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Molecular refinement of the taxonomy of *Pyrgulopsis* springsnails in western North America

Michael K. Young[1,*], Kristine L. Pilgrim[1], Eric S. Miskow[2], Chante L. Lundskog[3], Kathryn E. Perez[4], and Michael K. Schwartz[1]

[1]*USDA Forest Service, National Genomics Center for Wildlife and Fish Conservation, Rocky Mountain Research Station, 800 E. Beckwith Avenue, Missoula, MT 59801*
[2]*Nevada Division of Natural Heritage, Department of Conservation and Natural Resources, 901 S. Stewart Street, Suite 5002, Carson City, NV 89701*
[3]*Utah Division of Wildlife Resources, 1594 West North Temple, Salt Lake City, UT 84116*
[4]*School of Integrative Biological & Chemical Sciences, University of Texas Rio Grande Valley, Edinburg, TX 78542*

Abstract.—Springsnails in the genus *Pyrgulopsis* in western North America have long been a dilemma for taxonomists. Defining species boundaries has been complicated by the presence of undiscovered or cryptic taxa, range over- or underestimation, species complexes representing an unknown number of species, and named species synonymous with other taxa. To address these issues, we conducted an array of molecular species delimitation analyses using sequences from up to 2 mitochondrial and 2 nuclear genes from over 5000 specimens representing 140 of the 151 currently recognized taxa. These analyses reaffirmed most species hypotheses, favored synonymy for some taxa and redrawing of species boundaries for others, and provided evidence of the presence of myriad new or cryptic taxa. There were occasional disagreements among methods and genes with respect to species boundaries, particularly within the *P. pilsbryana* species complex, and we also observed instances of recent hybridization and potential taxa of hybrid origin. Although these analyses clarified many of the species hypotheses within the genus, further taxonomic resolution of this group will require more spatially comprehensive and intensive field sampling coupled with greater genetic representation of individuals. We suspect that the complex evolutionary histories of members of *Pyrgulopsis* will constitute an ongoing challenge for characterizing the biodiversity of this group.

Resumen.—Los caracoles de manantial del género *Pyrgulopsis* en el oeste de América del Norte han representado un desafío para los taxónomos durante mucho tiempo. La delimitación de los límites entre especies se ha visto complicada por la presencia de taxones crípticos o aún por descubrir, la sobreestimación o subestimación de sus áreas de distribución, la existencia de complejos de especies que representan un número desconocido de estas, y la presencia de especies descritas que resultan ser sinónimas de otros taxones. Para abordar estos problemas, se llevaron a cabo un conjunto de análisis moleculares de delimitación de especies utilizando secuencias de hasta dos genes mitocondriales y dos nucleares de más de 5000 ejemplares que representaron 140 de los 151 taxones actualmente reconocidos. Estos análisis reafirmaron la mayoría de las hipótesis de especie, respaldaron la sinonimia de algunos taxones y la redefinición de límites entre especies en otros, y aportaron evidencia sobre la presencia de numerosos taxones nuevos o crípticos. Surgieron algunas discrepancias entre métodos y genes respecto a los límites entre las especies, particularmente dentro del complejo de especies *P. pilsbryana*. Además, observamos casos de hibridación reciente y posibles taxones de origen híbrido. Aunque estos análisis aclararon muchas de las hipótesis sobre las especies dentro del género, una mayor resolución taxonómica de este grupo requerirá un muestreo de campo más intensivo y con una cobertura espacial más amplia, combinado con una mayor representación genética de individuos. Se prevé que la compleja historia evolutiva de los miembros de *Pyrgulopsis* represente un reto constante para la caracterización de la biodiversidad de este taxón.

Pyrgulopsis is the most speciose genus in the family Hydrobiidae and among endemic North American gastropods, with 19 fossil species and approximately 151 currently or recently extant species (https://www.molluscabase.org/aphia.php?p=taxdetails&id=717226, accessed 22 April 2025). Commonly referred to as springsnails, these taxa are almost exclusively restricted to perennial headsprings and spring-fed channels in arid portions of western North America, often where

*Corresponding author: troutflora@hotmail.com

MKY orcid.org/0000-0002-0191-6112 KLP orcid.org/0000-0003-3726-6980 ESM orcid.org/0009-0005-0953-0694
KEP orcid.org/0000-0003-3888-4756 MKS orcid.org/0000-0003-3521-3367

carbon dioxide or calcium levels are high (Hurt 2004). Most species have been described since 1980 (Supplementary Material 1), and additions to the genus are ongoing (e.g., Perez et al. 2021, 2022). Before 2000, delineation of new taxa relied on diagnostic morphological characteristics, typically involving shell shape, pigmentation, penial ornamentation and morphology, and radular characteristics (e.g., Hershler 1998). These morphological characteristics, however, are known to be relatively plastic and of questionable value for characterizing phylogenetic relationships and sometimes even species boundaries in *Pyrgulopsis* (Hershler and Sada 2002, Hurt 2004, Morningstar et al. 2014) and other springsnail genera (e.g., *Bythinella*; Jaszczyńska et al. 2025). Subsequently, the interpretation of mitochondrial genetic sequences became part of many species descriptions, with specimens for comparison drawn from (and primarily confined to) congeners thought to be near relatives of the taxa being newly described (e.g., Hershler et al. 2007, 2016b). Although some analyses touched on aspects of phylogenetic patterns across the genus (e.g., Hershler and Liu 2017, Liu et al. 2018), an understanding of broader patterns remained elusive, in part because analyses rarely included nuclear sequences (but see Delicado et al. 2024).

How well the current taxonomy approximates the true diversity within *Pyrgulopsis* is uncertain, as are the distributions of many species in this genus. Historically, the majority of taxa were thought to be local endemics restricted to a single spring or spring complex (Johnson et al. 2013). Given that virtually any relatively pristine perennial spring in interior western North America has the potential to serve as springsnail habitat and that sampling is far from complete (Hershler et al. 2014a, McKelvey et al. 2020, Perez et al. 2023), it is likely that additional species remain to be discovered (Hershler and Liu 2012). That said, the incidence of local endemism may be overestimated because broader sampling has expanded the known range of some species (e.g., *P. bacchus*; McKelvey et al. 2020). At the opposite extreme, a handful of species are found in springs separated by hundreds of kilometers. Such widely distributed species have occasionally been resolved as species complexes consisting of some combination of named and undescribed taxa (e.g., *P. micrococcus*, Hershler et al. 2013; *P. gilae*, Hershler et al. 2014b). In other cases, small groups of putatively valid taxa have instead been found to be phenotypic variants of a single species (e.g., *P. robusta*, Hershler and Liu 2004; *P. pilsbryana*, Perez et al. 2025). Finally, some taxa are so ambiguously defined (e.g., *P. kolobensis* and its close relatives from Nevada, Utah, and Idaho) that the number and location of putative species remain speculative (Hershler et al. 2017, Liu et al. 2018, but see Perez et al. 2025). There is particular urgency to resolve these taxonomic uncertainties because many populations of *Pyrgulopsis* have been extirpated and several species are believed to have gone extinct in recent decades (Sada 2008, Varela-Romero et al. 2013, Hershler et al. 2016a, 2017, Perez et al. 2023), and nearly every species is regarded as being of conservation concern (Johnson et al. 2013), including 7 species listed under the U.S. Endangered Species Act (https://www.ecfr.gov/current/title-50/chapter-I/subchapter-B/part-17/subpart-B/section-17.11#p-17.11(h), accessed 2 May 2025). A precursor to many conservation actions, however, is reaffirming that the organisms of interest constitute valid species (Young et al. 2019).

Taxonomically comprehensive molecular species delimitation coupled with extensive field sampling has been used to address similar issues in other taxa in western North America (Young et al. 2022). Here, we took a comparable approach by conducting a suite of molecular species delimitation analyses on nearly all extant species of *Pyrgulopsis*. Our analyses were based on over 1300 specimens with publicly available sequences and over 4000 new specimens from the zoogeographic center of diversity of this genus in Nevada and Utah (Hershler et al. 2014a). We sequenced 2 mitochondrial genes commonly examined in previous analyses, complemented by sequences of 2 nuclear genes to make our conclusions more robust. Our objective was to assess the completeness and validity, on molecular grounds, of the currently accepted taxonomy of *Pyrgulopsis*.

METHODS

Specimens and Sequencing

New samples for molecular analysis consisted of 4270 specimens from 644 locations (Fig. 1, Supplementary Material 2). These locations were generally chosen to address questions about particular species of interest, to obtain samples from at or near the type locations for some taxa, or to represent undersampled regions that might harbor new species.

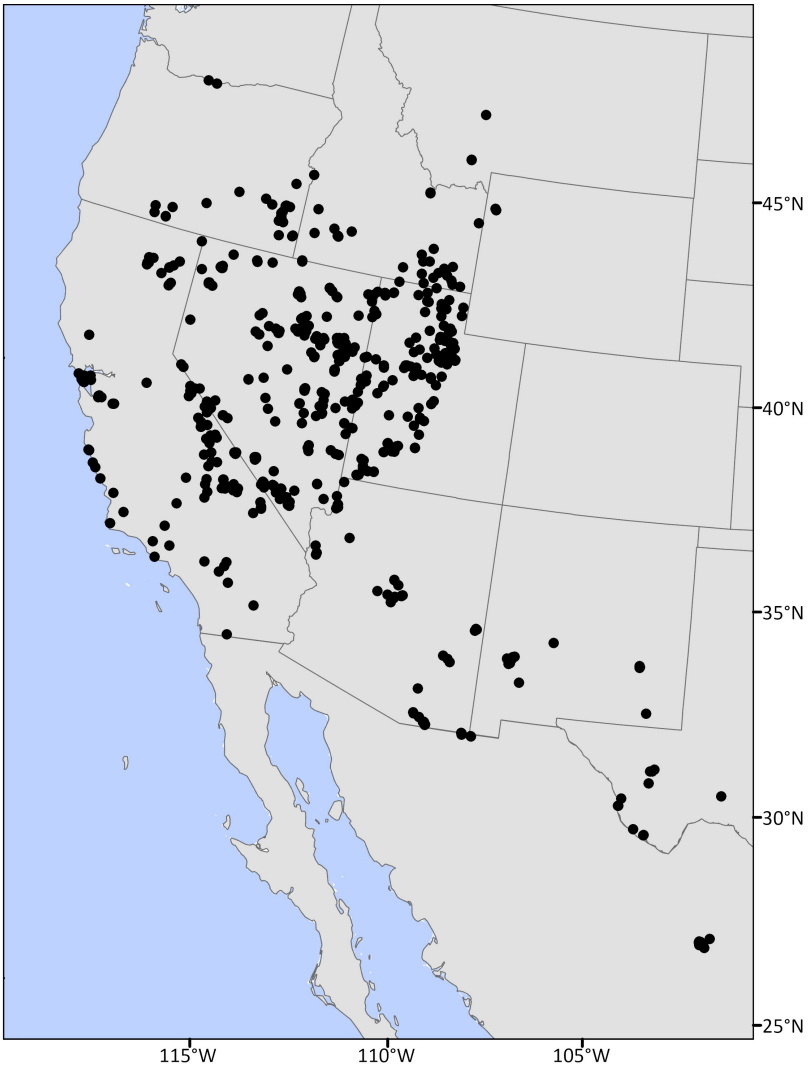

Fig. 1. Distribution of *Pyrgulopsis* specimens used in this study.

We used the QIAGEN DNeasy Blood and Tissue Kit to extract genomic DNA from tissues, following the manufacturer's instructions for tissue. We sequenced 2 mitochondrial regions, cytochrome c oxidase subunit 1 (COI) and NADH dehydrogenase subunit 1 (ND1), and 2 nuclear genes, the second internal transcribed spacer (ITS2) and the large ribosomal subunit (28S). These 4 genes were chosen because they exhibited a range of evolutionary rates and exposure to selection, and represented the broadest taxonomic array of reference sequences for *Pyrgulopsis*. Because of their linkages, we assumed the phylogenetic signal would be similar between COI and ND1 and between ITS2 and 28S. Not all individuals were sequenced at all genes. We began by sequencing all individuals at COI, and from each COI clade exhibiting a marked level of divergence and represented by more than one individual, we selected one or more individuals to sequence at the 3 additional genes.

We amplified the 4 regions using previously published primers and primer-specific annealing temperatures (Supplementary Material 3). Reaction volumes of 30 µL contained 50–100 ng DNA, 1× reaction buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 1 µM each primer, and 1 U Taq

polymerase (Thermo Fisher Scientific, MA). The PCR program was 94 °C/5 min, [94 °C/60 s, annealing temperature/60 s, 72 °C/90 s] × 34 cycles, 72 °C/5 min. Because both reverse primers for the ITS2 and 28S regions were published as "LSU3," we added "Benke" to the reverse primer for the 28S region to distinguish them in the lab. We evaluated PCR products using electrophoresis of 1.6% agarose gel. Those products were purified using ExoSap-IT (Thermo Fisher, MA) according to the manufacturer's instructions. Reactions were sequenced bidirectionally at Eurofins Genomics (Louisville, KY) using the primers employed for amplification and standard Sanger sequencing protocols.

Sequences of COI and ND1 were aligned by eye in MEGA 7.0 (Kumar et al. 2016). All lacked indels and were translated into amino acids to verify that stop codons were absent and the reading frame remained consistent. The nuclear sequences had multiple indels and were initially aligned using the online version of MAFFT (Katoh et al. 2019; https://mafft.cbrc.jp/alignment/server/). In some portions of the nuclear sequences, these subsequent alignments were nonsensical (but obviously so, because the indel-rich portions of the nuclear sequences were bounded by highly conserved regions that were sometimes incorrectly split by the alignment algorithm), and we subjected these to further manual modification. Highly ambiguous portions of these sequences remained, which we identified and removed using Noisy (Dress et al. 2008) with more conservative settings (Tan et al. 2015) implemented via the NGPhylogeny website (Lemoine et al. 2019; https://ngphylogeny.fr/). The final alignments still contained gaps, which we chose to retain because they can be useful for species delimitation and elucidating deeper phylogenetic relationships (Nagy et al. 2012, Tan et al. 2015). These sequence gaps were coded using FastGap (Borchsenius 2009) with the gap codes appended to the respective nuclear sequences. We sought to compare newly collected samples to reference sequences already present in public databases, so we adjusted new sequence fragments to maximize overlap. One of the nuclear alignments consisted of 198 positions of ITS2, 46 gap-coded positions, and the first 307 positions in 28S (hereafter referred to as ITS2), whereas the second nuclear alignment consisted of 944 positions in 28S and 44 gap-coded positions (hereafter, 28S). These nuclear alignments shared 233 bases of 28S.

Species Delimitation and Specimen Assignment

Using the sequences from the newly collected specimens as well as those from public sequence repositories (n = 1362 specimens from 499 locations; Fig. 1, Supplementary Materials 2, 4), we evaluated molecular support for the species hypotheses in the current taxonomy. MolluscaBase (accessed 25 April 2025) lists 151 species (after subtracting 3 entries that are misspellings of taxa already present, but retaining *P. carinata*, which is not an available name for an extant species, but whose members constitute a potential taxon). Of these, 140 are thought to be represented in the dataset by sequences from specimens collected at or near their type locations (Supplementary Material 1). Our objectives were to determine whether (1) all current species hypotheses were supported, and if not, whether (2) some were candidates for synonymy; (3) some appeared to conceal additional cryptic taxa; or (4) some constituted as yet unrecognized species. Much of the present taxonomy is based on a form of the evolutionary species concept that emphasized morphological diagnosability, reciprocal monophyly, and substantial molecular divergence (Hershler et al. 2013). In our analyses, we did not directly assess morphological variation (other than organizing specimens by name, if they were identified; Supplementary Material 2). Instead, we followed the phylogenetic species concept (Nixon and Wheeler 1990), which emphasizes reciprocal monophyly among all potential taxa. And similar to Hershler et al. (2013), we added an additional requirement for species-level rather than population-level divergence to avoid the taxonomic inflation associated with the strict application of this concept (Dufresnes et al. 2023). Requiring reciprocal monophyly for the *P. pilsbryana* species complex (see below), however, would have resulted in recognizing all specimens as members of the type species, despite many populations exhibiting levels of divergence that were characteristic of valid species among other members of this genus. Consequently, we required monophyly only for terminal taxa within this complex, interpreted the results of the species delimitation analyses as we did for all other *Pyrgulopsis* (a form of the unified species concept; De Queiroz 2007), and regarded the resultant para- and polyphyly within the *P. pilsbryana* species complex as a consequence of incomplete lineage sorting. Please note that although previous work labeled this group

as the *P. kolobensis* species complex (Hershler et al. 2017, Liu et al. 2018), the first species to be described that is a member of this complex is *P. pilsbryana*, hence the change in name.

We developed separate datasets for species delimitation and for specimen assignment to species. For species delimitation, we built separate alignments for each of the 4 genes (COI, 4921 sequences, 588 bases; ND1, 943 sequences, 464 bases; ITS2, 489 sequences, 551 positions; 28S, 521 sequences, 998 positions) because different numbers of specimens and potential species were present in each. Given the variability in species delimitation results for the *P. pilsbryana* species complex, we also built a concatenated COI-ND1 dataset (866 sequences, 1052 bases) to better resolve species boundaries therein. We retained only those public and newly generated sequences lacking missing or ambiguous sites. The COI and ND1 alignments contained 1 sequence of *Marstonia* as an outgroup. The alignment for 28S contained several additional outgroups from the Nymphophilinae, whereas outgroup sequences were unavailable for ITS2, for which a sequence of *P. texana* served as an outgroup. For specimen assignment to species, we used a dataset based on all *Pyrgulopsis* COI sequences ($n = 5481$) with at least 408 bases, which ensured sufficient sequence overlap to permit tree construction and provided the broadest taxonomic coverage.

We followed a stepwise approach for species delineation. First, we built maximum-likelihood (ML) phylogenies for each of the 4 genes in the online version of IQ-TREE (Nguyen et al. 2015). We assigned 3 preliminary partitions based on codon position for COI and ND1, 3 partitions for ITS2 (ITS2 positions, ITS2 gap codes, and 28S bases), and 2 partitions for 28S (28S positions and gap codes). We then selected edge-linked partitions and the TESTMERGE setting to determine the best-fitting substitution models for each gene (for which all partitions were merged): COI and 28S, GTR + F + I + G4; ND1, TIM + F + I + G4; ITS2, SYM + I + G4. We assigned support values using 1000 ultrafast bootstraps to each consensus maximum-likelihood tree. Because ultrafast bootstrap values are relatively unbiased (Minh et al. 2013), we assumed that values >85 reflected acceptable support. We compared well-supported species or species blocks—suites of candidate species composed of one or more recognized species and associated lineages—from the nuclear maximum-likelihood trees to well-supported and divergent individual lineages (i.e., those we deemed candidate species, as derived below) from the mitochondrial trees. We gave less weight to nuclear phylogenies because of the uncertainties associated with deletion of putatively ambiguous blocks of sequences and the inclusion of gaps and gap codes. Individuals with nuclear sequences that were assigned to 1 species (or species block) and mitochondrial sequences that were assigned to another were assumed to be hybrids (or errors for some public sequences; see Supplementary Material 2).

Next, we used each of the mitochondrial datasets to delimit species using 4 approaches. First, we analyzed these data in TCS 1.21 (Clement et al. 2000) to construct 95% statistical parsimony networks (SPN). Independent networks were regarded as candidate species (Hart and Sunday 2007), although this method is conservative because separate networks at this threshold are likely to underestimate species diversity (Hart and Sunday 2007, Chen et al. 2010). Second, we used the online version of ASAP (Assemble Species by Automatic Partitioning; https://bioinfo.mnhn.fr/abi/public/asap/#; Puillandre et al. 2020), in which genetic distances are used to identify the transition from intraspecific variation to interspecific divergence, and which includes a scoring system to identify the best-fitting set of partitions (i.e., candidate species). We adopted the default values and distances based on the K80 substitution model because of its similarity to the traditional distance metric used in comparable analyses (Ratnasingham and Hebert 2013). The analysis was run 10 times with different initial seeds for which the highest-scoring set of partitions did not vary. We again assumed that species counts would be conservative relative to other methods (cf. Puillandre et al. 2020). Third, we primarily used the single-rate maximum-likelihood implementation of Poisson tree processes (PTP; Zhang et al. 2018), which uses phylogenetic trees (from the aforementioned maximum-likelihood analysis) to identify the transition between population- and species-level divergence. Fourth, we used MEGA 7.0 (Kumar et al. 2016) to build a pairwise distance matrix based on the absolute number of differences between sequences, and then examined the maximum genetic distance among members of a candidate species and the minimum distance to a nonmember (Meier et al. 2008). When the latter exceeded the former (constituting a barcode gap; Hebert et al. 2003) and was >1.5% in COI sequences (a difference characteristic of the

majority of interspecific differences among *Pyrgulopsis* in the current taxonomy; Liu et al. 2018) or 2.5% in ND1 (which tended to be more divergent in previous studies of this genus; Liu and Hershler 2005), we considered this as support for a candidate species. For 19 otherwise well-supported taxa (Supplementary Material 10), sequences were available in COI but not ND1. To estimate potential divergence in ND1 in those taxa, we used regression analysis of well-supported taxa ($n = 71$) with measures of the minimum nearest-neighbor distance for both genes to estimate that distance for the 19 taxa without ND1 sequences. Finally, we indirectly used phenotypic and geographic information to inform species delimitation, in that publicly available specimens had been morphologically identified and georeferenced, and in that many of the newly added specimens from Nevada were tentatively identified in the field based on morphology and location.

For the COI dataset, preliminary results from the delimitation algorithms were highly variable. Inspection suggested that patterns within the *P. pilsbryana* species complex were responsible, so the analyses were rerun on 2 datasets: one restricted to members of that complex and one without them. These analyses showed greater consensus among the different algorithms and with results using ND1, but required further adjustments in PTP (use of the multi-rate, not single-rate, model) and ASAP (selecting a model that appeared in the top model set rather than the overall best-scoring model for the *P. pilsbryana* complex) to yield species hypotheses consistent with the aforementioned distance thresholds. We also considered other forms of overall analysis. These were, however, either unsuccessful (e.g., Bayesian models failed to converge regardless of the number of iterations or whether the datasets were split) or unwarranted (e.g., taxonomic coverage was too poor to merit using species delimitation based on all 4 genes in a multispecies coalescent context (e.g., STACEY [Jones 2017]).

The final step in species delimitation was to propose a set of species hypotheses. When a plurality of methods concurred (because consensus among all methods was unlikely; Dellicour and Flot 2018) and did not violate the phylogenetic species concept, we designated these as candidate species. As noted earlier, we singled out highly divergent clades and lumped less divergent ones as candidate species within the *P. pilsbryana* species complex (akin to the quasi-independent evolutionary unit approach of Nekola and Horsák 2022). Consequently, we did not assign the remaining members of this complex to a currently recognized species or propose a candidate species because we lacked the evidence to do so (i.e., molecular delineation of species boundaries was weak or unstable). Because we did not morphologically evaluate individual specimens, we did not propose names for the candidate species found within existing taxa or that were newly delimited (cf. Hershler et al. 2013). We did, however, propose names for groups of species that warranted synonymy, following article 24.2.1 of the International Code for Zoological Nomenclature. To assign specimens to a candidate species, we built neighbor-joining trees (based on the simple number of pairwise differences) using the specimen assignment COI alignment and examined clade membership. Specimens grouping with a clade containing a more comprehensively evaluated candidate species were assigned to that species. Specimens forming novel clades exclusive of named or candidate taxa, or associated with unresolved members of the *P. pilsbryana* complex, were not assigned.

RESULTS AND DISCUSSION

Phylogenetic Results and Limitations

None of the phylogenies based on different genes were concordant despite the known linkages between the 2 mitochondrial genes and 2 nuclear genes (Supplementary Materials 5–8). The topology and branch support varied widely among deeper branches in each phylogeny, such that evolutionary relationships among well-supported lineages remained uncertain. Variation in deep-branch topology emerged even for single genes analyzed using different methods (i.e., maximum-likelihood versus neighbor-joining; Supplementary Materials 5, 9). These observations are consistent with those from previous analyses of smaller sets of species within *Pyrgulopsis* (Hershler and Liu 2008, 2009, Hershler et al. 2015, 2016b, 2017, Liu and Hershler 2005) and from broader analyses of gastropods (Delicado et al. 2024). Both the weak support for deep phylogenetic branches and gene tree heterogeneity (including issues with Bayesian inference) may be attributable to rapid radiation from a common ancestor (Chafin et al. 2021, Pardo-De la Hoz et al. 2023) as well as subsequent

evolutionary histories that are highly complex and idiosyncratic (Sober 2004). That said, most terminal nodes considered to represent candidate taxa or blocks of taxa were well defined in nearly all gene trees (cf. Hurt 2004) and amenable to molecular species delimitation.

There was broad agreement among most methods with respect to the membership and number of candidate species. Counts of candidate species were similar among methods (range 111 [ND1, SPN] to 133 [COI, ASAP]). Nearly all candidate species (or blocks of them) constituted strongly supported clades in the maximum-likelihood analysis of each gene. Typically, they also exhibited a barcode gap in both mitochondrial genes and differed from their nearest interspecific neighbor by the prescribed mitochondrial thresholds (Supplementary Materials 10–13). Analyses based on the ND1 gene tended to result in higher support for species hypotheses and greater divergence among them. The regression equation relating minimum nearest-neighbor divergence (as a percentage) between the mitochondrial genes was

$$\text{ND1 divergence} = 1.0406 + 1.0871(\text{COI divergence})$$

($P << 0.001$, $r^2 = 0.658$). The mitochondrial results were largely corroborated by the nuclear phylogenies, which generally delimited, with high bootstrap support, the same candidate species or blocks of species identified in mitochondrial analyses. An exception was the *P. pilsbryana* complex. Although this complex and its members were relatively consistently identified, the location of species boundaries varied among mitochondrial and nuclear genes (Fig. 2, Supplementary Materials 10–13). This made the designation of candidate species or evaluation of the existing taxonomy within that complex problematic.

Taxonomic Implications

Analyses of this comprehensive molecular dataset from specimens across a broad geographic area suggested that portions of the taxonomy of *Pyrgulopsis* require substantial revision. The validity of the majority of species ($n = 90$) was supported (Table 1, Supplementary Material 10), reaffirming the utility of morphological analyses as an initial screen for recognizing potential taxa. For other species, however, the morphological plasticity or homoplasy common in *Pyrgulopsis* (and many other gastropods; Nekola and Horsák 2022, Jaszczyńska et al. 2025) coupled with a less stringent standard for species divergence (Hershler et al. 2016a, 2017, Liu et al. 2018) may have led to discrepancies between the current taxonomy and our interpretation of species boundaries; these fell into 4 categories.

Although Hershler et al. (2014a) considered it unlikely that the *Pyrgulopsis* taxonomy was oversplit, we found evidence that 31 currently recognized taxa may instead represent only 10 valid species (Table 1, Supplementary Material 11). In most instances, there were sets of 2 or 3 named taxa for which the mitochondrial and nuclear analyses indicated that only a single taxon was present. In nearly every instance, these species sets exhibited levels of interspecific divergence consistent with differences among populations of a single species, and sometimes no divergence at all (e.g., *P. bryantwalkeri*, *P. aurata*, and *P. pictilis*, but see below). In the case of the *P. pilsbryana* complex, 9 taxa were candidates for synonymy (including some that were previously proposed; Liu et al. 2018, Perez et al. 2025). Although pooling these complex members led to high estimates of intraspecific divergence in COI, this taxonomically conservative approach was more consistent with our treatment of all other members of *Pyrgulopsis*.

The remaining 3 differences generally contributed to an overall increase in species diversity. First, some named taxa constituted species complexes that concealed divergent lineages distinct enough to merit recognition (Table 1, Supplementary Material 12). Often this involved a widely distributed taxon (e.g., 3 candidate species within *P. californiensis*; also see Hershler and Liu 2010), but sometimes an additional

Fig. 2 (page 339). Identification of candidate species for most members of the *Pyrgulopsis pilsbryana* species complex, structured following highly supported clades (branches with red dots and all collapsed nodes have bootstrap support >85) from the maximum-likelihood tree for the COI gene fragment. Taxa in bold are hypothesized candidate species; those marked with a dagger are not considered part of this complex (see text). Each column depicts the results of 1 species delimitation method (A, ASAP; P, Poisson tree processes; S, statistical parsimony networks) for mitochondrial data (COI, ND1, or the concatenated sequences [CON]) or diagnosability (D) and substantial molecular support (B, bootstrap support ≥85) for nuclear data (ITS2 or 28S). Cell fill denotes whether a COI clade was delimited as a species (orange, yes; gray, no; white, no data). A cell with an asterisk denotes that more than 1 taxon within the clade was delimited. Vertically oriented labels indicate informal group membership for specimens not assigned to a candidate species.

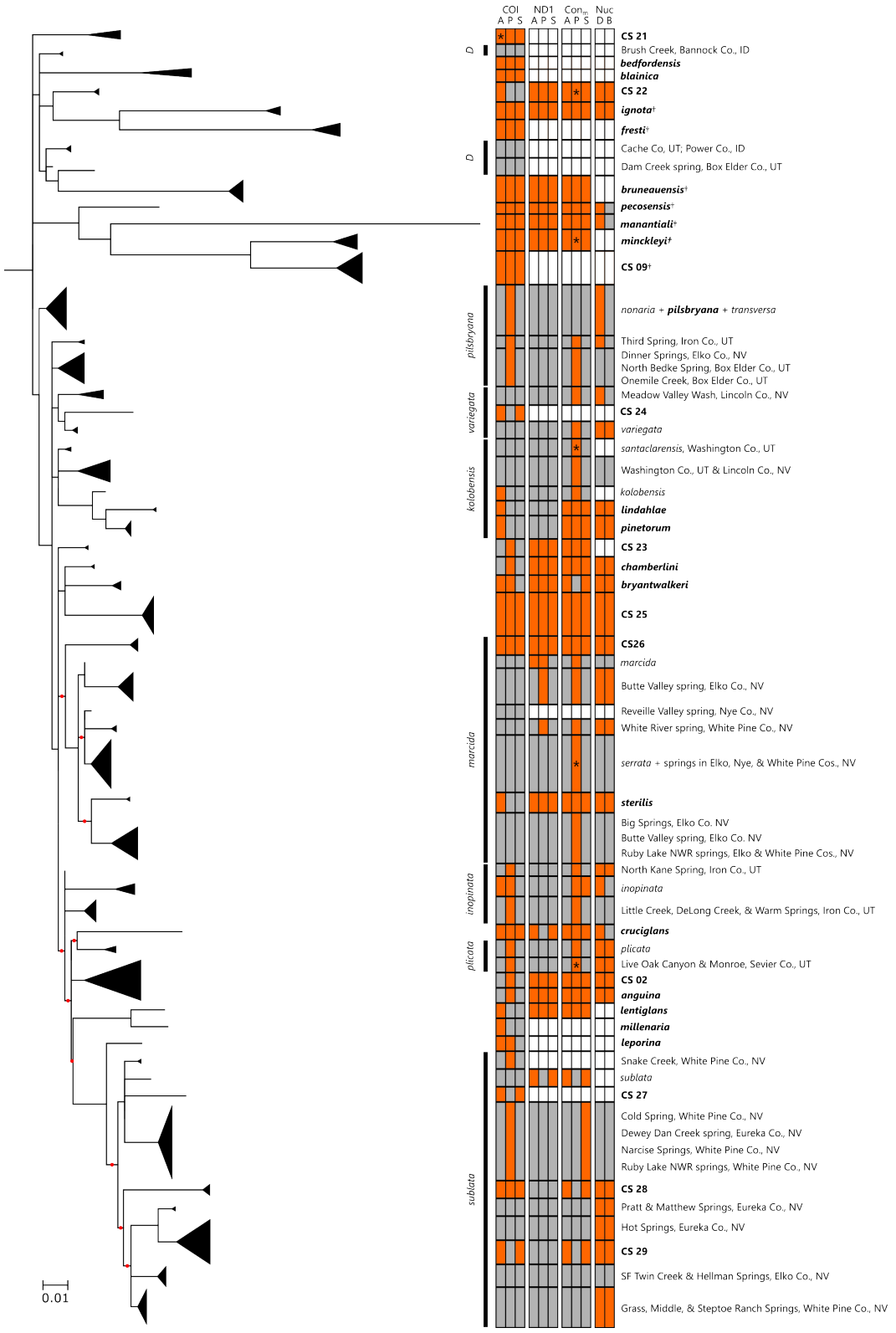
COI
A P S
ND1
A P S
Con$_{In}$
A P S
Nuc
D B
D.
D.
pilsbryana
variegata
kolobensis
marcida
inopinata
plicata
sublata
CS 21
Brush Creek, Bannock Co., ID
bedfordensis
blainica
CS 22
ignota†
fresti†
Cache Co, UT; Power Co., ID
Dam Creek spring, Box Elder Co., UT
bruneauensis†
pecosensis†
manantiali†
minckleyi‡†
CS 09†
nonaria + pilsbryana + transversa
Third Spring, Iron Co., UT
Dinner Springs, Elko Co., NV
North Bedke Spring, Box Elder Co., UT
Onemile Creek, Box Elder Co., UT
Meadow Valley Wash, Lincoln Co., NV
CS 24
variegata
santaclarensis, Washington Co., UT
Washington Co., UT & Lincoln Co., NV
kolobensis
lindahlae
pinetorum
CS 23
chamberlini
bryantwalkeri
CS 25
CS26
marcida
Butte Valley spring, Elko Co., NV
Reveille Valley spring, Nye Co., NV
White River spring, White Pine Co., NV
serrata + springs in Elko, Nye, & White Pine Cos., NV
sterilis
Big Springs, Elko Co. NV
Butte Valley spring, Elko Co. NV
Ruby Lake NWR springs, Elko & White Pine Cos., NV
North Kane Spring, Iron Co., UT
inopinata
Little Creek, DeLong Creek, & Warm Springs, Iron Co., UT
cruciglans
plicata
Live Oak Canyon & Monroe, Sevier Co., UT
CS 02
anguina
lentiglans
millenaria
leporina
Snake Creek, White Pine Co., NV
sublata
CS 27
Cold Spring, White Pine Co., NV
Dewey Dan Creek spring, Eureka Co., NV
Narcise Springs, White Pine Co., NV
Ruby Lake NWR springs, White Pine Co., NV
CS 28
Pratt & Matthew Springs, Eureka Co., NV
Hot Springs, Eureka Co., NV
CS 29
SF Twin Creek & Hellman Springs, Elko Co., NV
Grass, Middle, & Steptoe Ranch Springs, White Pine Co., NV
0.01

Table 1. Taxonomic recommendations for potentially extant members of the genus *Pyrgulopsis* (from MolluscaBase, accessed 22 April 2025). In the "Status" column, codes are as follows: **Amb,** ambiguous because of taxonomic or sequence issues; **Mul,** a taxon appears to represent multiple species; **ND,** no data to support taxonomic evaluation; **Syn,** synonymy with another taxon appears warranted; **Valid,** a taxon is valid as currently recognized; **Valid (Syn),** a taxon is valid when synonymized with another taxon. Note that 29 new candidate taxa were also delineated. See Supplementary Materials 1 and 10–13 for more details.

Species	Status
Pyrgulopsis aardahli Hershler, 1989	ND
Pyrgulopsis acarinata (Hershler, 1985)	Syn
Pyrgulopsis aloba Hershler, 1998	Mul
Pyrgulopsis amargosae Hershler, 1989	Valid
Pyrgulopsis anatina Hershler, 1998	Valid
Pyrgulopsis anguina Hershler, 1998[a]	Mul
Pyrgulopsis archimedis S.S. Berry, 1947	Valid
Pyrgulopsis arizonae (D.W. Taylor, 1987)	Valid
Pyrgulopsis augustae Hershler, 1998	ND
Pyrgulopsis aurata Hershler, 1998[a]	Syn
Pyrgulopsis avernalis (Pilsbry, 1935)	Valid
Pyrgulopsis bacchus Hershler, 1988	Valid
Pyrgulopsis basiglans Hershler, 1998	Syn
Pyrgulopsis bedfordensis Hershler & Gustafson, 2001[a]	Valid
Pyrgulopsis bernardina (D.W. Taylor, 1987)	Mul
Pyrgulopsis bifurcata Hershler, 1998	Valid (Syn)
Pyrgulopsis blainica Hershler, H.-P. Liu & Gustafson, 2008[a]	Valid
Pyrgulopsis brandi (Drake, 1953)	ND
Pyrgulopsis breviloba Hershler, 1998	Valid (Syn)
Pyrgulopsis bruesi Hershler & Sada, 2000	Syn
Pyrgulopsis bruneauensis Hershler, 1990	Valid
Pyrgulopsis bryantwalkeri Hershler, 1994[a]	Valid (Syn)
Pyrgulopsis californiensis (Gregg & D.W. Taylor, 1965)	Mul
Pyrgulopsis carinata Hershler, 1998	Amb[b]
Pyrgulopsis carinifera (Pilsbry, 1935)	Valid
Pyrgulopsis castaicensis Hershler & H.-P. Liu, 2010	Valid
Pyrgulopsis cedrosensis (Pilsbry, 1927)	ND
Pyrgulopsis chamberlini Hershler, 1998[a]	Valid
Pyrgulopsis chihuahua (Pilsbry, 1928)	ND
Pyrgulopsis chupaderae D.W. Taylor, 1987	Valid
Pyrgulopsis cinerana Hershler, Frest, H.-P. Liu & Johannes, 2003	Valid
Pyrgulopsis coloradensis Hershler, 1998	Valid
Pyrgulopsis conica Hershler, 1988	Valid
Pyrgulopsis cruciglans Hershler, 1998[a]	Valid
Pyrgulopsis crystalis Hershler & Sada, 1987	Syn
Pyrgulopsis cybele Hershler & H.-P. Liu, 2012	Valid
Pyrgulopsis davisi (D.W. Taylor, 1987)	Valid
Pyrgulopsis deaconi Hershler, 1998	Valid
Pyrgulopsis deserta (Pilsbry, 1916)	Valid
Pyrgulopsis diablensis Hershler, 1995	Syn
Pyrgulopsis dixensis Hershler, 1998	Valid (Syn)
Pyrgulopsis eremica Hershler, 1995	Valid (Syn)
Pyrgulopsis erythropoma (Pilsbry, 1899)	Valid
Pyrgulopsis fairbanksensis Hershler & Sada, 1987	Mul
Pyrgulopsis falciglans Hershler, Frest, H.-P. Liu & Johannes, 2003	Valid
Pyrgulopsis fausta Hershler, 1998	Valid
Pyrgulopsis fresti Hershler & H.-P. Liu, 2009	Valid
Pyrgulopsis fusca Hershler, 1998	Valid
Pyrgulopsis gibba Hershler, 1995	Valid (Syn)
Pyrgulopsis gilae (D.W. Taylor, 1987)	Valid
Pyrgulopsis giulianii Hershler & W.L. Pratt, 1990	Syn
Pyrgulopsis glandulosa Hershler, 1988	Valid
Pyrgulopsis gracilis Hershler, 1998	Valid
Pyrgulopsis greggi Hershler, 1995	Valid
Pyrgulopsis hamlinensis Hershler, 1998[a]	Syn
Pyrgulopsis harrymilleri K.E. Perez, 2021	Valid
Pyrgulopsis hovinghi Hershler, 1998	ND
Pyrgulopsis hualapaiensis Hershler, H.-P. Liu & Stevens, 2016	Valid
Pyrgulopsis hubbsi Hershler, 1998	Valid
Pyrgulopsis humboldtensis Hershler, 1998	Valid
Pyrgulopsis ignota Hershler, H.-P. Liu & B.K. Lang, 2010	Valid
Pyrgulopsis imperialis Hershler, 1998	Valid
Pyrgulopsis inopinata Hershler, 1998[a]	Syn
Pyrgulopsis intermedia (Tryon, 1865)	Valid
Pyrgulopsis isolata Hershler & Sada, 1987	Syn
Pyrgulopsis kolobensis (D.W. Taylor, 1987)[a]	Syn
Pyrgulopsis landyei Hershler, 1998	Valid
Pyrgulopsis lasseni Hershler, Frest, H.-P. Liu & Johannes, 2003	Syn
Pyrgulopsis lata Hershler, 1998	Syn
Pyrgulopsis lentiglans Hershler, 1998[a]	Valid
Pyrgulopsis leporina Hershler, 1998[a]	Valid
Pyrgulopsis licina Hershler, H.-P. Liu & Bradford, 2013	Valid
Pyrgulopsis limaria Hershler, 1998	Syn
Pyrgulopsis lindae Hershler, H.-P. Liu, Babbitt, Kellogg & J.K. Howard, 2016	Valid
Pyrgulopsis lindahlae Hershler, H.-P. Liu, Forsythe, Hovingh & K. Wheeler, 2017[a]	Valid
Pyrgulopsis lockensis Hershler, 1998	Valid
Pyrgulopsis longae Hershler, 1995	ND
Pyrgulopsis longiglans Hershler, 1998	Valid
Pyrgulopsis longinqua (A. Gould, 1855)	Valid
Pyrgulopsis madridensis K.E. Perez, 2022	Valid
Pyrgulopsis manantiali (Hershler, 1985)	Valid
Pyrgulopsis marcida Hershler, 1998[a]	Syn
Pyrgulopsis marilynae Hershler, Ratcliffe, H.-P. Liu, B.K. Lang & Hay, 2014	Amb[c]
Pyrgulopsis merriami (Pilsbry & Beecher, 1892)	Mul
Pyrgulopsis metcalfi (D.W. Taylor, 1987)	Valid
Pyrgulopsis micrococcus (Pilsbry, 1893)	Valid
Pyrgulopsis militaris Hershler, 1998	Valid
Pyrgulopsis millenaria Hershler, 1998[a]	Valid
Pyrgulopsis milleri Hershler & H.-P. Liu, 2010	Valid
Pyrgulopsis minckleyi (D.W. Taylor, 1966)	Mul
Pyrgulopsis montana Hershler, 1998[a]	Valid (Syn)
Pyrgulopsis montezumensis Hershler, 1988	Valid
Pyrgulopsis morrisoni Hershler, 1988	Valid
Pyrgulopsis nana Hershler & Sada, 1987	Valid
Pyrgulopsis neomexicana (Pilsbry, 1916)	ND

TABLE 1. Continued.

Species	Status
Pyrgulopsis neritella Hershler, 1998	Valid
Pyrgulopsis nevadensis (Stearns, 1883)	ND
Pyrgulopsis nonaria Hershler, 1998[a]	Syn
Pyrgulopsis notidicola Hershler, 1998	Mul
Pyrgulopsis nuwuvi Hershler, H.-P. Liu, Forsythe, Hovingh & K. Wheeler, 2017[a]	Valid
Pyrgulopsis ojaiensis Hershler, H.-P. Liu, Babbitt, Kellogg & J.K. Howard, 2016	Valid
Pyrgulopsis orbiculata Hershler, 1998	Amb[d]
Pyrgulopsis owensensis Hershler, 1989	Syn
Pyrgulopsis owyheensis Hershler & H.-P. Liu, 2009	Valid
Pyrgulopsis palomasensis (Pilsbry, 1895)	ND
Pyrgulopsis papillata Hershler, 1998	Valid
Pyrgulopsis patzcuarensis Pilsbry, 1891	ND
Pyrgulopsis pecosensis (D.W. Taylor, 1987)	Valid
Pyrgulopsis peculiaris Hershler, 1998	Valid
Pyrgulopsis pellita Hershler, 1998	Valid
Pyrgulopsis perforata Hershler, H.-P. Liu & Bradford, 2013	Valid
Pyrgulopsis perturbata Hershler, 1989	Valid (Syn)
Pyrgulopsis pictilis Hershler, 1998[a]	Syn
Pyrgulopsis pilsbryana (J.L. Baily & R.I. Baily, 1952)[a]	Valid (Syn)
Pyrgulopsis pinetorum (D.W. Taylor, 1987)[a]	Valid
Pyrgulopsis pisteri Hershler & Sada, 1987	Valid (Syn)
Pyrgulopsis planulata Hershler, 1998	Amb[e]
Pyrgulopsis plicata Hershler, 1998[a]	Syn
Pyrgulopsis robusta (B. Walker, 1908)	Valid
Pyrgulopsis roswellensis (D.W. Taylor, 1987)	Valid
Pyrgulopsis rubra K.E. Perez, 2021	Valid
Pyrgulopsis ruinosa Hershler, 1998	Valid
Pyrgulopsis rupinicola Hershler, Frest, H.-P. Liu & Johannes, 2003	Valid
Pyrgulopsis sadai Hershler, 1998	Valid
Pyrgulopsis sanchezi Hershler, H.-P. Liu & Bradford, 2013	Valid
Pyrgulopsis santaclarensis Hershler, H.-P. Liu, Forsythe, Hovingh & K. Wheeler, 2017[a]	Syn
Pyrgulopsis sathos Hershler, 1998	Valid
Pyrgulopsis saxatilis Hershler, 1998	Valid
Pyrgulopsis serrata Hershler, 1998[a]	Syn
Pyrgulopsis similis Hershler, Ratcliffe, H.-P. Liu, B.K. Lang & Hay, 2014	Valid
Pyrgulopsis simplex Hershler, 1988	Valid
Pyrgulopsis sola Hershler, 1988	Valid
Pyrgulopsis stearnsiana (Pilsbry, 1899)	Mul
Pyrgulopsis sterilis Hershler, 1998[a]	Valid
Pyrgulopsis sublata Hershler, 1998[a]	Syn
Pyrgulopsis sulcata Hershler, 1998	Valid
Pyrgulopsis taylori Hershler, 1995	Valid
Pyrgulopsis texana (Pilsbry, 1935)	Valid
Pyrgulopsis thermalis (D.W. Taylor, 1987)	Mul
Pyrgulopsis thompsoni Hershler, 1988	Valid
Pyrgulopsis torrida Hershler, H.-P. Liu, Babbitt, Kellogg & J.K. Howard, 2016	Valid
Pyrgulopsis transversa Hershler, 1998[a]	Syn
Pyrgulopsis trivialis (D.W. Taylor, 1987)	Valid
Pyrgulopsis turbatrix Hershler, 1998	Valid
Pyrgulopsis umbilicata Hershler, 1998	Valid (Syn)
Pyrgulopsis variegata Hershler, 1998[a]	Syn

TABLE 1. Continued.

Species	Status
Pyrgulopsis varneri Hershler, H.-P. Liu & Sada, 2007	Valid
Pyrgulopsis ventricosa Hershler, 1995	Valid
Pyrgulopsis villacampae Hershler, 1998	Valid
Pyrgulopsis vinyardi Hershler, 1998	Syn
Pyrgulopsis wongi Hershler, 1989	Valid

[a]*P. pilsbryana* species complex.
[b]Specimens with this name appear to represent a candidate species, but this name is not available. See Supplementary Material 10.
[c]COI sequences were too short for inclusion in the species delimitation analysis but constituted a divergent clade in the specimen identification tree.
[d]COI sequences were too short for inclusion in the species delimitation analysis but were synonymous with *P. sulcata* in the specimen identification tree.
[e]COI sequences were too short for inclusion in the species delimitation analysis but were synonymous with *P. landyei* in the specimen identification tree

taxon was delimited within a locally distributed one (e.g., new candidate species split from *P. aloba*, *P. anguina*, and *P. merriami*). Second, in some instances, named taxa constituted lineages that accounted for little of the variance within a larger species complex (e.g., *P. diablensis* and *P. giulianii* within *P. stearnsiana*; *P. isolata* within *P. fairbanksensis*), whereas unnamed lineages within each complex were highly divergent and likely to constitute candidate taxa. Third, there were 17 previously unrecognized clades (or recognized but not described; Hershler et al. 2013, Liu et al. 2018) that were delimited as candidate species (Table 1, Supplementary Material 13). Of these, 9 were members of the *P. pilsbryana* species complex.

Assignment of most specimens to a candidate species based on their COI sequences was straightforward (Supplementary Materials 2, 9). Candidate taxa nearly always assigned to a single clade or were members of multiple (and adjacent) clades and remained diagnosable. Even the many members of the *P. pilsbryana* species complex not assigned to a named taxon were readily distinguished as belonging to this complex. In several instances, clades appeared to constitute candidate taxa, but their COI sequences were too short to include in the overall analysis. This included all members of *P. marilynae* (Hershler et al. 2014b), 2 clades of putative *P. bernardina* (from Arroyo Agua Fria and Ojo Caliente in Sonora; Varela-Romaro et al. 2013), 2 putative specimens of *P. thompsoni* from Cochise County, Arizona (Hurt 2004, Lavretsky et al. 2021), 3 unidentified clades in central Arizona (from La Cebadilla Cienega,

Cold Water Spring, and Grapevine Spring; Lavretsky et al. 2021), and a clade composed of a single specimen (AY485535; Hurt 2004) from Green Spring in Washington County, Utah. Broader genetic or genomic characterization, as well as additional sampling, is critical to understanding whether these also constitute candidate taxa.

Range Limits and Dispersal

These efforts expanded the known ranges of some candidate species, sometimes substantially (cf. Johnson et al. 2013). Although many species had restricted ranges extending only a couple hundredths of a degree latitudinally or longitudinally, a relatively small proportion were restricted to a single site (Supplementary Material 2). More often, species occupied a modest number of sites within a spring system and its outflow, or in a handful of springs. And the distribution of a few species—such as *P. archimedis*, *P. bryantwalkeri*, *P. gibba*, *P. robusta*, *P. sanchezi*, *P. stearnsiana*, *P. turbatrix*, and *P. wongi*—in springs separated by tens to hundreds of kilometers and one or more basin divides demonstrates that these taxa are mobile at evolutionarily relevant timescales but not in ways that comport with modern or older hydrological connections (Hurt 2004, Hershler and Liu 2008, McKelvey et al. 2020). That populations of some species occupy springs that would have been inundated by rivers or lakes associated with the Last Glacial Maximum (Reheis et al. 2002, Oviatt 2015) is further evidence of relatively recent dispersal, given that springsnails are algal grazers for which the occupation of deeply submerged springs deprived of sunlight seems unlikely (Hershler et al. 2007). Because bird-assisted transport is regarded as the most probable mode of dispersal in *Pyrgulopsis* (Liu et al. 2003, Hershler et al. 2015, Lavretsky et al. 2021) and other small gastropods (Coughlan et al. 2017, Boulaassafer et al. 2020), a species' presence in disjunct and often distant spring systems is unsurprising. It also suggests that field sampling to delineate species ranges (and consequently their conservation status) may require systematic and intensive surveys well beyond the single watersheds or spring complexes thought to support a taxon. And given the fairly frequent co-occurrence of taxa—at 100 of the 1101 sites in this study, which is a minimum estimate—more thorough collecting and analysis of specimens from single sites is also warranted. Ensuring that all species from any particular site are detected can be challenging, in part because of difficulties in recognizing specimens in the field and because springsnails can achieve extremely high densities in suitable habitat (>1000–10,000/m^2; Hershler 1998, Stagliano 2016, Johnson et al. 2019). To reduce the burden of sequencing large numbers of individuals from every site (or the intensity of sampling at individual sites when the presence of any species is unknown), one approach could be to rely on eDNA sampling coupled with metabarcoding using primers specific to *Pyrgulopsis* to determine community membership, including the discovery of new species (Vanderpool et al. 2024). A logical follow-up would then be targeted field collections and genetic or genomic and morphological assessments of specimens of interest.

Ongoing Issues and Future Directions

Unfortunately, there were also instances where the new data did not resolve previous taxonomic uncertainties, primarily within the *P. pilsbryana* species complex (Hershler et al. 2017, Liu et al. 2018). Membership in this clade—distributed from western Montana (*P. blainica* and *P. bedfordensis*) to southern Nevada and Utah (*P. anguina* and many unassigned individuals)—was unambiguous for many taxa, but the inclusion of highly divergent forms varied. For example, the COI maximum-likelihood phylogeny placed a group of southwestern species (*P. ignota*, *P. manantiali*, *P. minckleyi*, and *P. pecosensis*) as well as *P. bruneauensis* and *P. fresti* in this complex, but that result may instead reflect long-branch attraction. Because we lacked data from other genes (or the limited available data conflicted with this placement), we did not regard these as members of this complex. Alternatively, *P. montana* (and by association, *P. hamlinensis*; Supplementary Material 11) and *P. nuwuvi* had nuclear sequences identical to other members of this complex, and so were included within it, despite that neither mitochondrial gene supported this placement.

Also problematic was that named taxa within the *P. pilsbryana* complex often failed to constitute lineages that met criteria for recognizing them as candidate species. Many were neither monophyletic nor diagnosable in nuclear phylogenies and were rarely or never delimited in analyses of the mitochondrial genes, including *P. kolobensis* from its type location, for which this complex was previously named (Fig. 2). In some instances, currently accepted taxa (e.g.,

P. sublata or *P. inopinata*) were distinguishable, but no more so than a host of other specimens in minor clades that represented collections from one or a handful of locations. These results suggest that the *P. pilsbryana* species complex constitutes a broadly distributed and weakly cohesive lineage, featuring clades composed of often widely disjunct populations exhibiting little divergence (indicative of recent and far-reaching dispersal; Liu et al. 2015) juxtaposed with clades that were highly divergent and often narrowly distributed. Although the existence of the latter has been argued as evidence of vicariance of great antiquity (Liu and Hershler 2005, Hershler et al. 2007, 2008), perhaps a more parsimonious explanation is that most clades are of similar age but some have undergone more rapid evolution. Some of these lineages (e.g., *P. bedfordensis*; Hershler and Gustafson 2001) occupy thermal springs (mean water temperature >20 °C), and higher water temperatures have been argued to lead to higher metabolic rates and greater mutation rates in ectotherms (April et al. 2013). Other species complexes from thermal springs often exhibit very high levels of divergence among their members (Hershler et al. 2007, Liu et al. 2013), but this would not explain the marked divergence of certain lineages restricted to cold-water springs (e.g., *P. blainica*; Hershler et al. 2008). Nevertheless, the failure of molecular data to exhibit clocklike behavior among other groups of *Pyrgulopsis* (Liu and Hershler 2005, Hershler and Liu 2008) indicates that clade-specific evolutionary rates are not unusual.

Although we believe the circumstances above explain the existence and distribution of members of the *P. pilsbryana* species complex, labeling many of its members remains challenging. Liu et al. (2018) recommended considering the bulk of specimens from southern Idaho and northern Utah as *P. pilsbryana* (also see Perez et al. 2025), although these are geographically interspersed and sometimes sympatric with a variety of members of other minor clades within this complex that are more common elsewhere. Consequently, we adopted the conservative approach of applying *P. pilsbryana* as the placeholder name for most members of this species complex while recognizing well-delimited clades as candidate species, hopefully avoiding the taxonomic inflation associated with recognizing every clade as a different taxonomic entity (Agapow et al. 2004). That said, within the newly circumscribed *P. pilsbryana*, there were weakly divergent lineages that exhibited some geographic affinities; we regarded these as groups (Fig. 2, Supplementary Material 2) within this species complex that may be in the incipient stages of speciation. More thorough field surveys of *Pyrgulopsis* in this region coupled with additional genetic or genomic exploration may provide a more meaningful resolution of these issues. Alternatively, it may be that the evolutionary history of this complex will be a difficult fit for most species concepts and require compromise in recognizing species boundaries.

A taxonomically conservative strategy may also be warranted for some of the species hypotheses we delimited in this study, particularly for candidate taxa poorly represented in public databases or museum collections. In most cases for species outside of Nevada and Utah, there were no nuclear sequences (and often few ND1 sequences) available, and often these species were represented by singleton COI sequences in public databases. Thus, these species hypotheses should be regarded as tentative, pending confirmation with additional samples and analyses. We also acknowledge that such sampling may not be possible for locations for which access cannot be obtained or for locations where spring habitats no longer exist; for these, museum samples may be the only viable option. Finally, some pairs of highly divergent but sister lineages were sympatric but also recognized as separate candidate species using molecular data (e.g., candidate species 03 and 04; *P. minckleyi* and candidate species 09; *P. notidicola* and candidate species 10; *P. thermalis* and candidate species 12). For these species hypotheses, more thorough genomic inventories are in order to assess the potential for admixture. More fundamentally, we acknowledge that applying a different species concept emphasizing other criteria, such as morphological diagnosability or reproductive isolation, would likely result in a different interpretation of species hypotheses. We encourage such alternative analyses, which can only lead to a more robust taxonomy for these species and perhaps resolve some of the issues we have identified regarding their evolutionary history.

A problem for all analyses, however, involves errors in species status or specimen identification. For example, *P. ruinosa* was presumed to be extinct because it was not detected in later surveys of its type location, a spring system which had undergone substantial alteration (Hershler 1998). Our more recent sampling

revealed that a highly divergent lineage of *Pyrgulopsis* was present at that location, which we hypothesized was this species. Whether this is attributable to a remnant population rebuilding to detectable levels or recent dispersal from nearby locations is unknown, but it suggests the value of revisiting formerly occupied locations (cf. Lavretsky et al. 2021, Johnson et al. 2022). There was a more nuanced problem involving *P. hubbsi* and *P. sathos* in public sequence databases. In the paper describing both, Hershler (1998) noted that *P. sathos* was found in Flag Springs (the type location) and Camp Spring, Nye County, Nevada, whereas *P. hubbsi* was found in Crystal Spring and Hiko Spring (the type location), Lincoln County, Nevada. Hershler (1998) distinguished them based on a multitude of morphological differences, and later used a sequence of a specimen from Flag Springs to represent *P. sathos* (Hershler and Liu 2008) and one from Crystal Spring to represent *P. hubbsi* (Liu and Hershler 2005). Our subsequent resampling and analysis revealed that the specimens from Flag Springs, Camp Spring, and Crystal Spring represented 1 candidate species, which we interpreted as *P. sathos*. Samples from Hiko Spring were highly divergent at all genes, and we suspect these actually represent *P. hubbsi*. A different issue involved synthetically concatenated mitochondrial and nuclear sequences of many *Pyrgulopsis* specimens (i.e., sequences of mitochondrial genes and nuclear genes thought to represent 1 species that were taken from different individuals and combined for analysis; Delicado et al. 2024). In a handful of cases (Supplementary Material 2), the sequences appeared to represent chimeras composed of mitochondrial sequences from 1 species and nuclear sequences from another. Because in most instances the problematic sequences were based on museum collections at sites where both species were present, we suspect that this reflected errors in the identification of preserved specimens rather than rampant hybridization.

We did, however, also observe occasional hybrids in our broader sampling. In southern Nye County, Nevada, a single specimen (NGC1407) with mitochondrial sequences of *P. fairbanksensis* exhibited an ITS2 sequence identical to that of *P. erythropoma* (minimum COI pairwise distance, 7.65%). In 2 isolated springs on Duckwater Tribal Lands, Nye County, Nevada, we sampled specimens of *P. bryantwalkeri* (field-identified as *P. aurata*) and *P. anatina*, which are highly divergent in both mitochondrial and nuclear trees. One individual (NGC2107), however, had the mitochondrial haplotype of the former and nuclear sequence of the latter and appeared to be a hybrid. In a tributary to Cain Spring, Lander County, Nevada, 3 specimens (NGC1931–3) had the mitochondrial haplotype of *P. bryantwalkeri* (field-identified as *P. pictilis*, for which the type location is in the same drainage 660 m west) and a nuclear sequence related to that of *P. gibba*. At this site, we did not obtain nuclear sequences of additional specimens with the *P. bryantwalkeri* haplotype ($n = 6$) or observe any individuals with the *P. gibba* mitochondrial haplotype, although the species' ranges overlap in this area. A second species was present based on mitochondrial sampling (*P. dixensis*; $n = 6$), and the single specimen with a nuclear sequence also assigned to *P. dixensis*. Elsewhere, other specimens labeled as *P. bryantwalkeri* or *P. aurata* (NGC0567, 0571), and falling within that mitochondrial clade, had nuclear sequences similar to those of *P. gibba*. A possible explanation for these observations that warrants further study is that one or all of the taxa described as *P. aurata*, *P. bryantwalkeri*, and *P. pictilis* (and perhaps *P. augustae*; Hershler 1998) may be of hybrid origin. Regardless, the appearance of hybrids between taxa with markedly divergent mitochondrial lineages (COI pairwise distances from *P. bryantwalkeri*: *P. gibba*, 5.61%; *P. anatina*, 6.97%) demonstrates the need for nuclear sequences in taxonomic assessments of *Pyrgulopsis*.

In summary, our molecular analyses of the nearly complete taxonomic inventory of *Pyrgulopsis* revealed a taxonomy that is neither fully sampled nor entirely resolved. The deeper phylogenetic history of this genus remains poorly described, and although we estimated that the total number of species present was similar to that from the presently accepted taxonomy, we propose notable changes in species boundaries. Although the majority of previously recognized species were validated, a number of others appear to warrant further revision, and there are many potential new additions to this genus. There remains a fundamental need to include morphological evaluations of proposed taxa, but just as important is their more complete genetic or genomic characterization, especially given the possibility of hybridization and the complex and recent evolutionary histories of some clades. Finally, with the potential for long- and short-term habitat changes leading to the ongoing

loss of some populations or whole species, we encourage continued efforts to find new populations, monitor readily accessible ones, and seek to resample those previously discovered but not recently visited or fully characterized. An understanding of species taxonomic and geographic boundaries, coupled with the effectiveness of habitat protection at maintaining populations and restoration and translocation at rebuilding them (Sada 2008, Johnson et al. 2013, 2022), suggests a means for recognizing and conserving this iconic aquatic fauna of western North America.

DATA AVAILABILITY

Sequence information has been deposited in GenBank for COI (PX790011–PX794277), ND1 (PX787943–PX788463), ITS2 (PX782595–PX783020), and 28S (PX810320–PX810784). Alignments are available in Dryad (https://doi.org/10.5061/dryad.95x69p8zn).

SUPPLEMENTARY MATERIAL

Thirteen online-only supplementary files accompany this article (https://scholarsarchive.byu.edu/wnan/vol86/iss2/10).

SUPPLEMENTARY MATERIAL 1. Recently extant (nonfossil) species in *Pyrgulopsis* (from MolluscaBase, accessed 22 April 2025).

SUPPLEMENTARY MATERIAL 2. Specimens and sequences of *Pyrgulopsis* and outgroups used in this study.

SUPPLEMENTARY MATERIAL 3. Gene, primer name and sequence, annealing temperature, and source for primers used to obtain *Pyrgulopsis* sequences.

SUPPLEMENTARY MATERIAL 4. Published sources of sequences used in this study.

SUPPLEMENTARY MATERIAL 5. The maximum-likelihood phylogenetic tree based on COI sequences.

SUPPLEMENTARY MATERIAL 6. The maximum-likelihood phylogenetic tree based on ND1 sequences.

SUPPLEMENTARY MATERIAL 7. The maximum-likelihood phylogenetic tree based on ITS2 sequences.

SUPPLEMENTARY MATERIAL 8. The maximum-likelihood phylogenetic tree based on 28S sequences.

SUPPLEMENTARY MATERIAL 9. *Pyrgulopsis* specimen assignment via placement on a neighbor-joining phylogenetic tree based on COI sequences.

SUPPLEMENTARY MATERIAL 10. Species from the present taxonomy of *Pyrgulopsis* that were generally supported.

SUPPLEMENTARY MATERIAL 11. Groups of species that were candidates for synonymy, including the preferred overall epithet and rationale for its choice.

SUPPLEMENTARY MATERIAL 12. Species complexes representing multiple candidate taxa. Candidate taxa are either denoted by a currently accepted name or delimited as a new candidate species.

SUPPLEMENTARY MATERIAL 13. New candidate species. Specimens are from Supplementary Material 2. Other entries are as in Supplementary Material 10.

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New candidate species. Specimens are from Supplementary Material 2. Other entries are as in Supplementary Material 10.
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