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Taxonomic Reappraisal of Species Assigned to the North American Freshwater Gastropod Subgenus *Natricola* (Rissooidea: Hydrobiidae)

ROBERT HERSHLER

Department of Systematic Biology, Smithsonian Institution, P.O. Box 37012, NHB W-305, MRC 163, Washington, D.C. 20013-7012

AND

HSIU-PING LIU

Department of Biological Sciences, University of Denver, Denver, Colorado 80208, USA

Abstract. The hydrobiid gastropod subgenus *Natricola* (currently placed in synonymy with *Pyrgulopsis*) was erected for three species from the northwestern United States which have a large shell and long accessory process on the penis. Two of these are restricted to the Snake River basin (*P. idahoensis, P. robusta*) while the third ranges among Snake River and Great Basin drainages (*P. hendersoni*). A possible additional species of this subgenus was recently discovered in the Columbia River. In this paper we evaluate the taxonomic status of these closely similar and little studied gastropods. Our review of morphology demonstrated extensive overlap or only slight differences in taxonomic characters previously utilized to diagnose these snails. Our analyses of mitochondrial (cytochrome *c* oxidase) and nuclear (first internal transcribed spacer region) DNA sequences congruently depicted a strongly supported but weakly structured clade composed of these species. Sequence divergences among these species were markedly lower than those documented for other species of *Pyrgulopsis* and for other congeneric freshwater mollusks. Based on the results of our morphological and genetic studies, *P. idahoenis* (Pilsbry, 1933), *P. hendersoni* (Pilsbry, 1933), and the Columbia River population are placed in synonymy with *P. robusta* (Walker, 1908), which is redescribed herein. Our genetic data also suggest that the middle Snake River populations which have been ruled endangered (*P. idahoensis*) may be appropriately treated as a management unit of *P. robusta.*

INTRODUCTION

The hydrobiid gastropod subgenus *Natricola* Gregg & Taylor, 1965, which is currently placed in synonymy with *Pyrgulopsis* Call & Pilsbry, 1886 (Hershler & Thompson, 1987) was erected for three Recent species from the northwestern United States (Figure 1) which have a large shell and long accessory process on the penis. One of these, *P. robusta* (Walker, 1908) is endemic to spring-fed headwaters of the Snake River in Wyoming (Beetle, 1989) whereas *P. idahoensis* (Pilsbry, 1933) lives in the middle Snake River in southern Idaho (Taylor, 1982; USFWS, 1995). The third species, *P. hendersoni* (Pilsbry, 1933), is distributed among springs in the Great Basin and Snake River drainage of southeastern Oregon (Taylor & Smith, 1981; Hershler, 1998). A fossil member of *Natricola* was subsequently described from Pliocene lake beds of northeastern California (Taylor & Smith, 1981) and a possible fourth Recent species was recently discovered in the Columbia River (Frest & Johannes, 1995).

The subgenus *Natricola* has been a focus of biogeographic inquiry in which its distribution was interpreted as evidence of an ancestral route of the Snake River to the Pacific through northeastern California (Taylor & Smith, 1981; Taylor, 1982). (Note that *Natricola* is used herein to conveniently refer to this group of snails, while species names instead are coupled with their correct generic name, *Pyrgulopsis.*) *Natricola* has also received attention from conservation biologists owing to the various threats to its habitats. *Pyrgulopsis idahoensis* has been ruled endangered by the USFWS (1992), *P. robusta* is a former candidate for addition to this list (USFWS, 1994) (and is currently threatened by introduced *Potamopyrgus antipodarum* (Gray, 1853); Richards, 2001), and *P. hendersoni* is listed as critically imperiled (Oregon Natural Heritage Program, 2001). Despite the interest in these species, they have not been studied in depth, and their taxonomic status is in need of further investigation. As is the case for many North American freshwater gastropods, these were originally described based on shells. However, they are closely similar in this aspect of morphology (Figure 2; also see Taylor & Smith, 1981; Taylor, 1982). Taylor (1982) suggested that they are instead differentiated by the shape and glands of the penis and documented variation of these features within *P. idahoensis.* However, the other two Recent species of *Natricola* have not been studied in similar detail and the diagnostic utility of the

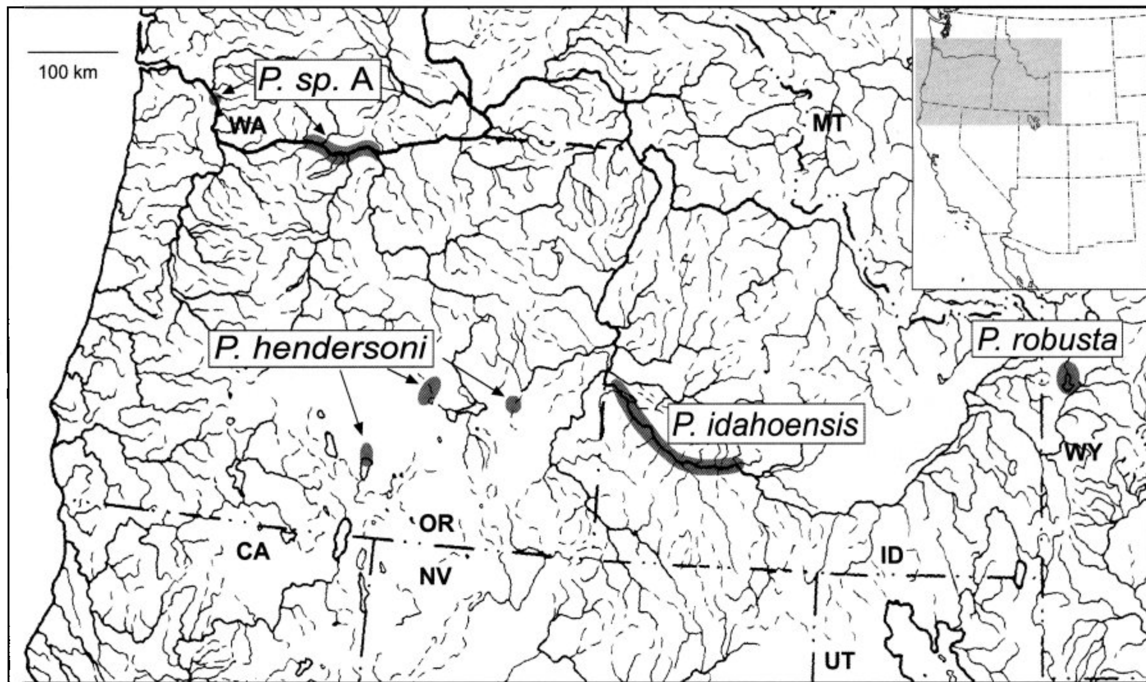

Figure 1. Map showing the distribution of *Natricola* species.

penial characters described by Taylor (1982) is therefore uncertain. Although other aspects of morphology have also been used to differentiate *Natricola* species (radula, Pilsbry, 1933; female genitalia, Hershler, 1994), relatively few specimens were examined in those studies.

In this paper we present new data to evaluate the extent that *Natricola* species can be distinguished on the basis of previously utilized meristic and morphological characters. We also analyze mitochondrial and nuclear genetic markers to assess the phylogenetic relationships of these snails and to quantify the degree of differentiation among them. The taxonomic status of *Natricola* species is reexamined based on the results of these studies.

MATERIALS AND METHODS

Morphology

The total number of shell whorls was counted and standard shell parameters were measured for a series of adult shells (e.g., having a fully formed inner apertural lip) of the Recent species of *Natricola* and the undescribed population from the Columbia River (referred to herein as *P.* sp. A). Ratios were calculated to estimate overall shell shape (shell height/width), relative size of body whorl (shell height/body whorl height), and relative size of aperture (shell height/aperture height). Shells, opercula, and radula were extracted, cleaned with commercial bleach, and mounted on cover slips for study and photography using a scanning electron microscope. For each species the number of cusps was counted on 6–12 examples of each radular tooth type. Shape of the central radular tooth was measured as tooth width/tooth height from the base of the lateral margins to the dorsal edge of the cutting edge. The penial characters used by Taylor (1982: table 1) to describe variation in *P. idahoensis* were scored for one to three samples of each of the other species of *Natricola.* These data were obtained from alcohol-preserved specimens that had been relaxed with menthol prior to fixation so that their penes were in a well extended state, enabling ready scoring of characters. Since suitably prepared alcohol-preserved material of *P. idahoensis* is not available in museum collections, we instead used the data that Taylor [1982] provided for this species. The distal genitalia of five females of each species were dissected and drawn (using a camera lucida) to evaluate variation in characters utilized by Hershler (1994:41, 63). Shell parameters were evaluated by analysis of variance (ANOVA) with Tukey pairwise post-hoc testing of differences among means. Statistical analyses were performed using SYSTAT (Systat Software Inc., 2002). Institutional abbreviations are as follows: Orma J. Smith Museum of Natural History, Albertson College of Idaho (ALBRCIDA); Academy of Natural Sciences of Philadelphia (ANSP); University of Illinois Museum of Natural History (UIMNH), National Museum of Natural History (formerly the United States National Museum) (USNM).

Genetics

We analyzed multiple samples of each species of *Natricola* (spanning most or all of their geographic ranges)

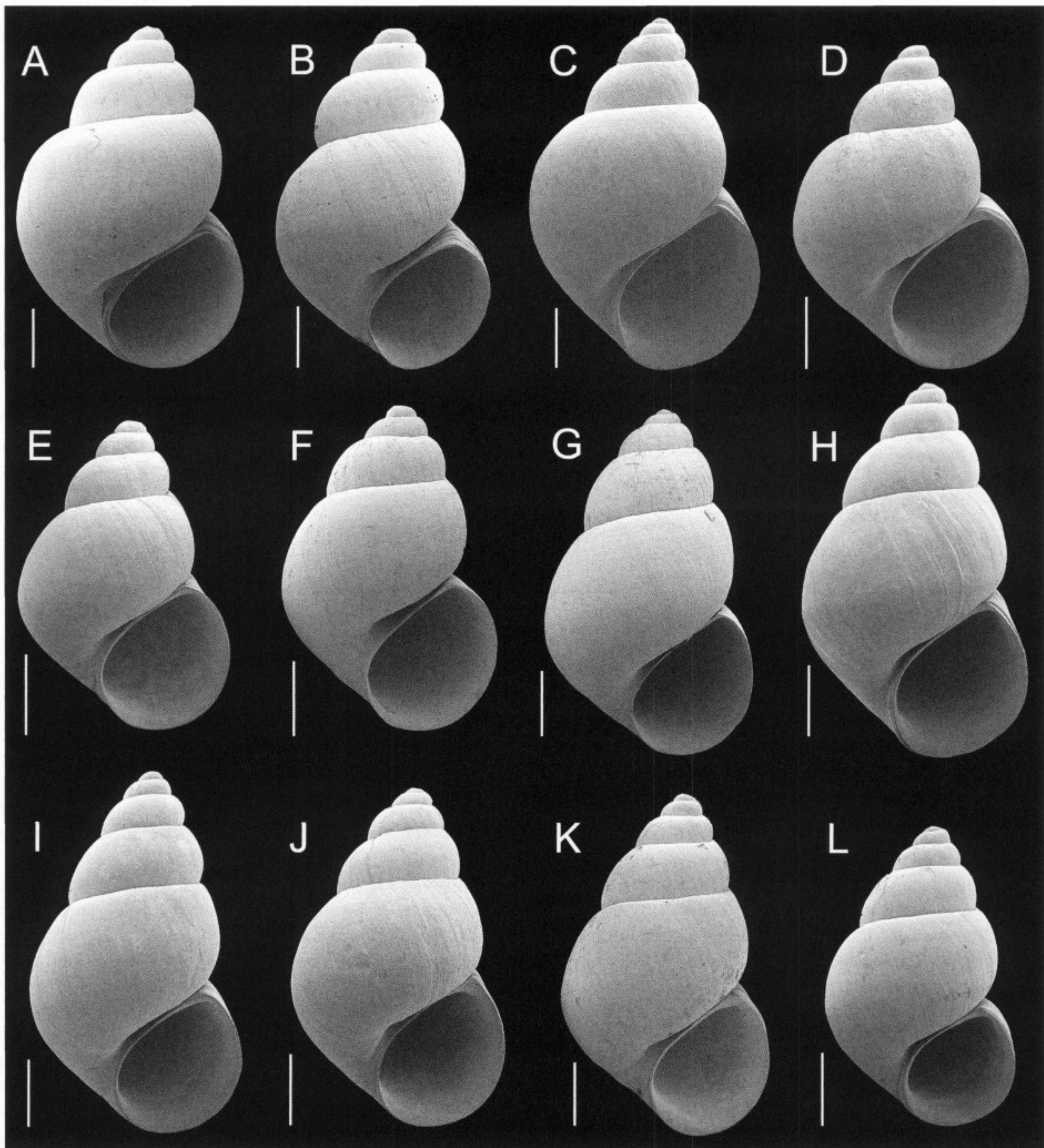

Figure 2. Shells of *Natricola* species. A, B. *P. robusta,* USNM 874185. C. *P. hendersoni,* USNM 874386. D, E. *P. hendersoni,* USNM 892179. F, G. *P. hendersoni,* USNM 884547. H–J. *P. idahoensis,* ALBRCIDA 7568. K, L. *P.* sp. A, USNM 883873. Scales = 1.0 mm.

except for narrowly distributed *P. robusta.* Outgroups consisted of two large congeners from the region, *P. kolobensis* (Taylor, 1987) and an undescribed species from the upper Snake River drainage (referred to herein as *P.* sp. B); and two species, *P. imperialis* Hershler, 1998, and *P. intermedia* (Tryon, 1865), which were shown to be most closely related to *Natricola* in an analysis of mtDNA sequences of 68 species of *Pyrgulopsis* (Liu & Hershler, in preparation). As is commonly done in studies of molecular systematics (e.g., Martin & Bermingham, 1998; Morando et al., 2003), we sequenced two specimens from each sample, except for *P. imperialis,* whose

Table 1

Samples used for DNA sequencing.

Species	Code	Locality	Voucher
Natricola			
P. hendersoni	1	Hughet Spring, Harney basin, Harney Co., OR	USNM 863508
	2	South Fork Malheur Reservoir, Malheur Cave Road crossing, Harney Co., OR	USNM 863509
	3	Unnamed spring, XL Ranch, Lake Abert basin, Lake Co., OR	USNM 1010682
P. idahoensis	1	Bruneau Arm of C.J. Strike Reservoir, (Bruneau) river mile 3.8, Owyhee Co., ID	USNM 1004890
	2	Snake River, river mile 538.0, Elmore Co., ID	USNM 1010689
	3	Snake River, river mile 365.9, 366.3, 367.2, Malheur Co., ID	ALBRCIDA 12268
P. robusta		Spring tributary to Polecat Creek, Teton Co., WY	USNM 1009842
P. sp. A	1	Columbia River, East Mayer State Park, Wasco Co., OR	USNM 1010683
	2	Columbia River, Celilo State Park, Wasco Co., OR	USNM 894695
Outgroups			
P. imperialis		Unnamed spring, Thacker Pass, Kings River Valley, Humboldt Co., NV	USNM 1002354
P. intermedia	1	Crooked Creek, US 95 crossing, Owyhee River basin, Malheur Co., OR	USNM 863511
	2	Skylight Spring, Barren Valley, Malheur Co., OR	USNM 863510
P. kolobensis		Big Malad Spring, Malad Valley, Oneida Co., ID	USNM 1003673
P. sp. B		Teton River, Buxton Bridge crossing, Teton Co., ID	USNM 1003706

single exemplar was used to root all trees. Specimens were collected from 1997–2002 and preserved in 90% ethanol.

We analyzed the first subunit of the cytochrome *c* oxidase (COI) region of mitochondrial DNA because this gene was useful in discriminating among species of *Pyrgulopsis* in previous studies (Hershler et al., 2003; Liu et al., 2003). We also analyzed the first internal transcribed spacer region (ITS-1) between the 5.8S and 18S ribosomal DNA genes to provide a perspective from the nuclear genome.

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.

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, 1 μL of template (ca. 100 ng double-stranded DNA), and 13.8 μL of sterile water. The temperature profile for the PCR reaction consisted of an initial 2 min denaturation step at 94°C, followed by 30 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 ITS-1 region, MUSSEL18S and WHITE5.8 (White et al., 1994; WHITE5.8 5′AGCTRGCTGCGTTCTTCATCGA3′) were used to amplify an approximately 680 bp fragment by PCR. MUSSEL18S (5′TCCCTGCCCTTTGTACACACCG3′) was designed by Liu based on the conserved 18S ribosomal DNA genes which flank the spacer region in mollusks. 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, 1 μL of template (ca. 50 ng double-stranded DNA), and 13.8 μL of sterile water. The temperature profile for the PCR reaction consisted of an initial 2 min denaturation step at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C, and a final extension step at 72°C for 7 min.

Amplified DNA was resolved by electrophoresis on 1.5% agarose gel that was stained with ethidium bromide to check for fragment size, quality, and quantity. The amplified PCR product was incubated at 37°C for 30 min and then at 85°C for another 15 min with 5 units of Exonuclease I (ExoI, Amersham) and 0.5 unit Shrimp Alkaline Phosphatase (SAP, Amersham) to cleave nucleotides one at a time from an end of excess primers and to inactivate single nucleotides. Approximately 10–30 ng of cleaned PCR product was used as a template in a cycle sequencing reaction using the CEQ DTCS Quick Start Kit (Beckman Coulter, Inc.). The following cycling conditions were used: 96°C for 2 min, then 30 cycles of 96°C for 20 sec 45°C for COI and 50°C for ITS-1 for 20 sec, and 60°C for 4 min. The cycle-sequenced product was cleaned following the Beckman Coulter protocol. Fluorescent dye-labeled 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

Table 2

Variation in shell parameters. Sample sizes are in parentheses. Values shown are mean ± standard deviation and range. ANOVA results for comparisons among samples are given on the right (asterisked F values significant, $P < 0.001$). The asterisked mean differs significantly ($P < 0.05$) from those of all other species (Tukey post-hoc test). AH = aperture height, HBW = height of body whorl, SH = shell height, SW = shell width, WH = number of shell whorls.

Variable	*P. robusta* (14)	*P. hendersoni* (13)	*P. hendersoni* (16)	*P. hendersoni* (9)	*P. idahoensis* (16)	*P.* sp A (15)	ANOVA
	USNM 874185 Snake River headwaters	USNM 874386 Harney basin	USNM 883547 Lake Abert basin	USNM 892179 Malheur River	ALBRCIDA 7568 Snake River	USNM 883873 Columbia River	
WH	5.04 ± 0.257	5.21 ± 0.172	5.16 ± 0.27	4.92 ± 0.125	5.57 ± 0.291	5.40 ± 0.184	*F = 13.168
	4.50–5.50	5.00–5.50	4.75–5.75	4.75–5.00	5.25–6.25	5.25–5.75	df = 5, 72
SH	5.72 ± 0.484	5.78 ± 0.479	5.86 ± 0.639	4.95 ± 0.302	6.01 ± 0.572	5.48 ± 0.365	*F = 6.249
	4.94–6.61	4.96–6.58	5.04–7.46	4.61–5.65	5.32–7.88	4.99–6.17	df = 5, 78
SH/SW	1.58 ± 0.063	1.59 ± 0.063	1.70 ± 0.108	1.59 ± 0.056	1.84 ± 0.092	1.71 ± 0.084	*F = 21.569
	1.47–1.71	1.49–1.71	1.54–1.88	1.52–1.67	1.71–2.03	1.58–1.82	df = 5, 78
SH/HBW	1.37 ± 0.048	1.36 ± 0.039	1.41 ± 0.059	1.35 ± 0.043	*1.52 ± 0.061	1.49 ± 0.054	*F = 26.256
	1.31–1.47	1.31–1.45	1.30–1.48	1.29–1.40	1.40–1.62	1.42–1.59	df = 5, 78
SH/AH	2.31 ± 0.116	2.16 ± 0.091	2.37 ± 0.170	2.17 ± 0.117	2.67 ± 0.162	2.61 ± 0.143	*F = 32.474
	2.16–2.57	1.99–2.30	2.12–2.72	1.99–2.33	2.40–2.96	2.36–2.83	df = 5, 78

cold 95% (v/v) ethanol/water. Fluorescent dye-labeled 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. Sequences were determined for both strands and were edited and aligned using Sequencher™. Collecting localities, sample sizes, and voucher information are summarized in Table 1.

Phylogenetic trees based on distance, parsimony, and maximum-likelihood methods were generated using PAUP* 4.0b10 (Swofford, 2002). Maximum-parsimony (MP) analyses were conducted with equal weighting, using the heuristic search option with tree bisection reconnection branch-swapping and 10 random additions. Given their critical importance in phylogenetic reconstruction, gaps in the ITS-1 data set were weighted using three different methods, as suggested by Vogler & DeSalle (1994). First, gaps were treated as missing data and excluded from the analysis. Second, gaps were treated as a "fifth" nucleotide base. Third, each insertion/deletion event was treated as a single character, regardless of the length of gaps. The aligned data matrix is available from authors upon request. Since no such option is available in PAUP, the ITS-1 data matrix was recoded manually. Each method was used to generate the most parsimonious trees, and the resulting topologies were compared and found to be closely similar. In our final parsimony analysis, gaps were treated as a "fifth" nucleotide base. Bootstrapping with 1000 replications was used to evaluate node support. The HKY model and the JC model with variable sites assumed to follow a discrete gamma distribution (e.g., HKY + G; Hasegawa et al. 1985; JC + G; Jukes & Cantor, 1969) were selected as the best fits to the COI and the ITS-1 data, respectively, based on the results of Modeltest 3.06 (Posada & Crandall, 1998). HKY distances were used to generate a neighbor-joining (NJ) tree for the COI data based on the clustering method of Saitou & Nei (1987). Gaps in the aligned ITS-1 matrix were treated as missing in the NJ analysis. Jukes-Cantor (JC) distances were used to generate a NJ tree for the ITS-1 data. Node support was assessed by completion of 1000 bootstrap replications (Felsenstein, 1985) in PAUP, using the fast-search option. Maximum-likelihood (ML) analyses were based on the HKY + G model with empirical base frequencies for COI data and the JC + G model for ITS-1 data using a heuristic search algorithm. A NJ tree with appropriately corrected genetic distances was used as the initial topology for branch-swapping. Node support was evaluated by 100 bootstrap pseudoreplicates.

RESULTS

Morphology

Univariate comparisons among samples of the *Natricola* species were significantly heterogeneous ($P <$

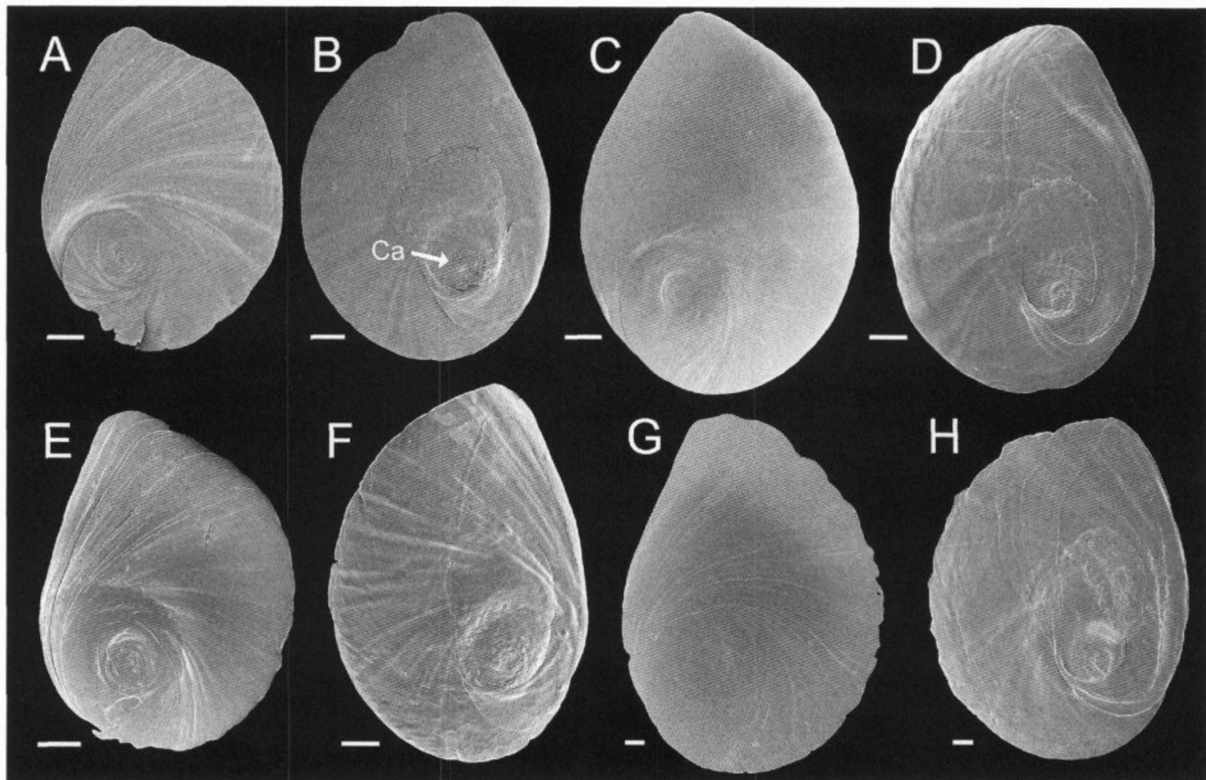

Figure 3. Opercula of *Natricola* species. A, B. *P. robusta* (USNM 874185). C, D. *P. hendersoni* (USNM 874386). E, F. *P. idahoensis* (ALBRCIDA 7568). G, H. *P.* sp. A (USNM 883873). A–F, scales = 200 μm; G, H, scales = 100 μm. Ca = callus.

0.001) for each shell parameter (Table 2). However, the only case in which a given species differed significantly from all others involved the overall shape of *P. idahoensis*, which is congruent with the original diagnosis of this snail based on its slender shell (Pilsbry, 1933). The operculum of *Natricola* species is closely similar in all respects (Figure 3) and our study of this structure did not confirm a previous assertion that the "callus" on the inner surface is consistently weaker in *P. robusta* (Figure 3B) than in the other snails (Hershler, 1994:63). Our findings indicated that the shape of the central cusp of the central teeth (generally rounded to weakly pointed) overlapped among species (Figure 4) and does not provide a basis for distinguishing *P. robusta* (contra Hershler, 1994: 63). We also determined that the number of cusps on the cutting edge of the central teeth broadly overlapped among these species (*P. robusta* and *P. hendersoni*, 3–5; *P. idahoensis*, 3–7; *P.* sp. A., 4–6) and is of no diagnostic utility (contra Pilsbry, 1933:11). However, our findings confirmed that the central teeth of *P. robusta* (Figures 4A–C) have a broader shape (2.85–3.12, n = 4) than those of *P. hendersoni* (per Pilsbry, 1933:11) and the other species (1.79–3.55, n = 13, Figures 4D–L). Our study showed that the inner marginal teeth of *Natricola* species do not differ in terms of "notching" along the edge of the cusp row (contra Pilsbry, 1933:12) or in any other respect (Figures 5A–D). We also determined that the number of cusps on the outer marginal teeth (Figures 5E–H) broadly overlaps among these species (*P. robusta*, 13–29; *P. hendersoni*, 18–28; *P. idahoensis*, 18–22; *P.* sp. A., 20–24) and cannot be used to distinguish among them (contra Pilsbry, 1933:12). The meristic data for penial features overlapped extensively (Table 3) and provided no clear basis for differentiating these snails (contra Taylor, 1982; Hershler, 1994:41), which are closely similar in this aspect of morphology (Figure 6). Our study also indicated that members of *Natricola* are closely similar in female genitalia (Figure 7). *Pyrgulopsis hendersoni* and *P. idahoensis* differed little if at all in three of the characters previously used to distinguish these species (shape of bursa copulatrix, position of bursal duct and seminal receptacle; Hershler, 1994:41). The fourth character, a second well developed oviduct coil, was found to be more commonly present in *P. idahoensis* (all five specimens) than in *P. hendersoni* (three of five specimens) (per Hershler, 1994). Our findings did not confirm that *P. robusta* has a broader bursa copulatrix than other *Natricola* species (contra Hershler, 1994:63). We also determined that the "anterior capsule gland vestibule" used to distinguish *P. robusta* (Hershler, 1994:63) was variably

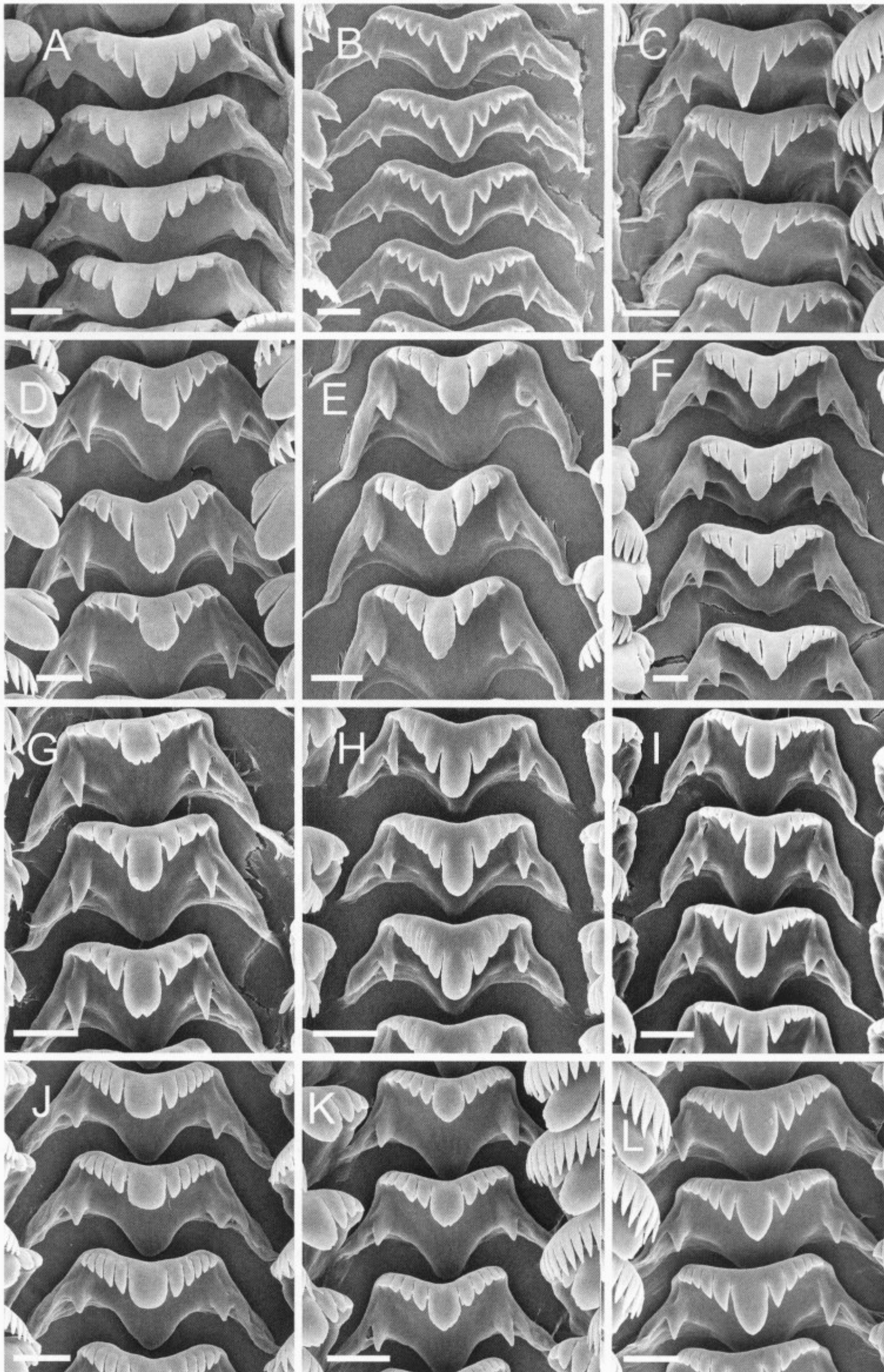

Figure 4. Central radular teeth of *Natricola* species. A–C. *P. robusta,* USNM 874185. D–F, *P. hendersoni,* USNM 874386. G–I, *P. idahoensis,* ALBRCIDA 7568. J–L, *P.* sp. A, USNM 883873. Scales = 10 μm.

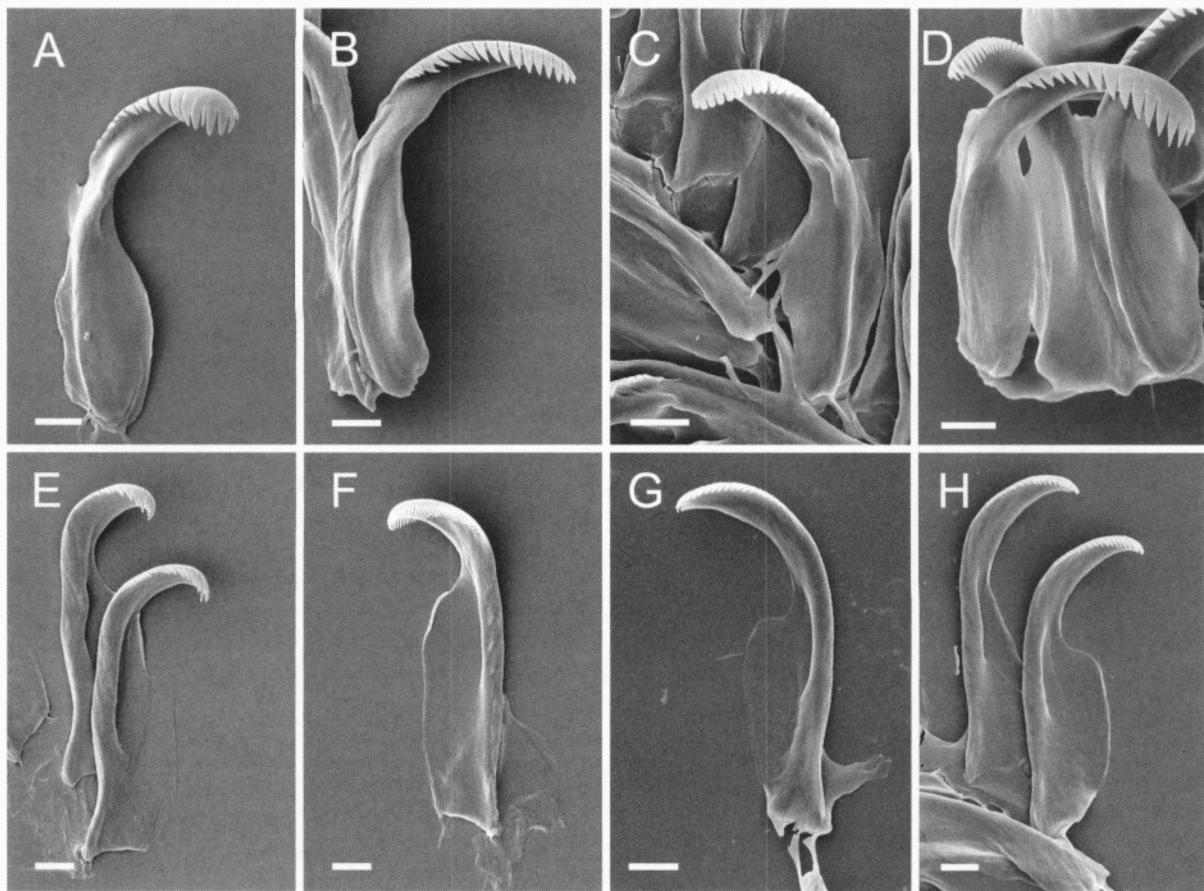

Figure 5. Marginal (A–D, inner; E–H, outer) radular teeth of *Natricola* species. A, E. *P. robusta,* USNM 874185. B, F. *P. hendersoni,* USNM 874386. C, G. *P. idahoensis,* ALBRCIDA 7568. D, H. *P.* sp. A, USNM 883873. Scales = 10 μm.

expressed in this and the other species of *Natricola* (not figured). Other potentially useful female genitalic characters (e.g., length and width of bursal duct) were variable within and overlapping among species (Figure 7).

Genetics

Sequences are deposited in GenBank (Accession Numbers AY379424–AV379477). The alignment of sequences from the partial COI gene yielded 619 bp, of which 62 sites were variable (10.0%), and 53 were parsimony informative (8.6%). The MP analysis of these data yielded six trees of 75 steps (CI = 0.85), one of which is shown in Figure 8. All of these trees depicted a strongly supported *Natricola* clade that was sister to *P. intermedia,* which lives in Snake River drainage of southeast Oregon (Tryon, 1865). Groupings of populations within the *Natricola* clade varied among these trees and were neither strongly supported, nor entirely consistent with species assignments (e.g., varied positions of *P.* sp. A sequences). The NJ and ML (not figured) topologies were entirely congruent in all of the above respects. Differences between haplotypes of species of *Natricola* ranged from *P* = 0.0–0.8% (0–5 bp). In one case a (single) haplotype was shared among two species (*P. robusta, P.* sp. A). Species assigned to *Natricola* were differentiated from *P. intermedia* by 2.6–3.1% (16–19 bp) and from the other outgroups by 3.4–6.9% (21–43 bp).

Homologous nucleotide sequences from the partial 18S rDNA gene, the complete ITS-1 region, and the partial 5.8S gene were obtained from each specimen. The 3″ end of the 18S ribosomal gene is highly conserved and therefore easily recognizable. Only one of the 133 nucleotides of this gene was variable. Average base frequencies for 18S were 27.1% A, 24.8% T, 18.0% C, and 30.0% G. Of the 52–54 nucleotides of the 5.8S rRNA gene, four were variable (two substitutions and two indels). Average base frequencies for 5.8S were 26.4% A, 29.6% T, 26.8% C, 17.2% G. The length of ITS-1 varied from 382 bp in *P. imperialis* to 451 bp in *P. intermedia.* Average base frequencies for ITS-1 were 20.1% A, 25.7% T, 25.6% C, and 28.8% G. The total aligned data matrix including indels (insertion/deletions) was 689 bp, of which 67 sites

Table 3

Meristic variation in penial features. N = 30 for all samples. Data for *P. idahoensis* are from Taylor (1982). DDL = dorsal distal lobule, DPL = dorsal proximal lobule with glandular strip, Dr = anterior glandular strip, VL = ventral lobule. * includes specimens in which DPL did not extend to base of filament and Dr was absent. ** includes specimen in which DDL lacked glands. *** includes specimen(s) having lobule but lacking ventral glands.

	P. robusta	*P. hendersoni*	*P. hendersoni*	*P. hendersoni*	*P. idahoensis*	*P. idahoensis*	*P.* sp. A
Variable	Snake River headwaters	Harney Lake basin	Lake Albert basin	Malheur River	Snake River, Bancroft Springs	Snake River, south of Hammett	Columbia River
Terminal gland (T)							
(a) long strip	29	27	28	28	29	29	30
(b) divided into 2 strips	1	3	2	2	1	1	0
Dorsal glands							
Strong DPL with long strip							
(a) DPL continuous onto base of free por-	30	30*	30	30*	30	30	30
tion	29	20	27	23	28	30	26
(b) DPL separate from all or part of Dr	1	7	3	6	2	0	4
Glands on medial side of DPL-Dr							
0	23	6	28	17	12	13	19
1	6	13	2	10	10	9	5
2	1	6	0	3	4	5	5
3	0	3	0	0	3	2	1
4	0	1	0	0	1	1	0
5	0	1	0	0	0	0	0
Strong dorsal distal lobule (DDL)	26**	30	29	30	30	30	30
(a) strip on DDL separate	25	24	28	22	20	18	21
(b) strip on DDL continued proximally	4	6	1	8	10	12	9
Number of glands separate from DDL							
0	2	1	19	1	7	10	3
1	16	12	9	12	13	9	15
2	10	7	1	13	8	6	11
3	1	7	1	2	0	4	1
4	0	3	0	2	2	1	0
Ventral glands				***	***	***	***
(a) no glands, no lobule	2	11	0	13	16	19	17
(b) gland on VL	26*	16	27	5	6	8	6
(c) minor gland on VL site	0	3	3	9	7	1	6
# additional minor glands							
1	4	4	5	0	4	2	3
2	2	1	1	0	1	2	1

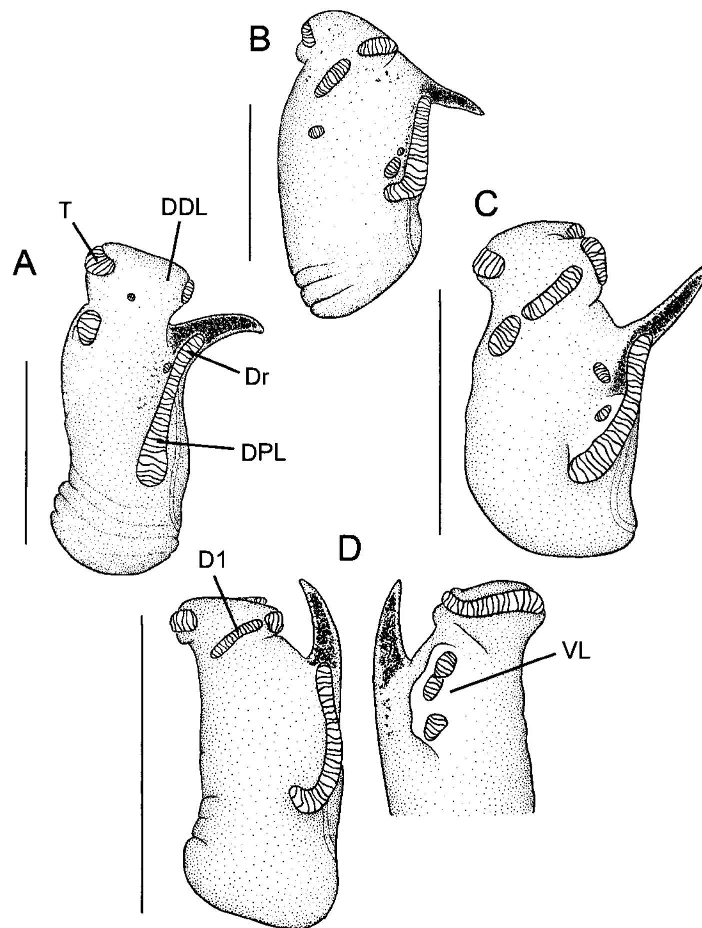

Figure 6. Penis of *Natricola* species. A. *P. robusta,* USNM 874185. B. *P. hendersoni,* USNM 874386. C. *P. idahoensis,* USNM 1011032. D. *P.* sp. A, USNM 883873. Scales = 1.0 mm. D1 = gland on DDL, DDL = dorsal distal lobule, DPL = dorsal proximal lobule with glandular strip, Dr = anterior glandular strip, T = terminal gland, VL = ventral lobule.

were variable (9.7%) and 49 (7.1%) were parsimony informative. The MP analysis of these data yielded a single tree of 84 steps (CI = 0.93), which is shown in Figure 9. As was the case for the COI dataset, a *Natricola* clade was resolved with high bootstrap support (98%) and was depicted as sister to *P. intermedia.* The *Natricola* clade was weakly structured and sub-groupings were little congruent with species assignments. The NJ and ML trees (not figured) also depicted a strongly supported yet weakly structured *Natricola* clade, and differed from the MP tree only in terms of relationships among outgroups. Percent uncorrected p distances (with gaps treated as missing data) among genotypes of *Natricola* species ranged from 0.0–0.6% (0–4 bp). Each of these species shared one or more genotypes with at least one other member of the subgenus. Species of *Natricola* were differentiated from *P. intermedia* by 0.47–0.79% (3–5 bp) and from the rest of the outgroups by 2.81–4.28% (17–24 bp).

Inasmuch as COI and ITS-1 sequences yielded highly congruent results, we did not perform a combined analysis of these datasets. Noncoding DNA sequences frequently are more variable than coding regions and therefore have been considered an appropriate marker for studies of lower taxonomic levels (Smith & Klein, 1994). Note, however, that in our study ITS-1 was less variable than the COI coding region with respect to members of the *Natricola* clade (five vs. eight substitutions, respectively) and provided a weaker signal in resolving the phylogenetic relationships among these snails.

DISCUSSION

Our findings indicated that species of *Natricola* are very closely similar morphologically. The only significant difference that we found in external features typically used to diagnose species of *Pyrgulopsis* (e.g., shell, penis; Hershler, 1994) was the slender shell of *P. idahoensis.* However, the taxonomic significance of this difference may be debated given its overlap among species (Table 2; also see Taylor & Smith, 1981: table 1, fig. 6) and the possi-

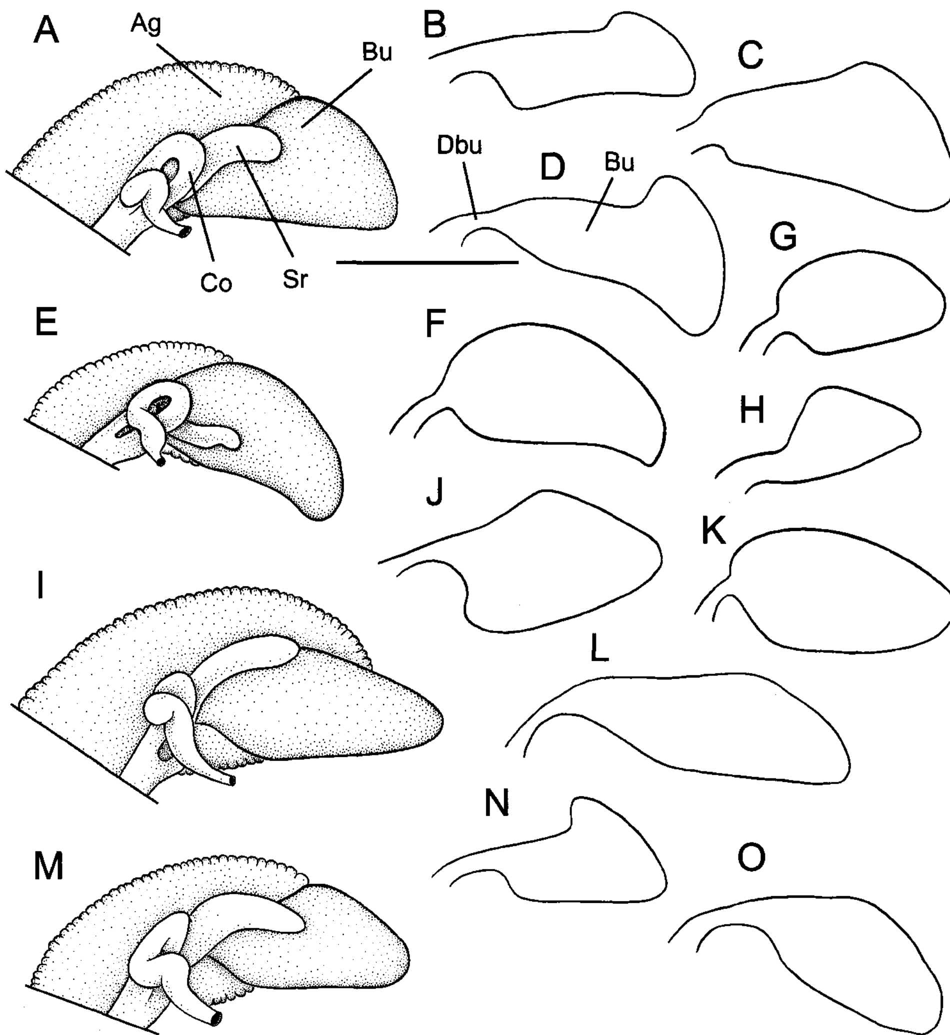

Figure 7. Distal female genitalia of *Natricola* species. Bursa copulatrix variation is shown to the right. A–D. *P. robusta,* USNM 874185. E–H. *P. hendersoni,* USNM 883549. I–L. *P. idahoensis,* USNM 1011032. M–O. *P.* sp. A, USNM 883873. Scale = 0.5 mm. Ag = albumen gland; Bu = bursa copulatrix, Co = coiled oviduct; Dbu = bursal duct; Sr = seminal receptacle.

bility that variation in shell features within *Natricola* may be influenced by ecological factors (e.g., spring vs. riverine habitat; Taylor & Smith, 1981). We also found only a single consistent anatomical difference among these snails, the broad central radular teeth of *P. robusta.*

Our findings from two independent sources of genetic variation confirmed a close phylogenetic relationship between the species of *Natricola,* which were congruently resolved as a strongly supported clade. However, these species were not well supported as monophyletic units, and the level of distinctiveness among them (0.0–0.8%, based on COI) is within the range of intra-specific genetic distances of other species of *Pyrgulopsis.* (Note that these same patterns were observed in our [unpublished] analysis of a substantially larger number of sequences of these species; Hershler & Liu, 2003). In comparison, sequence divergence among 68 other congeners for which we have COI data ranged from 1.18–12.21% (Liu & Hershler, in preparation). COI divergence between species of *Natricola* is also low relative to values documented for other freshwater molluscan congeners—e.g., 1.3–14.8% in *Tryonia* (Gastropoda: Cochliopidae) (Hershler et al., 1999), 1.2–14.5% in *Potamilus* (Bivalvia: Unionidae) (Roe & Lydeard, 1998), and 3.8–15.0% in *Lasmigona* (Bivalvia: Unionidae) (King et al., 1999). Divergence of *Natricola* species based on ITS-1 sequences (0–0.6%) is also very low in comparison to values among other congeners (5.9–20.4% for 16 species; Kepes, 2003).

A recent mtDNA survey of another widely distributed congener, *P. micrococcus,* revealed significant sequence divergence (up to 10.59%) among 65 specimens from 29 populations in five different drainage basins (Liu et al.,

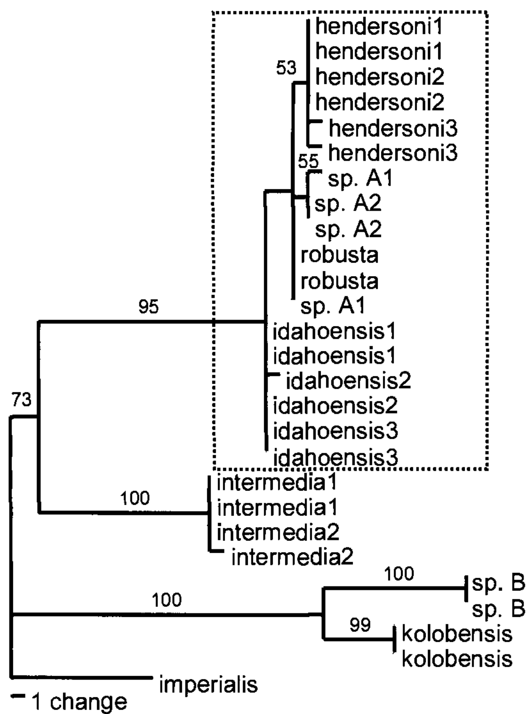

Figure 8. One of six minimum length topologies generated by maximum parsimony analysis of the COI nucleotide matrix. Bootstrap percentages > 50% are shown. The *Natricola* clade is indicated by dotted lines.

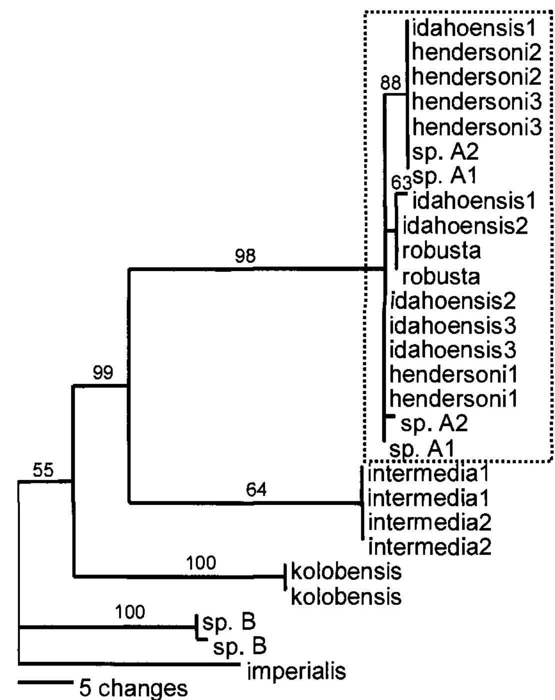

Figure 9. Tree topology produced by maximum parsimony analysis of the ITS-1 nucleotide matrix. Bootstrap percentages > 50% are shown. The *Natricola* clade is indicated by dotted lines.

2003). Haplotypes were resolved among five well supported clades. Based on the amount of sequence divergence and the inferred phylogenetic relationships of these populations, the authors concluded that *P. micrococcus* is a complex which includes as many as seven or eight previously undescribed species. This study demonstrated the utility of the COI gene for phylogenetic resolution of *Pyrgulopsis* at the species level.

Three independent data sets (morphology, mitochondrial, and nuclear DNA sequences) congruently suggest that these four *Natricola* snails do not merit recognition as distinct species according to various currently applied concepts of this taxonomic rank (summarized in Mayden, 1997). These snails instead should be treated as a single species, whose correct (e.g., oldest available) name is *P. robusta* (= *Pomatiopsis robusta* Walker, 1908). As newly constituted herein, this species has a considerably broader distribution than most other congeners. Note, however, that in contrast with this snail, which has had opportunities to disperse within relatively continuous riverine habitat, most other members of *Pyrgulopsis* are restricted to small, isolated springs and streams. Although a treatment of biogeographic history of these species is beyond the scope of this paper (and will be presented elsewhere), we nonetheless note that spread of progenitors among currently inhabited areas may also have been facilitated by regional flooding (Baker & Bunker, 1985; O'Connor, 1993) and integration of Snake River and Oregon Great Basin drainages (Taylor, 1985:309) during the late Pleistocene.

The taxonomic change proposed herein has important conservation implications given the federally listed status of *P. idahoensis* (Idaho springsnail). This taxon, which is restricted to portions of the middle Snake River, is distributed in an allopatric fashion with respect to other populations that we consider to be conspecific (Figure 1). Although it is possible that the distribution of snails in the Snake-Columbia River is underestimated because of incomplete sampling, genetic exchange between the middle Snake River and other populations is nonetheless presumably precluded by the presence of natural (e.g., falls) and man-made (e.g., dams) barriers. The absence of contemporary gene flow is further suggested by two fixed nucleotide differences in COI which distinguish the middle Snake River populations. Application of a recently proposed COI calibration rate for hydrobiid snails (1.8% divergence per million years; Wilke, 2003) implies that divergence of the middle Snake River populations (0.3–0.8% for COI) minimally occurred 0.16 Ma. However, despite this possibly substantial history of isolation, divergence of this and the other three groups of populations has not yet proceeded to the point of reciprocal monophyly and thus the Idaho springsnail does not merit rec-

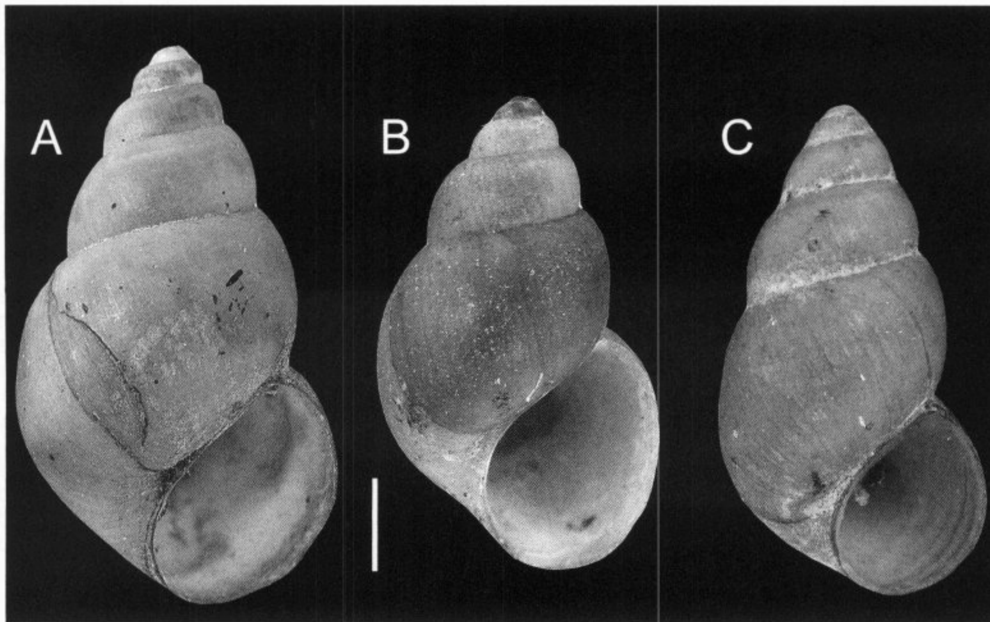

Figure 10. Type specimens of *Pyrgulopsis robusta* and its junior synonyms. A. *P. robusta,* Jackson Lake, Wyoming (holotype, UIMNH Z17982). B. *P. hendersoni,* south of Burns, Oregon (holotype, ANSP 145951). C. *P. idahoensis,* Homedale, Idaho (lectotype, ANSP 152677). Scale = 1.0 mm.

ognition as a separate evolutionarily significant unit (ESU) in accordance with the commonly applied definition of Moritz (1994). The middle Snake River populations may more appropriately be treated as a "management unit" (of *P. robusta*), which has been defined as populations having significant divergence at nuclear or mitochondrial loci irrespective of phylogenetic distinctiveness (Moritz, 1994).

TAXONOMY

Superfamily Rissooidea

Family Hydrobiidae

Pyrgulopsis Call & Pilsbry, 1886

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

Fontelicella Gregg & Taylor, 1965:103 [type species: *Fontelicella californiensis* Gregg & Taylor, 1965, by original designation].

Natricola Gregg & Taylor, 1965:108 [type species: *Pomatiopsis robusta* Walker, 1908, by original designation; proposed as a subgenus of *Fontelicella*].

Microamnicola Gregg & Taylor, 1965:109 [type species: *Amnicola micrococcus* Pilsbry in Stearns, 1893, by original designation; proposed as a subgenus of *Fontelicella*].

Savaginius Taylor, 1966:130 [type species: *Paludestrina nanna* Chamberlain & Berry, 1933; original designation].

Mexistiobia Hershler, 1985:46 [type species: *Mexistiobia manantiali* Hershler, 1985; original designation].

Apachecoccus Taylor, 1987:32 [type species: *Apachecoccus arizonae* Taylor, 1987; original designation].

Yaquicoccus Taylor, 1987:34 [type species: *Yaquicoccus bernardinus* Taylor, 1987; original designation].

Pyrgulopsis robusta (Walker, 1908) Jackson Lake Springsnail

Pomatiopsis robusta Walker, 1908:97, unlabeled figure (shell) (Jackson Lake, Wyoming; holotype, UIMNH Z17982, 6.3 × 3.5 mm, Figure 10A).

Amnicola robusta.—Pilsbry, 1933:9–10, plate 2, figs. 1 (radula), 7, 8 (shells).

Amnicola hendersoni Pilsbry, 1933:10–11, plate 2, figs. 2 (radula), 9, 10 (shells) (south of Burns, Oregon; holotype, ANSP 145951, 4.95 × 2.85 mm, Figure 10B).

Amnicola idahoensis Pilsbry, 1933:11–12, plate 2, figs. 3 (radula), 4, 5 (shells) (Homedale, Owyhee County, Idaho; lectotype, ANSP 152677, 6.1 × 2.95 mm, Figure 10C).

Fontelicella (*Natricola*) *robusta.*—Gregg & Taylor, 1965: 109.

Fontelicella (*Natricola*) *hendersoni.*—Gregg & Taylor, 1965:109.—Taylor & Smith, 1981:table 1, figure 6 (shell data).

Fontelicella (*Natricola*) *idahoensis.*—Gregg & Taylor, 1965: 109.—Taylor & Smith, 1981:table 1, figure 6 (shell data).—Taylor, 1982:1–12, table 1 (penial data), unlabeled figure (shell, operculum, penis).

F.[*ontelicella*] [*Natricola*] sp.—Taylor & Smith, 1981:352 (Abert Lake basin record).

Pyrgulopsis hendersoni.—Hershler & Thompson, 1987: 29.—Hershler, 1994:40–41, figs. 16 (a, b, shell; c, d,

operculum), 36a (central radular teeth), 46f (penis).—Hershler, 1998:98–99 (new record, Abert Lake basin).
Pyrgulopsis idahoensis.—Hershler & Thompson, 1987: 29.—Hershler, 1994:41–42, figs. 16 (e, shell; f,g, operculum), 36b (central radular teeth), 47a (penis).
Pyrgulopsis robusta.—Hershler & Thompson, 1987:30.—Hershler, 1994:62–63, figs. 5f (female genitalia), 24 (d, shell; e, f, operculum), 39c (central radular teeth), 50d (penis).

Diagnosis: Shell large for genus; usually ovate- to narrow-conic, rarely subglobose; whorls weakly to moderately convex. Penial lobe and filament about equal in length; dorsal proximal lobule well developed, usually overlapping base of filament and often borne on weak swelling proximally; terminal gland elongate, transverse; dorsal distal lobule well developed, usually bearing one or a series of small glands; ventral lobule usually well developed and bearing a large gland.

Description: Emended from that of Hershler (1994). Shell ca. 4.6–7.5 mm tall, shell height/shell width 1.48–2.10, shell height/aperture height 1.98–3.38, whorls 4.5–6.25 (Table 2). Shell clear-white, periostracum tan. Protoconch 1.3–1.4 whorls, smooth throughout or with apical section weakly wrinkled. Teleoconch whorls often shouldered, typically sculptured with well developed, collabral growth lines; body whorl rarely having weak basal angulation (Figure 2H), often sculptured with numerous faint spiral threads. Aperture ovate, weakly angled above. Inner lip complete in larger specimens, often slightly thickened, broadly adnate to slightly disjunct. Outer lip orthocline or prosocline. Shell narrowly umbilicate.

Operculum ovate, multispiral; nucleus eccentric. Outer surface smooth or with whorl edges weakly frilled (Figure 3A). Edges of attachment scar sometimes moderately thickened (Figure 3D); central portion of attachment region (callus) sometimes slightly raised (Figure 3B).

Central radular teeth (Figure 4) with hoelike to weakly pointed central cusps; lateral cusps, three to seven; single pair of basal cusps well developed. Basal tongue of central teeth broad V-shaped, base a little shorter than distal ends of lateral margins. Central cusp of lateral teeth broad, hoelike, lateral cusps, one to four (inner side), two to five (outer side); outer wing a little longer to a little shorter than height of tooth face. Inner marginal teeth with 16–25 cusps. Outer marginal teeth with 17–29 cusps; outer edge having long rectangular wing (Figures 5E–H).

Dark pigment present on head, pallial roof, and visceral coil. Ctenidium well developed; filaments 32–36, broad; osphradium elongate. Stomach with large posterior caecum.

Testis large, overlapping stomach anteriorly. Prostate gland with large pallial section; pallial vas deferens with prominent proximal bend. Penis medium to large; base rectangular, sometimes weakly folded along inner edge (Figure 6A); filament medium length, tapering, horizontal (Figure 6D) or oblique (Figure 6A); lobe medium length, broad. Terminal gland often largely restricted to ventral surface of distal edge of lobe, rarely divided into multiple units. Dorsal proximal lobule (= Dg1 of Hershler, 1994) elongate, rarely split into multiple units, often flanked on inner side by one-five small glandular units. Dorsal distal lobule usually raised and bearing a small glandular unit (= Dg3 of Hershler, 1994), often accompanied by additional glandular units extending to inner edge (Figure 6C). Ventral lobule sometimes having second glandular unit (Figure 6D); additional gland also sometimes present on distal portion of ventral penis.

Ovary fairly large (> 1.0 whorl), overlapping stomach anteriorly. Renal oviduct of two small, broadly overlapping, posterior oblique loops, proximal coil sometimes only weakly kinked (Figure 7E). Bursa copulatrix medium sized (relative to albumen gland); ovate, pyriform, or elongate; largely overlapped by albumen gland. Bursal duct short, variable in width (Figures 7C, L). Seminal receptacle narrow or fingerlike, variably positioned on albumen gland, overlapping anterior half of bursa copulatrix. Albumen gland entirely visceral or with very short pallial section. Capsule gland of two glandular units. Genital opening a subterminal slit.

Material examined: IDAHO. *Elmore County*: Snake River, river mile 473.8 (ALBRCIDA 7568).—Snake River, river mile 489.5 (USNM 1011032). **OREGON.** *Harney County:* South Fork Malheur River, below South Fork Reservoir (USNM 892179).—Lower Sizemore Spring, Harney Lake basin (USNM 874386).—Barnyard Spring, Harney Lake basin (USNM 883549). *Lake County:* spring, XL Ranch, Lake Abert basin (USNM 1010534).—spring, near northwest corner of Lake Abert (USNM 883547). *Wasco County*: Columbia River, Celilo Park (USNM 883873, USNM 1010533). **WYOMING.** *Teton County*; Polecat Creek, west of Flagg Ranch (USNM 874185).

Distribution: Broadly ranging in the northwestern United States, including parts of the Snake-Columbia River basin and several closed basins in southeastern Oregon. Habitats include springs and spring-fed streams as well as large rivers. Does not co-occur with other species of *Pyrgulopsis,* but is sometimes sympatric with other hydrobiid snails (e.g., *Fluminicola, Taylorconcha*).

Remarks: The synonymy given above is not intended to be complete. The reader is referred to Taylor (1975) and Hershler (1994) for additional references to *P. robusta* and its junior synonyms. Note that we are also treating the previously undescribed snail from the Columbia River (Frest & Johannes, 1995:202–203) as *P. robusta. Pyrgulopsis robusta* is readily distinguished from *P. intermedia,* which is depicted as its sister species based on molecular evidence (Figures 8, 9), by several penial features, including a larger penial lobe, presence of a long gland along the outer edge of the penis behind the filament (dorsal proximal lobule), more elongate transverse

gland, and well developed dorsal distal lobule with one or more glands.

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