of sample points against generation time). The ln likelihood scores started at around −19,000 and quickly converged upon a stable value of about −5,350 after ~ 50,000 generations. Metropolis-coupled Markov chain Monte Carlo simulations were then run with four chains (using the model selected by Modeltest) 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. In order to provide a readable tree and reduce computation time, only one sequence of each haplotype per population was used in our phylogenetic analyses.

Alternative hypotheses concerning the phylogenetic position of the Bog Hot Valley population of *P. militaris* were explicitly tested using the Kishino-Hasegawa test (Kishino & Hasegawa, 1989). A molecular clock hypothesis for the COI dataset was tested using the likelihood ratio test (Felsenstein, 1981), based on the ML topology under the best model selected with and without the constraint of a molecular clock.

RESULTS

New sequences were deposited in GenBank under accession numbers EF119076–EF119210 (Table 1; note that we only deposited one sequence per haplotype per population). One hundred fifty specimens of Soldier Meadow springsnails were sequenced for COI. A total of 658 bp of COI was analysed, of which 168 sites were variable (25.5%) and 113 were parsimony informative (17.2%). Average base frequencies for this gene were 24.97% A, 39.58% T, 16.86% C and 18.60% G. There was no significant base frequency bias among species ($\chi^2 = 45.55$, df = 222, $P = 1.00$). One hundred thirty seven specimens were sequenced for NDI. The alignment of NDI sequences yielded 530 bp, of which 186 sites were variable (35.1%) and 151 were parsimony informative (28.5%). Overall nucleotide composition was biased towards adenine (A) (28.60%) and thymine (T) (37.39%), followed by cytosine (C) (17.97%) and guanine (G) (16.04%). Base frequencies were homogeneous among species ($\chi^2 = 20.56$, df = 225, $P = 1.00$). The likelihood ratio test failed to reject clocklike behaviour of the COI dataset ($\chi^2 = 66.3$, df = 71, $P = 0.64$).

Mean uncorrected sequence divergence (Table 2) between the Soldier Meadow springsnails and outgroup species was substantial, ranging from 6.79–10.36% for COI and 10.30–15.88% for NDI. Sequence divergence among Soldier Meadow species ranged up to 8.1% for COI (*P. notidicola* – *P.* sp.) and 11.61% for NDI (*P. notidicola* – *P. umbilicata*).

The ILD tests did not reveal significant incongruence between COI and NDI ($P = 0.23$) and thus we performed phylogenetic analyses using the combined (1,188 bp) dataset. Modeltest selected the General Time Reversible (GTR) model (Tavare, 1986), with some sites assumed to be invariable and with variable sites assumed to follow a discrete gamma distribution (e.g. GTR + I + G), as the best fit for the combined dataset using the Akaike Information Criterion. The optimized parameters were base frequencies of A = 0.2849, C = 0.1662, G = 0.1562, T = 0.3927; Rmat = {4.5025 58.0507 1.0014 3.6862 46.1946}; shape of gamma distribution = 3.9778; and proportion of invariant sites = 0.6327.

Phylogenetic analyses of the combined dataset using different methods (MP, ML, NJ and Bayesian) were congruent in all but minor details. A representative (Bayesian) tree is shown in

Table 2. Uncorrected sequence divergences (mean ± standard deviation) for NDI (above) and COI (below) among members of the Soldier Meadow clade and between these species and outgroups.

	P. militaris	*P.* sp.	*P. limaria*	*P. umbilicata*	*P. notidicola*	*P. gibba*	*P. imperialis*	*P. lockensis*	*P. longiglans*	*P. villacampae*
P. militaris	0.79 ± 0.24	–	–	–	–	–	–	–	–	–
	1.10 ± 0.29									
P. sp.	3.45 ± 0.69	0.40 ± 0.17	–	–	–	–	–	–	–	–
	2.36 ± 0.53	0.14 ± 0.08								
P. limaria	9.98 + 1.23	9.93 ± 1.24	1.34 ± 0.28	–	–	–	–	–	–	–
	6.95 ± 0.92	6.69 ± 0.92	0.46 ± 0.13							
P. umbilicata	10.15 + 1.29	10.05 ± 1.31	1.64 ± 0.39	0.32 ± 0.13	–	–	–	–	–	–
	6.71 ± 0.92	6.40 ± 0.92	0.40 ± 0.13	0.10 ± 0.05						
P. notidicola	10.64 + 1.19	10.97 ± 1.22	11.17 + 1.24	11.61 ± 1.31	1.21 ± 0.28	–	–	–	–	–
	7.31 ± 0.94	8.11 ± 1.03	6.44 ± 0.89	6.26 ± 0.89	0.63 ± 0.17					
P. gibba	11.29 ± 1.28	12.03 ± 1.34	13.63 ± 1.48	13.82 ± 1.52	14.49 ± 1.44	–	–	–	–	–
	7.71 ± 1.00	7.17 ± 1.01	8.93 ± 1.07	8.67 ± 1.07	9.19 ± 1.11					
P. imperialis	11.99 ± 1.35	12.23 ± 1.39	13.63 ± 1.42	13.79 ± 1.47	14.27 ± 1.43	11.13 ± 1.38	–	–	–	–
	8.16 ± 1.03	7.88 ± 1.03	9.19 ± 1.13	8.93 ± 1.12	9.68 ± 1.16	6.48 ± 0.97				
P. lockensis	10.57 ± 1.28	11.33 ± 1.34	14.23 ± 1.49	14.27 ± 1.55	14.58 ± 1.43	12.26 ± 1.43	13.58 ± 1.46	–	–	–
	8.24 ± 1.00	7.92 ± 0.99	9.42 ± 1.08	9.12 ± 1.08	8.37 ± 1.03	8.97 ± 1.08	8.18 ± 1.10			
P. longiglans	10.30 ± 1.25	11.46 ± 1.34	11.64 ± 1.32	12.12 ± 1.38	13.03 ± 1.34	8.68 ± 1.20	8.87 ± 1.20	11.13 ± 1.38	–	–
	6.79 ± 0.99	6.92 ± 1.01	7.63 ± 1.04	7.33 ± 1.03	7.47 ± 1.03	7.94 ± 1.09	7.79 ± 1.03	7.48 ± 0.99		
P. villacampae	11.51 ± 1.31	12.28 ± 1.36	14.07 ± 1.49	14.09 ± 1.53	14.28 ± 1.40	12.08 ± 1.40	13.77 ± 1.46	2.08 ± 0.60	11.32 ± 1.36	–
	9.44 ± 1.11	9.29 ± 1.12	10.36 ± 1.15	10.05 ± 1.15	9.10 ± 1.13	10.37 ± 1.22	10.05 ± 1.21	2.39 ± 0.61	7.97 ± 1.07	
F. winkleyi	12.35 ± 1.34	13.76 ± 1.39	15.57 ± 1.52	15.88 ± 1.56	15.53 ± 1.48	14.91 ± 1.48	13.58 ± 1.45	13.77 ± 1.50	11.13 ± 1.32	12.83 ± 1.48
	8.45 ± 1.04	8.69 ± 1.09	9.39 ± 1.12	9.15 ± 1.13	8.98 ± 1.08	10.49 ± 1.20	10.80 ± 1.24	9.88 ± 1.17	9.81 ± 1.16	10.69 ± 1.24

Figure 4. The Soldier Meadows fauna, together with the Bog Hot Valley sample of *P. militaris* (SM1A), formed a well supported monophyletic group that was sister to a clade composed of *P. gibba*, *P. imperialis* and *P. longiglans*. When the Bog Hot Valley haplotype was forced outside of the Soldier Meadow clade using the CONSTRAINT function in PAUP*, the shortest trees (TL = 706) were significantly different from the unconstrained, most parsimonious topologies (TL = 692) (t = 2.75, $P = 0.006$).

Three strongly supported subunits were recovered among the Soldier Meadow springsnails (Fig. 4). One clade (A) was composed of specimens of *P. umbilicata* and *P. limaria*. A second clade (B) contained *P. notidicola* and specimens of a minute (shell height, 1.0–1.5 mm), recently discovered springsnail that resembles this species (*P.* cf. *notidicola*). A third clade (C) was composed of *P. militaris* and another recently discovered springsnail (*Pyrgulopsis* sp.) that does not conform morphologically to any of the Soldier Meadow species.

DISCUSSION

Monophyly and biogeographic history of the Soldier Meadow springsnails

Phylogenetic analyses of our mitochondrial dataset consistently resolved the Soldier Meadow springsnails as a well differentiated monophyletic group. This fauna may thus be considered a species flock as defined by Greenwood (1984:18) – an 'aggregate of several species ... [whose] members are endemic to the geographically circumscribed area under consideration and are each other's closest relatives.' Our findings indicate that this clade is most closely related to three species (*P. gibba*, *P. imperialis*, *P. longiglans*) that also live in the northern portion of the Lahontan basin (see Hershler, 1998: Figs. 52–54). One of these species, broadly ranging *P. gibba*, contains a population that lives in the Soldier Meadow drainage (Tollhouse Canyon), well to the north of the thermal spring area (Hershler, 1998). All three of these species live in cold water springs

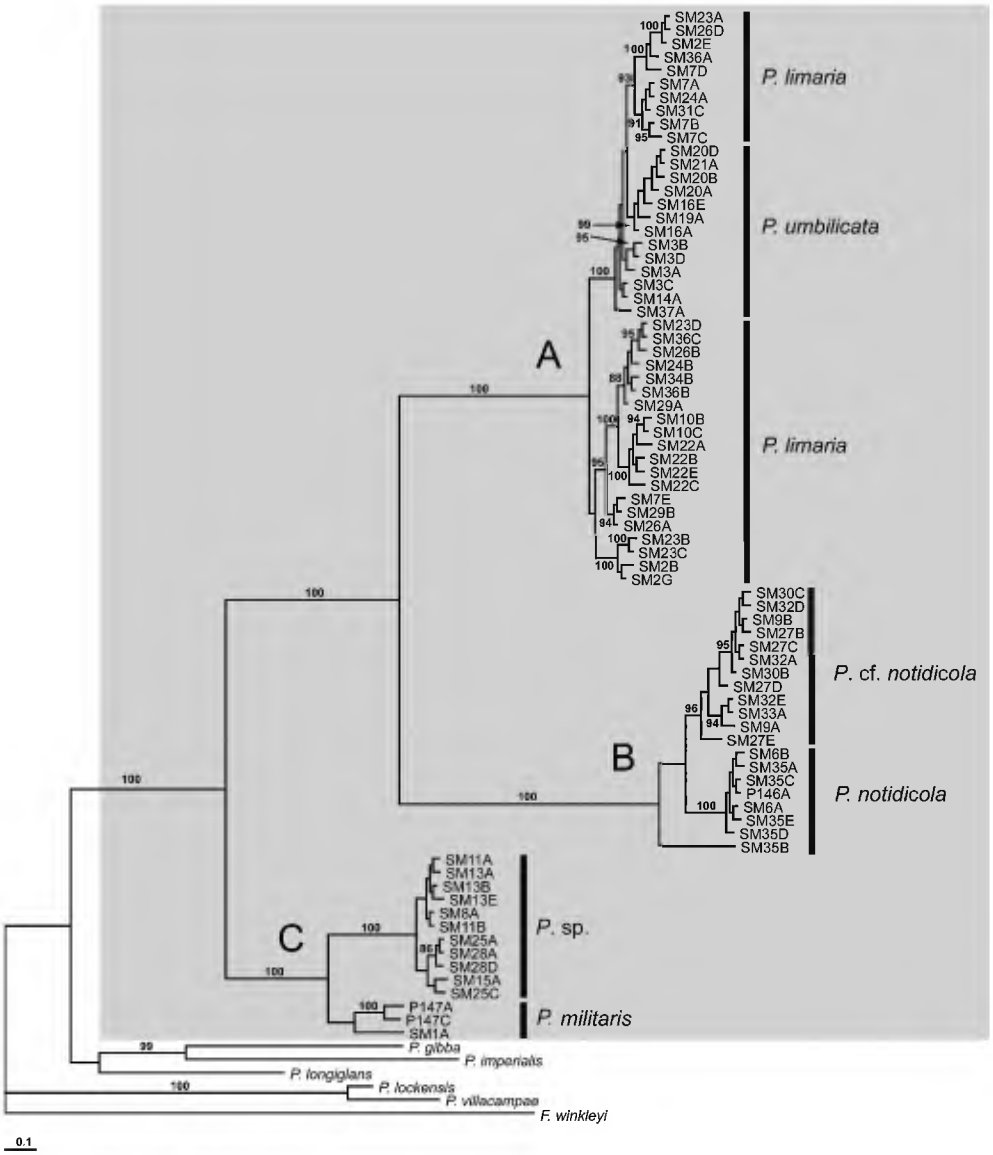

Figure 4. Bayesian tree based on the combined COI and NDI dataset. The Soldier Meadow clade is shaded grey. Numbers are posterior probabilities of well supported (>85%) nodes.

(D.W. Sada, unpubl.), thus implying that the Soldier Meadow fauna was derived independently of other springsnail radiations in thermal spring settings, including that of Railroad Valley (Fig. 4).

Although the application of a molecular clock is laden with well known difficulties (e.g. Arbogast *et al.*, 2002), it nonetheless provides a useful method of estimating divergence times (Bromham & Penny, 2003; Kumar, 2005) which we use herein because the Soldier Meadow springsnails do not have a fossil record. Based on the COI clock calibration previously derived for *Pyrgulopsis* (1.62% per million years; Liu & Hershler, 2007), the 8.25 ± 0.78% mean sequence divergence between the Soldier Meadow fauna and its sister clade implies that they split 5.09 ± 0.48 Ma (early Pliocene). This finding is congruent with molecular phylogenetic evidence that *Eremichthys acros* Hubbs & Miller, 1948, a monotypic genus of cyprinid fishes that is also endemic to thermal springs in Soldier Meadow (Nyquist, 1963; Vinyard, 1996), is likewise an old (Neogene) lineage (Smith *et al.*, 2002). Our result is also consistent with previously published molecular evidence (Liu & Hershler, 2005) that other springsnail radiations in thermal settings represent old lineages.

The splits within the Soldier Meadow lineage into three main clades (Fig. 4A–C) also appear to be old events, as the mean 5.78–8.54% COI divergence among these subunits suggests that they diverged 5.27–3.57 Ma. This finding suggests that the springsnail fauna of Soldier Meadow may be a product of a lengthy history of evolution within the basin and is supported by evidence that the thermal spring habitats of these snails, which are associated with geologic faults, are potentially ancient. Extensional geothermal systems in northern Nevada similar to that of Soldier Meadow have been shown to have 'lifespans' in the millions of years based on the amount of offset of spring deposits across faults having known slip rates (Coolbaugh *et al.*, 2005). Although the Soldier Meadow springs have not been dated in this manner, the faults with which they are associated are Tertiary in age (Dohrenwend & Moring, 1991; Sawyer, 1998).

The Quaternary history of the Soldier Meadow area is of particular interest with respect to the evolution of the springsnail flock. Soldier Meadow is topographically closed on three sides but is not separated from the Lahontan basin to the south (Fig. 1). A high shoreline site from just to the southeast of Soldier Meadow Reservoir suggests that during its final highstand 13,000 years ago (ka), Lake Lahontan locally rose to at least 1,329 m (4,360 ft) elevation (Adams, Wesnousky & Bills, 1999; site HRC-2) and thus flooded the southernmost portion of Soldier Meadow (note the 4,360 ft contour in Fig. 3). Recently discovered shoreline evidence from several places in the Smoke Creek Desert (southwest of Soldier Meadow) suggests that during several earlier periods (roughly 1.0–0.5 Ma) of the Pleistocene Lake Lahontan rose to a much higher (1,400 m, 4,593 ft) elevation locally (Reheis *et al.*, 2002), which must have inundated all of the currently inhabited springs in Soldier Meadow (see 4,600 ft contour in Fig. 3). Physical evidence of Lake Lahontan shorelines has not been found in Soldier Meadow. This may have been due to the limited fetch of the small Soldier Meadow embayment (*fide* Adams *et al.*, 1999). This raises the question of how the progenitors of the Soldier Meadow springsnail fauna, which presumably were adapted to thermal spring habitats in a manner similar to extant species (e.g. Mladenka & Minshall, 2001), survived flooding of the basin by a cold water lake. We think it likely that snails persisted in refuges provided by springs upslope of the lake margin, as previously hypothesized by Hubbs & Miller, (1948) in reference to locally endemic fishes. Studies in other parts of the Great Basin have shown that during prior, pluvial periods, groundwater discharged at elevations well above the modern water table (e.g. Quade *et al.*, 1995), but we are not aware of any geological mapping of the Soldier Meadow area that is sufficiently detailed to determine whether high elevation (>1,400 m) 'fossil' spring deposits are present in conformance with our hypothesis. Nonetheless, the relatively shallow structuring within the three Soldier Meadow clades (Fig. 4, also see below) suggests that much of this phylogeographic pattern could have been shaped after snails colonized (or recolonized) present day habitats following the early to middle Pleistocene extreme highstands. Alternatively, but less likely in our view, one may speculate that snails continued to live in the submerged springs, as the fairly shallow embayment of Lake Lahontan in Soldier Meadow could have been well oxygenated and may not have had sufficient hydrostatic pressure head to 'turn off' groundwater discharge beneath the lake.

The Bog Hot Valley population of *P. militaris* was consistently nested within the Soldier Meadow clade in our analyses, forming a subclade together with the other population of this species, which lives in the northernmost site in Soldier Meadow occupied by members of the flock (Fig. 3). This finding is intriguing because these broadly disjunct areas belong to different drainages and are separated by mountainous terrain that probably formed in the Miocene (*fide* Colgan, Dumitru & Miller, 2004; Colgan *et al.*, 2004, 2006; Lerch *et al*, 2005). We are not aware of any evidence of a prior aquatic connection between Soldier Meadow and Bog Hot Valley, although the headwaters of Soldier Creek formerly penetrated northward to the intervening Summit Lake basin (Fig. 1), perhaps only briefly (Mifflin & Wheat, 1979:30), prior to being truncated by a massive landslide between 19,000–7,840 ka (latest Pleistocene) (Curry & Melhorn 1990). The 1.79% COI sequence divergence between the two *P. militaris* populations suggests that they split 1.10 Ma, well prior to this geomorphic event. Hose & Taylor (1974) described a prominent lineament extending from Soldier Meadow into Bog Hot Valley, which they interpreted as the trace of an old fault. One may speculate that groundwater discharged along this fault during wetter periods of the late Quaternary and provided continuity of aquatic habitat between these areas, with divergence of Bog Hot Valley snails occurring after these springs dried. It is also possible that the Bog Hot Valley population was founded by 'jump' dispersal from Soldier Meadow, perhaps involving transport of snails on waterfowl (*fide* Liu *et al.*, 2003; Liu & Hershler, 2007).

Diversification of the Soldier Meadow springsnails

Each of the Soldier Meadow clades (Fig. 1A–C) is composed of two or more groups of differentiated, allopatric populations that (in our view) are in the process of diverging, or have only recently speciated, which poses a challenging situation for taxonomists (Funk & Omland, 2003). Clade A was composed of specimens of *P. umbilicata* and *P. limaria*, with the former consistently depicted as a paraphyletic group nested within the latter. These species are distributed in the eastern and western parts of Soldier Meadow, respectively and are not closely proximal (Fig. 3). The mean sequence divergence among specimens of these two species was only 0.40% for COI and 1.64% for NDI. Thus, our genetic evidence indicates that these snails are neither phylogenetically distinct nor strongly divergent. Although these two snails have closely similar shells (Fig. 2A, B), they are readily distinguished by penial characters (Hershler, 1998, Figs. 37H-I, 38B-E) which are consistent in all of the populations thus far studied. Given the very clear morphological differences between these species, we are reluctant to place them into synonymy at present, but this situation clearly merits additional study.

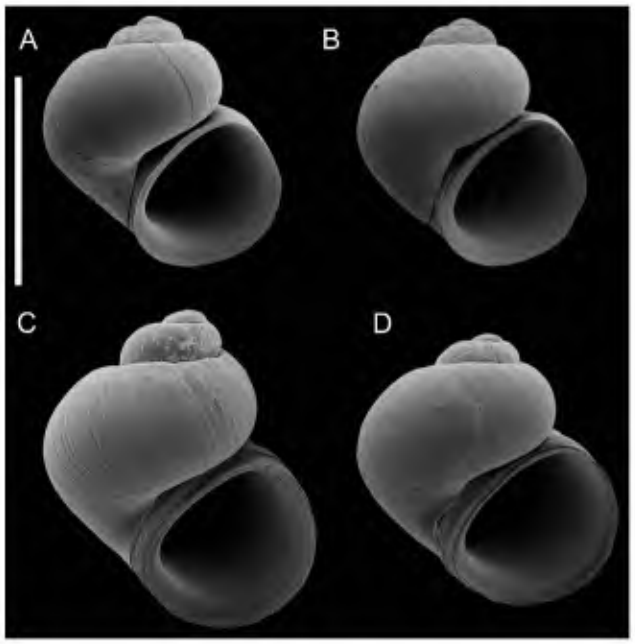

Figure 5. Scanning electron micrographs of shells of *P.* cf. *notidicola*. **A, B.** USNM 1083391. **C, D.** USNM 1083394. Scale bar = 1.0 mm.

Clade B was composed of *P. notidicola* and *P.* cf. *notidicola*, which are distributed in close proximity of each other in the Satellite Spring area (Fig. 3). This clade was subdivided into two groups conforming to these snails, with a single specimen of *P. notidicola* (SM35B) occupying a basal position relative to these subclades. In the separate COI and NDI analyses, this specimen was either basally positioned or grouped with *P.* cf. *notidicola* and one specimen of the minute form (SM27D) sometimes grouped with 'typical' *P. notidicola*. Divergence of SM35B relative to other members of clade B (2.56% for COI, 2.69% for NDI) was larger than the differences between *P. notidicola* and *P.* cf. *notidicola* (1.01% for COI, 1.91% for NDI). *Pyrgulopsis*

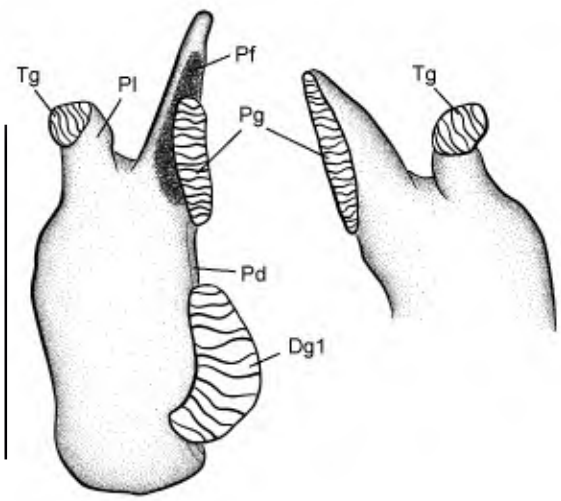

Figure 6. Penis of *P.* cf. *notidicola* (USNM 1083391). Dorsal surface shown on left, distal portion of ventral surface shown on right. Pigmented area darkly stippled. Abbreviations: Dg1, gland on proximal end of outer edge; Pd, penial duct; Pf, penial filament; Pg, penial gland; Pl, penial lobe; Tg, terminal gland. Scale bar = 250 μm.

cf. *notidicola* (Fig. 5) resembles *P. notidicola* (Fig. 2C) in shell shape but is smaller, has a larger body whorl and also differs in shape and glandular ornament of the penis (compare Fig. 6 with Hershler, 1998: Fig. 38 G, H). At the present time, we nonetheless suggest treating specimens belonging to this clade as a single species because the subgroups were not consistently depicted as monophyletic and their sequence divergence relative to each other was relatively low.

Clade C was composed of specimens of *P. militaris*, which is distributed in the northernmost part of Soldier Meadow as well as Bog Hot Valley and *P.* sp., which ranges widely within Soldier Meadow (Figs 1,3). Specimens of *P.* sp. formed a strongly supported, monophyletic group in all of our phylogenetic analyses; and differed from *P. militaris* by 2.36% for COI and 3.45% for NDI and from the other Soldier Meadow species by 6.69–8.11% for COI and 9.93–10.97 for NDI. The sequence divergence of this snail relative to its sister group (*P. militaris*) falls within the range of values documented for other species in this genus (1.1–13.1% for COI, 1.7–15.8% for NDI; Liu & Hershler, 2005). *Pyrgulopsis* sp. is also readily distinguished from *P. militaris* by both shell and anatomical features (see below). Based on this body of evidence, we consider this snail to be a new species (*P. varneri*), which is described below. The subclade composed of the two broadly disjunct populations of *P. militaris* was consistently resolved albeit without strong support e.g. posterior probability in the combined Bayesian tree = 72% ($P \geq 95\%$ is typically considered evidence of significant support). The two samples differed by 1.79% for COI and 1.60% for NDI. We have discerned no morphological differences between the two populations other than size and (perhaps correlative) shell shape (Fig. 2D, E). In this case we again favour retention of existing taxonomy, as there is substantial genetic but very little morphologic divergence.

We have recognized as a new species one lineage that is genetically distinct (monophyletic), substantially divergent and morphologically diagnosable. In other cases where previously described species or newly discovered differentiated populations do not fully meet these criteria, we have suggested that the former continue to be recognized as distinct and the latter be allocated to those species which they are most closely related to. We acknowledge that this is a conservative approach which, for example, precludes recognition of species that are morphologically cryptic or have haplotypes which have not yet sorted into reciprocally monophyletic lineages. It is hoped that our study will be followed by additional investigations that incorporate nuclear markers and other tools for evaluating the systematics of this locally complex springsnail radiation.

SYSTEMATIC DESCRIPTION

Genus *Pyrgulopsis* Call & Pilsbry, 1886

Pyrgulopsis Call & Pilsbry, 1886: 9 (type species, *Pyrgula nevadensis* Stearns, 1883, by original designation).

Pyrgulopsis varneri new species

Varner's Pyrg

Types: Holotype (Fig. 7), USNM 1083246, spring brook north of Mud Meadow Reservoir (150 m downflow from SM11), Soldier Meadow, Humboldt County, Nevada (N 4578270, E 318756, elevation 1320 m), 6/9/2005, coll. DWS. Paratypes (from same lot), USNM 1096917.

Etymology: For Matthew Varner, in recognition of his efforts (while working for the Bureau of Land Management, Winnemucca Field Office) to conserve, manage and support research on the endemic biota of Soldier Meadow.

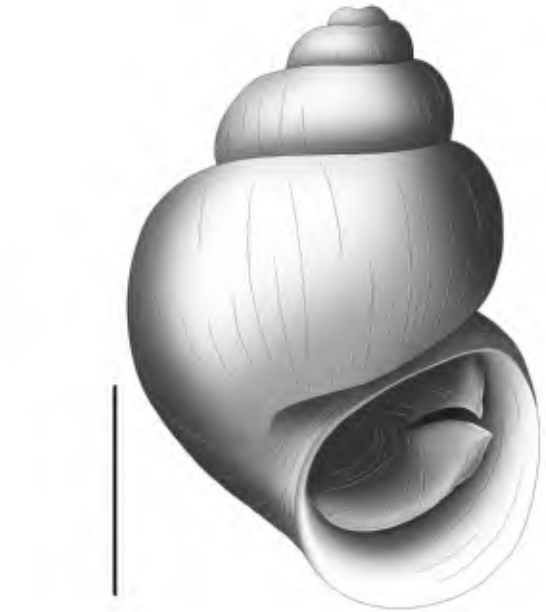

Figure 7. *P. varneri*, holotype (USNM 1083246). Scale bar = 1.0 mm.

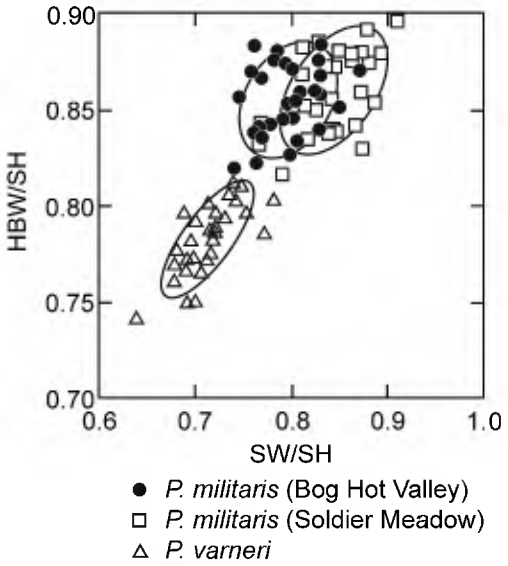

Figure 8. Scatterplot (SW/SH vs HBW/SH) showing differentiation between *P. varneri* (paratypes, USNM 1083246) and *P. militaris* (Soldier Meadow, paratypes, USNM 860704; Bog Hot Valley, USNM 883921). $n = 30$ for all samples (some symbols represent more than one specimen). Confidence ellipses centred on sample means ($P = 0.6827$).

Referred material: NEVADA. *Humboldt County* (all from Soldier Meadow, all collected by DWS): USNM 1083248, topotypes, 8/9/2005; USNM 1093065, topotypes, 10/8/2006; USNM 1072238, spring brook north of Mud Meadow Reservoir (N 4578295, E 318719, elevation 1326 m), 9/9/2004; USNM 1009230, spring brook north of Mud Meadow Reservoir (N 4578311, E 318695, elevation 1,433 m), 22/4/2002; USNM 1083195, spring 'B' in complex north of Mud Meadow Reservoir (N 4578404, E 318582, 1,320 m), 6/9/2005; USNM 1093064, *ibid.*, 8/10/2006; USNM 1083194, USNM 1083436, spring complex (two springs) north of Mud Meadow Reservoir (N 4578405, E 318616, elevation 1,320–1,321 m), 6/9/2005; USNM 1083392, Satellite Spring complex, spring 'G' (N 4580034, E 314212, elevation 1,348 m), 8/9/2005; USNM 1085670, Satellite Spring complex, spring 'E' (N 4580113, E 314098, elevation 1,374 m), 7/9/2005; USNM 1093583, Satellite Spring complex, spring 'F' (N 4580123, E 314086, elevation 1,346 m), 9/9/2004; USNM 1093584, 'Tole Spring,' 30 m downflow from source (N 4581296, E 314608, elevation 1,383 m), 7/9/2005; USNM 1083249, Spring near mouth of Warm Springs Canyon (N 4583071, E 317182, elevation data not available), 9/6/2005.

Diagnosis: A member of the Soldier Meadow species flock having a large, ovate- or narrow-conic shell. Penis having small lobe and short filament; penial ornament an elongate terminal gland, medium length penial gland, large Dg1 and Dg2, small Dg3, one–three additional dorsal glands and small ventral gland. *Pyrgulopsis varneri* is larger than its sister species (*P. militaris*) and is further differentiated by its narrower, lower spired shell (Fig. 8). *Pyrgulopsis varneri* is also distinguished from *P. militaris* by its less convex shell whorls; broader whorl shoulders; thicker shell lip; darker periostracum; and differences in penial morphology (larger Dg2, smaller lobe, smaller ventral gland), female genitalia (more elongate bursa copulatrix, shorter bursal duct, smaller seminal receptacle); and mitochondrial DNA sequences (see Discussion section).

Description: Shell (Figs 7,9) clear-white, ovate- or narrow-conic, width/height, 64–78%; height 1.72–2.96 mm; whorls 3.75–5.0. Periostracum brown or tan, thin. Apex blunt, slightly tilted; protoconch (Fig. 10A) 1.25 whorls, diameter about 300 μm, surface weakly wrinkled near apex. Teleoconch whorls weakly or medium convex, having well developed, narrow shoulders; sculpture of collabral growth lines and faint spiral striae. Aperture pyriform, lip often thickened adapically and along parietal wall. Parietal lip narrowly adnate or slightly disjunct; columellar lip narrow. Outer lip thin or slightly thickened, orthocline or slightly prosocline, sometimes weakly sinuate. Umbilicus absent or narrow. Operculum (Fig. 10B–D) amber, ovate, multispiral, having eccentric nucleus; edge of last half whorl sometimes frilled (Fig. 10C); attachment scar thickened along inner edge (Fig. 10D, E). Radula taenioglossate (Fig. 10F), having about 50 well-formed rows of teeth. Central teeth trapezoidal, about 35 μm wide, cutting edge slightly concave; lateral cusps 4–5; central cusp dagger-like (Fig. 10G) or pointed with parallel sides (Fig. 10H); basal cusp 1; basal tongue U-shaped, a little shorter than lateral margins. Lateral tooth (Fig. 10I, J) face rectangular, angled; central cusp broad, pointed or hoe-like, lateral cusps 2–3 (inner), 3 (outer); outer wing fairly broad, weakly flexed, 150–167% length of cutting edge; basal tongue weakly developed. Inner marginal teeth (Fig. 10K) having 16–24 cusps, third or fourth cusp from outer edge sometimes enlarged (Fig. 10F). Outer marginal teeth (Fig. 10L) having 24–34 small cusps, inner edge with long, rectangular wing. Cephalic tentacles dark brown dorsally, ventral surfaces pale. Snout dark brown, distal lips pale. Foot light grey or brown. Pallial roof, visceral coil dark brown dorsally, pigment lighter on pallial genital glands. Ctendium filling most of pallial cavity, positioned a little in front of pericardium; ctenidial filaments about 12, broader than tall, having pleats. Osphradium banana-shaped, positioned slightly posterior to middle of ctenidium. Prostate gland large, white coloured, bean-shaped, with about 33% of length in pallial roof. Anterior vas deferens opening from ventral edge of prostate gland a little in front of pallial wall, section of duct on columellar muscle having S-shaped loop. Penis (Fig. 11A, B) large, square-rectangular, without folds; penial filament (Pf) rather short, narrow, tapering, oblique; lobe small, usually twisted to outer side. Penial gland (Pg) positioned basally along inner edge of

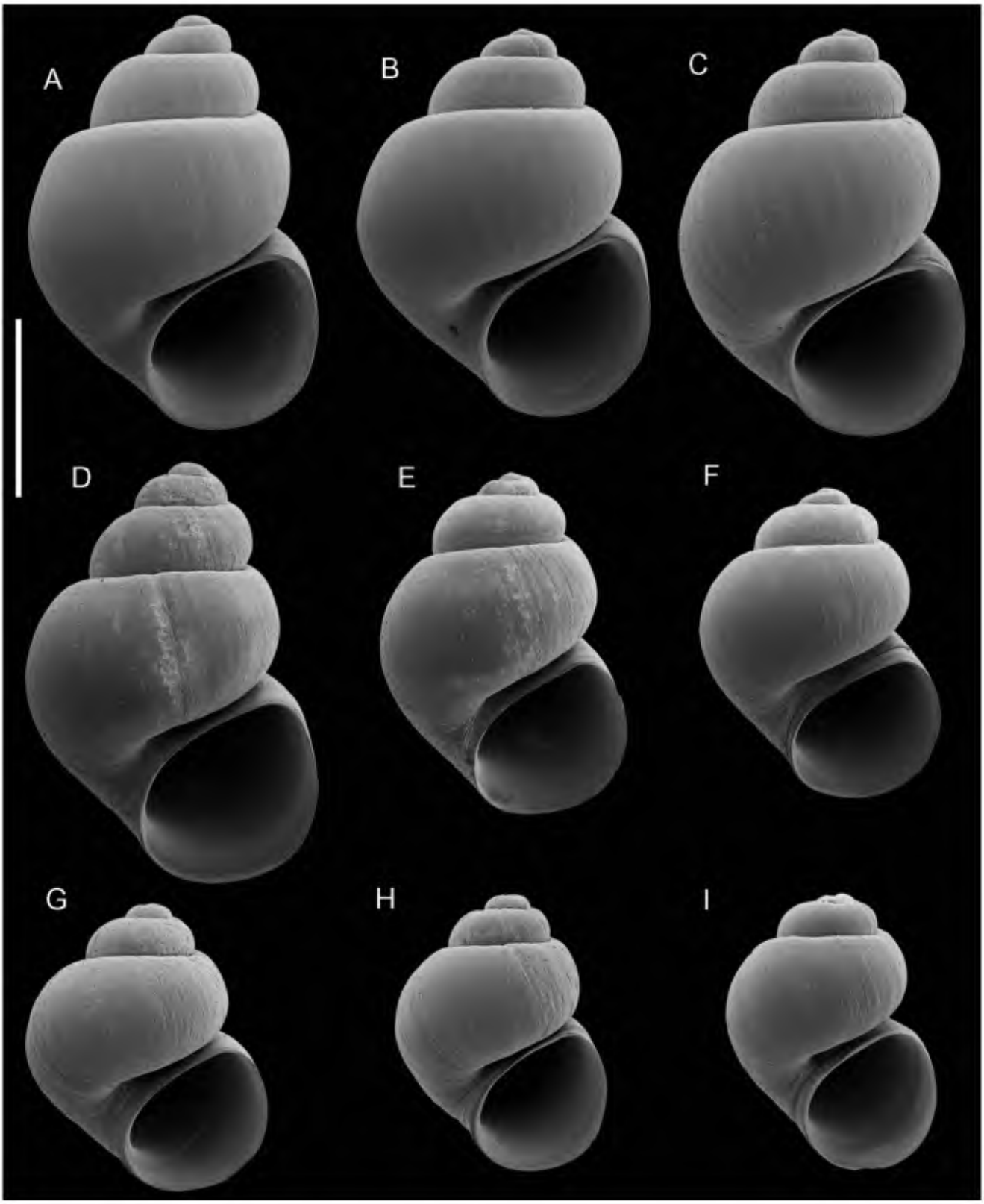

Figure 9. Scanning electron micrographs of shells of *P. varneri*. **A–C.** USNM 1096917. **D–F.** USNM 1083392. **G–I.** USNM 1083249. Scale bar = 1.0 mm.

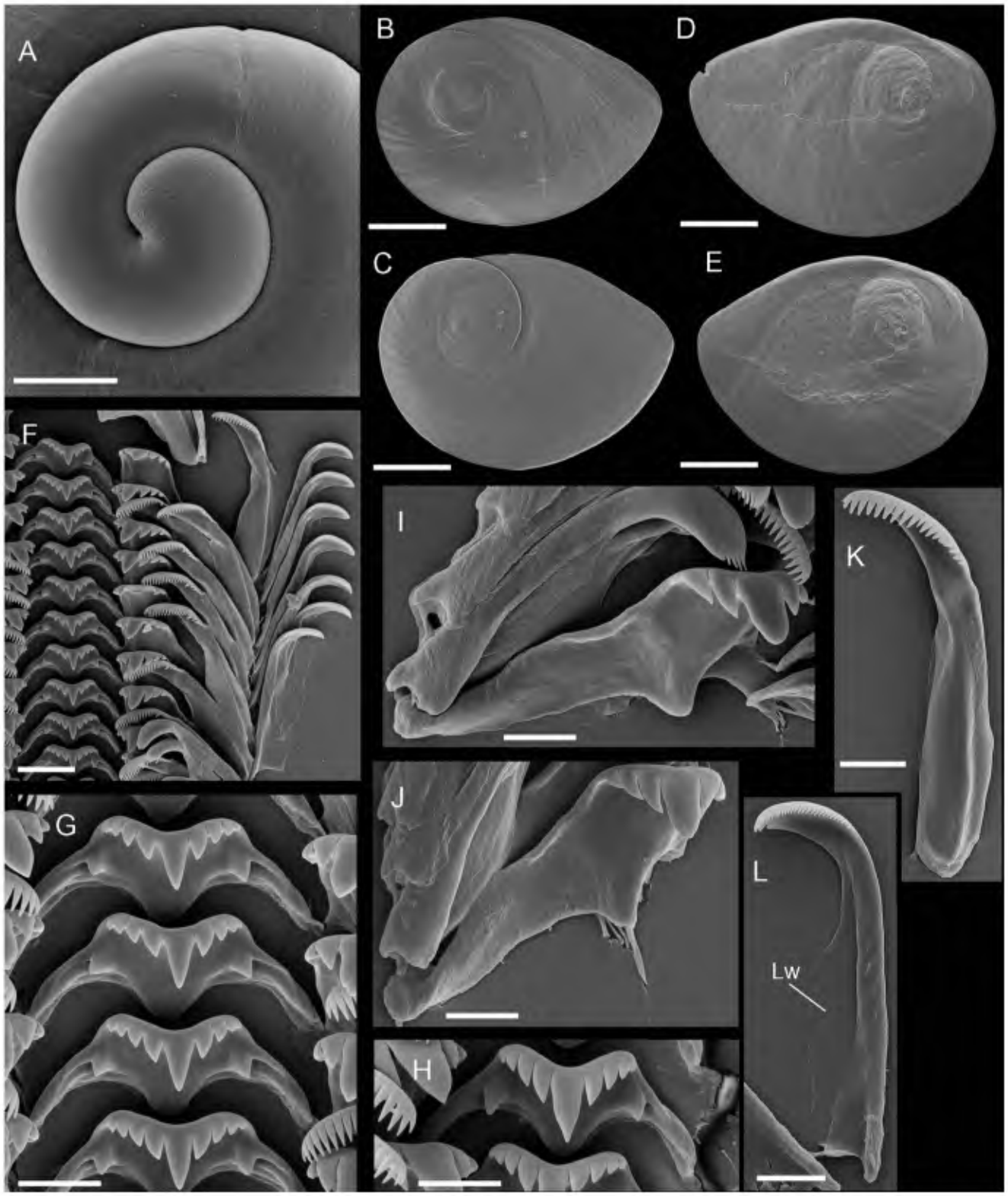

Figure 10. Scanning electron micrographs of shell, opercula and radula of *P. varneri*, USNM 1096917. **A.** Shell apex, showing protoconch sculpture. **B, C.** Opercula, outer side. **D, E.** Opercula, inner side. **F.** Portion of radular ribbon. **G, H.** Central radular teeth. **I, J.** Lateral radular teeth. **K.** Inner marginal tooth. **L.** Outer marginal tooth. Abbreviation: Lw, lateral wing of outer marginal tooth. Scale bars **A** = 100 μm; **B–E**, 250 μm; **F** = 20 μm; **G–K**, 10 μm.

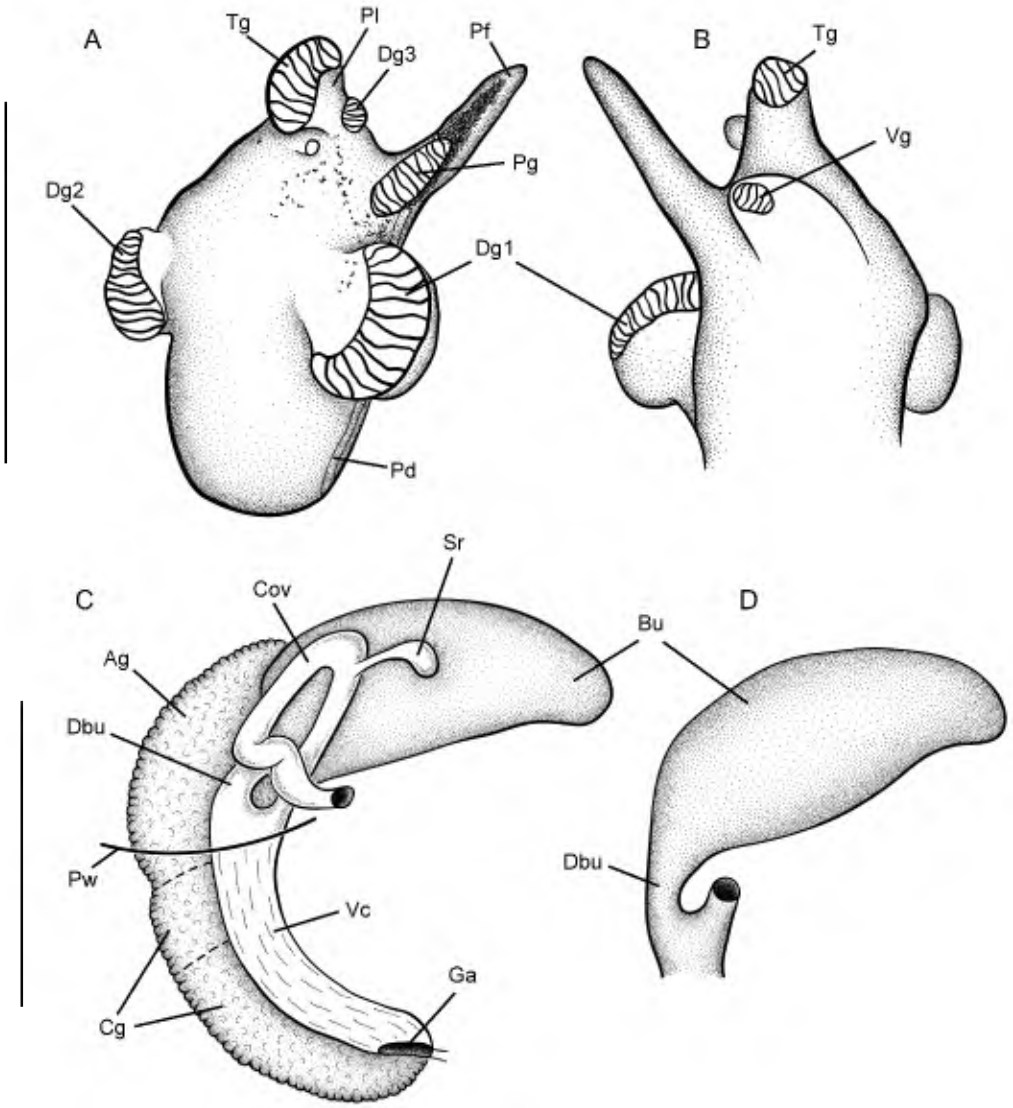

Figure 11. Reproductive anatomy of *P. varneri* (USNM 1093065). **A.** Penis, dorsal surface (pigment in penial filament darkly stippled). **B.** Penis, ventral surface. **C.** Female glandular oviduct and associated structures (viewed from left side). **D.** Bursa copulatrix. Abbreviations: Ag, albumen gland; Bu, bursa copulatrix; Cg, capsule gland; Cov, coiled oviduct; Dbu, bursal duct; Dg1, gland along outer edge of penis; Dg2, gland along inner edge of penis; Dg3, gland on outer edge of penial lobe; Ga, female genital aperture; Pd, penial duct; Pf, penial filament; Pg, penial gland; Pl, penial lobe; Pw, posterior wall of pallial cavity; Sr, seminal receptacle; Tg, terminal gland; Vc, ventral channel of capsule gland; Vg, ventral gland. Scale bars = 500 μm.

filament, filling about 50% of filament length. Terminal gland (Tg) elongate, curved, overlapping dorsal and ventral edges of penial lobe. Dg1 large, curved, borne on fleshy crest, positioned a little behind filament. Dg2 large, curved, borne on crest, positioned medially along inner edge. Dg3 a small circular unit, borne on distinct lobule. Dorsal surface of penis also ornamented with one–three small glands positioned between Dg2 and the terminal gland, glands sometimes seemingly fused into single, elongate unit. Ventral gland (Vg) small, circular, distally positioned on distinct lobe. Penial duct narrow, nearly straight, positioned alongside outer edge. Penial filament darkly pigmented along most of length; scattered pigment granules also present on distal penis. Ovary 1.25 whorls, overlapping stomach, consisting of simple lobes. Female glandular oviduct and associated structures shown in Fig. 11C, D. Coiled oviduct (Cov) an anteriorly kinked, posterior-oblique loop. Bursa copulatrix (Bu) large, pyriform or elongate, horizontal, partly overlapped by albumen gland. Bursal duct (Dbu) short, narrow, opening from distal edge, joining oviduct a little behind pallial wall. Seminal receptacle (Sr) a small folded sac, positioned near middle of bursa copulatrix posterior to edge of albumen gland; duct short. Albumen gland (Ag) having very short pallial section. Capsule gland (Cg) composed of two distinct sections. Genital aperture (Ga) a short, terminal slit. Ventral channel (Vc) rather broad.

Distribution and habitat: *Pyrgulopsis varneri* has been collected from three spring complexes in Soldier Meadow (Fig. 3, as '*P.* sp.'). The temperature of waters occupied by this species ranged from 18.5–37.5° C (D.W. Sada, unpubl.). *Pyrgulopsis varneri* is sympatric with *P. limaria* at several sites in southwestern Soldier Meadow.

Remarks: Shell measurements of *P. varneri* and its sister species, *P. militaris*, are given in Table 3. Note that samples of these two species differed significantly ($P < 0.001$) in all parameters.

Table 3. Shell parameters for *P. varneri* and *P. militaris* and results of *t* tests (separate variances) comparing samples of these species.

	WH	SH	SW	HBW	WBW	AH	AW	SW/SH	HBW/SH	AH/SH
P. varneri										
Holotype	4.50	2.85	1.97	2.18	1.78	1.24	1.11	0.692	0.766	0.433
Paratypes ($n = 30$)										
Mean	4.25	2.61	1.86	2.05	1.64	1.21	1.07	0.713	0.783	0.463
Range	4.00–4.50	2.37–3.01	1.75–2.01	1.90–2.29	1.55–1.77	1.10–1.30	0.970–1.18	0.639–0.782	0.742–0.812	0.421–0.497
SD	0.161	0.140	0.076	0.083	0.068	0.050	0.045	0.030	0.019	0.019
P. militaris										
Paratypes ($n = 30$)										
Mean	3.50	1.48	1.25	1.27	0.990	0.818	0.748	0.845	0.861	0.556
Range	3.25–3.75	1.35–1.68	1.13–1.39	1.14–1.40	0.900–1.14	0.733–0.964	0.680–0.834	0.765–0.905	0.818–0.898	0.489–0.624
SD	0.131	0.088	0.068	0.068	0.053	0.054	0.040	0.036	0.021	0.037
Bog Hot Valley ($n = 30$)										
Mean	3.52	1.84	1.46	1.57	1.22	0.919	0.850	0.796	0.855	0.501
Range	3.25–3.75	1.69–2.28	1.29–1.74	1.43–1.01	1.13–1.45	0.820–1.13	0.775–0.988	0.741–0.872	0.820–0.884	0.467–0.534
SD	0.130	0.119	0.068	0.091	0.072	0.055	0.045	0.032	0.019	0.018
*t**	−19.786	−37.806	−33.253	−39.591	−41.436	−29.225	−29.556	15.377	15.223	12.136
	−20.469	−23.254	−19.168	−21.182	−23.360	−21.509	−19.007	10.425	14.925	7.988
d.f.*	55.8	49.0	57.3	56.0	54.6	57.6	57.1	56.2	57.3	43.0
	51.9	56.6	57.1	57.5	57.8	57.5	58.0	57.8	58.0	57.8
*P**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

WH, total shell whorls; SH, shell height; SW, shell width; HBW, height of body whorl; WBW, width of body whorl; AH, aperture height; AW, aperture width; $t = t$ value; d.f., degrees of freedom; **P. varneri* – *P. militaris* (paratypes), above; *P. varneri* – *P. militaris* (Bog Hot Valley), below.

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REFERENCES

ADAMS, K.D., WESNOUSKY, S.G. & BILLS, B.G. 1999. Isostatic rebound, active faulting, and potential geomorphic effects in the Lake Lahontan basin, Nevada and California. *Geological Society of America Bulletin*, **111**: 1739–1756. [see ftp://rock.geosociety.org/pub/reposit/1999/9991.pdf for relevant supplementary data]

ARBOGAST, B.S., EDWARDS, S.V., WAKELEY, J., BEERLI, P. & SLOWINSKI, J.B. 2002. Estimating divergence times from molecular data on phylogenetic and population genetic timescales. *Annual Review of Ecology and Systematics*, **33**: 707–740.

BROMHAM, L. & PENNY, D. 2003. The modern molecular clock. *Nature Reviews Genetics*, **4**: 216–224.

BUCKLIN, A. 1992. Use of formalin-preserved samples for molecular analysis. *Newsletter of Crustacean Molecular Techniques*, **2**: 3.

CALL, R.E. & PILSBRY, H.A. 1886. On *Pyrgulopsis*, a new genus of rissoid mollusk, with descriptions of two new forms. *Proceedings of the Davenport Academy of Natural Sciences*, **5**: 9–14.

COLGAN, J.P., DUMITRU, T.A., McWILLIAMS, M. & MILLER, E.L. 2004. Timing of Cenozoic volcanism and Basin and Range extension in northwestern Nevada: new constraints from the northern Pine Forest Range. *Geological Society of America Bulletin*, **118**: 126–139.

COLGAN, J.P., DUMITRU, T.A. & MILLER, E.L. 2004. Diachroneity of Basin and Range extension and Yellowstone hotspot volcanism in northwestern Nevada. *Geology*, **32**: 121–124.

COLGAN, J.P., DUMITRU, T.A., REINERS, P.W., WOODEN, J.L. & MILLER, E.L. 2006. Cenozoic tectonic evolution of the Basin and Range Province in northwestern Nevada. *American Journal of Science*, **306**: 616–654.

COOLBAUGH, M.F., AREHART, G.B., FAULDS, J.E. & GARSIDE, L.J. 2005. Geothermal systems in the Great Basin, western United States: modern analogues to the roles of magmatism, structure, and regional tectonics in the formation of gold deposits. In: *Geological Society of Nevada Symposium 2005: window to the world* (H.N., Rhoden, R.C. Steininger & P.G. Vikre, eds), 1063–1081. Geological Society of Nevada, Reno.

CURRY, B.B. & MELHORN, W.N. 1990. Summit Lake landslide and geomorphic history of Summit Lake basin, northwestern Nevada. *Geomorphology*, **4**: 1–17.

DOHRENWEND, J.C. & MORING, B.C. 1991. Reconnaissance photogeologic map of young faults in the Vya 1° by 2° quadrangle, Nevada, Oregon, and California. *United States Geological Survey Miscellaneous Field Studies Map*, **MF-2174**.

DUVERNELL, D.D. & TURNER, B.J. 1998. Evolutionary genetics of Death Valley pupfish populations: mitochondrial DNA sequence variation and population structure. *Molecular Ecology*, **7**: 279–288.

ECHELLE, A.A. & DOWLING, T.E. 1992. Mitochondrial DNA variation and evolution of the Death Valley pupfishes (*Cyprinodon*, Cyprinodontidae). *Evolution*, **46**: 193–206.

ECHELLE, A.A. & KORNFIELD, I. 1984. *Evolution of fish species flocks*. University of Maine at Orono Press, Orono.

FARRIS, J.S., KÄLLERSJÖ, M., KLUGE, A.G. & BULT, C. 1994. Testing significance of incongruence. *Cladistics*, **10:** 315–319.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, **17**: 368–376.

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. & VRIJENHOEK, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**: 294–299.

FUNK, D. & OMLAND, K.E. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology and Systematics*, **34**: 397–423.

GARSIDE, L.J. & SCHILLING, J.H. 1979. Thermal waters of Nevada. *Nevada Bureau of Mines and Geology Bulletin*, **91**: 1–163.

GLAUBRECHT, M. & KÖHLER, F. 2004. Radiating in a river: systematics, molecular genetics and morphological differentiation of viviparous freshwater gastropods endemic to the Kaek River, central Thailand (Cerithioidea, Pachychilidae). *Biological Journal of the Linnean Society*, **82**: 275–311.

GREENWOOD, P.H. 1984. What *is* a species flock? In: *Evolution of fish species flocks* (A.A. Echelle & I. Kornfield, eds), 13–19. University of Maine at Orono Press, Orono.

GROSE, L.T. & KELLER, G.V. 1975. Colorado School of Mines Nevada geothermal study progress report no. 4 – for period February 1 – October 31, 1975. Report to National Science Foundation (NSF Grant GI 43866).

HERSHLER, R. 1989. Springsnails (Gastropoda: Hydrobiidae) of Owens and Amargosa River (exclusive of Ash Meadows) drainages, Death Valley system, California-Nevada. *Proceedings of the Biological Society of Washington*, **102**: 176–248.

HERSHLER, R. 1994. A review of the North American freshwater snail genus *Pyrgulopsis* (Hydrobiidae). *Smithsonian Contributions to Zoology*, **554**: 1–115.

HERSHLER, R. 1998. A systematic review of the hydrobiid snails (Gastropoda: Rissooidea) of the Great Basin, western United States. Part I. Genus *Pyrgulopsis*. *Veliger*, **41**: 1–132.

HERSHLER, R., FREST, T.J., LIU, H.-P. & JOHANNES, E.J. 2003. Rissooidean snails from the Pit River basin, California. *Veliger*, **46**: 275–304.

HERSHLER, R. & LIU, H.-P. 2004a. A molecular phylogeny of aquatic gastropods provides a new perspective on biogeographic history of the Snake River region. *Molecular Phylogenetics and Evolution*, **32**: 927–937.

HERSHLER, R. & LIU, H.-P. 2004b. Taxonomic reappraisal of species assigned to the North American freshwater gastropod subgenus *Natricola* Gregg & Taylor (Rissooidea: Hydrobiidae). *Veliger*, **47**: 66–81.

HERSHLER, R., LIU, H.-P. & THOMPSON, F.G. 2003. Phylogenetic relationships of North American nymphophiline gastropods based on mitochondrial DNA sequences. *Zoologica Scripta*, **32**: 357–366.

HERSHLER, R., LIU, H.-P., FREST, T.J. & JOHANNES, E.J. 2007. Extensive diversification of pebblesnails (Lithoglyphidae: *Fluminicola*) in the upper Sacramento River basin, northwestern United States. *Zoological Journal of the Linnean Society*, **149**, 371–422.

HERSHLER, R. & SADA, D.W. 2002. Biogeography of Great Basin aquatic snails of the genus *Pyrgulopsis*. In: *Great Basin aquatic systems history* (R. Hershler, D.B. Madsen & D.R. Currey, eds), 255–276. Smithsonian Institution Press, Washington, D.C. [*Smithsonian Contributions to the Earth Sciences* **33**].

HOSE, R.K. & TAYLOR, B.E. 1974. Geothermal systems of northern Nevada. *United States Geological Survey Open-File Report*, **74–271**: 1–27.

HOUGHTON, J.G., SAKAMOTO, C.M. & GIFFORD, R.O. 1975. Nevada's weather and climate. *Nevada Bureau of Mines and Geology Special Publication*, **2**: 1–78.

HUBBS, C.L. & MILLER, R.R. 1948. Two new, relict genera of cyprinid fishes from Nevada. *Occasional Papers of the Museum of Zoology, Museum of Michigan*, **507**: 1–30 + plates I–III.

HUELSENBECK, J.P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**: 754–755.

KISHINO, H. & HASEGAWA, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution*, **29**: 170–179.

KUMAR, S. 2005. Molecular clocks: four decades of evolution. *Nature Reviews Genetics*, **6**: 654–662.

KUMAR, S., TAMURA, K. & NEI, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**: 150–163.

LERCH, D., COLGAN, J.P., MILLER, E. & McWILLIAMS, M.O. 2005. Tectonic and magmatic evolution of the northwestern Basin and Range transition zone: mapping and geochronology from the Black Rock Range, NV. *Geological Society of America Abstracts with Programs*, **37**: 70.

LIU, H.-P. & HERSHLER, R. 2005. Molecular systematics and radiation of western North American nymphophiline gastropods. *Molecular Phylogenetics and Evolution*, **34**: 284–298.

LIU, H.-P. & HERSHLER, R. 2007. A test of the vicariance hypothesis of western North American freshwater biogeography. *Journal of Biogeography*, **34**: 534–548.

LIU, H.-P., HERSHLER, R. & CLIFT, K. 2003. Mitochondrial DNA sequences reveal extensive cryptic diversity within a western American springsnail. *Molecular Ecology*, **12**: 2771–2782.

MELHOP, P. 1996. Ecology and conservation needs of hydrobiid snails. *Biodiversity Network News*, **9**: 6–7.

MIFFLIN, M.D. & WHEAT, M.M. 1979. Pluvial lakes and estimated pluvial climates of Nevada. *Nevada Bureau of Mines and Geology Bulletin*, **94**: 1–57.

MLADENKA, G.C. & MINSHALL, G.W. 2001. Variation in the life history and abundance of three populations of Bruneau Hot Springsnails (*Pyrgulopsis bruneauensis*). *Western North American Naturalist*, **61**: 204–212.

NYQUIST, D. 1963. The ecology of *Eremichthys acros*, an endemic thermal species of cyprinid fish from northwestern Nevada. Unpublished MS thesis, University of Nevada, Reno. XII + 247 pp., 27 plates, 2 maps.

PALUMBI, S., MARTIN, A., ROMANO, S., McMILLAN, W.O., STICE, L. & GRABOWSKI, G. 1991. *The simple fools guide to PCR, version II*. University of Hawaii, Honolulu.

POSADA, D. & CRANDALL, K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**: 817–818. [program available from http://darwin.uvigo.es/software/modeltest.html]

QUADE, J., MIFFLIN, M.D., PRATT, W.L., McCOY, W. & BURCKLE, L. 1995. Fossil spring deposits in the southern Great Basin and their implications for changes in water-table levels near Yucca Mountain, Nevada, during Quaternary time. *Geological Society of America Bulletin*, **107**: 213–230.

REHEIS, M. 1999. Extent of Pleistocene lakes in the western Great Basin. *United States Geological Survey Miscellaneous Field Studies Map*, **MF-2323**. [http://pubs.usgs.gov/mf/1999/mf-2323/mf2323.pdf]

REHEIS, M.C., SARNA-WOJCICKI, A.M., REYNOLDS, R.L., REPENNING, C.A. & MIFFLIN, M.D. 2002. Pliocene to middle Pleistocene lakes in the western Great Basin: ages and correlations. In: *Great Basin aquatic systems history* (R. Hershler, D.B. Madsen & D.R. Currey, eds), 53–108. Smithsonian Institution Press, Washington, D.C. [*Smithsonian Contributions to the Earth Sciences* **33**].

SADA, D.W. & POWELL, H. 2001. Distribution, abundance, and habitat use of Soldier Meadow springsnails (family Hydrobiidae). Unpublished report to United States Bureau of Land Management, Winnemucca, NV.

SADA, D.W. & VINYARD, G.L. 2002. Anthropogenic changes in biogeography of Great Basin aquatic biota. In: *Great Basin aquatic systems history* (R. Hershler, D.B. Madsen & D.R. Currey, eds), 277–293. Smithsonian Institution Press, Washington, D.C. [*Smithsonian Contributions to the Earth Sciences* **33**].

SAITOU, N. & NEI, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**: 406–425.

SAWYER, T.L. 1998. Fault number 1486, unnamed faults in northern Black Rock Desert. In: *Quaternary fault and fold database of the United States* (United States Geological Survey website), http://earthquakes.usgs.gov/regional/qfaults (accessed 11/06/2006).

SHEVENELL, L. & GARSIDE, L.J. 2005. Soldier Meadow hot springs (chemistry). http://www.nbmg.unr.edu/geothermal/geochemdata/access/areachem/soldiermeadowshotsprings.xls (accessed 12/8/2006).

SMITH, G.R., DOWLING, T.E., GOBALET, K.W., LUGASKI, T., SHIOZAWA, D.K. & EVANS, R.P. 2002. Biogeography and timing of evolutionary events among Great Basin fishes. In: *Great Basin aquatic systems history* (R. Hershler, D.B. Madsen & D.R. Currey, eds), 175–234. Smithsonian Institution Press, Washington, D.C. [*Smithsonian Contributions to the Earth Sciences* **33**].

SULLIVAN, J.P., LAVOUÉ, S. & HOPKINS, C.D. 2002. Discovery and phylogenetic analysis of a riverine species flock of African electric fishes (Mormyridae: Teleostei). *Evolution*, **56**: 597–616.

SWOFFORD, D.L. 2002. *PAUP*: Phylogenetic analysis using parsimony (and other methods). Version 4.0b10*. Sinauer Associates, Sunderland, MA.

SYSTAT SOFTWARE, INC. (SSI), 2004. *Systat® for Windows®*. Richmond, CA.

TAVARE, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. *Lectures on Mathematics in the Life Sciences*, **17**: 57–86.

TAYLOR, D.W. 1985. Evolution of freshwater drainages and molluscs in western North America. In: *Late Cenozoic history of the Pacific Northwest, interdisciplinary studies on the Clarkia fossil beds of northern Idaho* (C.J. Smiley & A.J. Leviton, eds), 265–321. American Association for the Advancement of Science, San Francisco, CA.

UNITED STATES FISH AND WILDLIFE SERVICE (USFWS). 1997. Recovery plan for the rare species of Soldier Meadows. Portland, Oregon. [http://ecos.fws.gov/docs/recovery_plans/1997/970527.pdf]

UNITED STATES FISH AND WILDLIFE SERVICE (USFWS). 2002. Endangered and threatened wildlife and plants; review of species that are candidates or proposed for listing as endangered or threatened; annual notice of findings on recycled petitions; annual description of progress on listing actions. *Federal Register*, **67**: 40657–40679. [http://www.fws.gov/endangered/candidates/2002cnor.pdf]

VINYARD, G.L. 1996. Distribution of a thermal endemic minnow, the Desert Dace (*Eremichthys acros*), and observations of impacts of water diversion on its population. *Great Basin Naturalist*, **56**: 360–368.

WILLIAMS, J.E., BOWMAN, D.B., BROOKS, J.E., ECHELLE, A.A., EDWARDS, R.J., HENDRICKSON, D.A. & LANDYE, J.J. 1985. Endangered aquatic ecosystems in North American deserts with a list of vanishing fishes of the region. *Journal of the Arizona-Nevada Academy of Science*, **20**: 1–62.