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RESEARCH ARTICLE

Assessing species number and genetic diversity of the Mountainsnails (Oreohelicidae)

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Abstract

One of the current challenges facing conservation biologists is a lack of resolution of species boundaries in threatened groups residing in at-risk areas. This is particularly key for habitats like calcareous outcrops that are known to harbor a high degree of endemic species that may also possess extensive morphological variation. Here, we construct the first time-calibrated phylogeny and evaluate species number of the limestone endemic Mountainsnails (Oreohelicidae), a highly-threatened and phenotypically variable family of land snails from Western North America, using sequence fragments of the mitochondrial gene Cytochrome Oxidase subunit I (COI) from 50 recognized taxonomic species and subspecies. We found four highly supported clades that span wide geographic areas from southern Canada to northern Mexico. Using three species delimitation approaches, we identified a largely concordant set of 16 putative species, which represents less than a third the expected number of species given the current taxonomy and our dataset composition. Our results reveal that this is largely a result of two of the delimitation approaches lumping much of the taxonomic diversity of Oreohelicidae into a single species that possesses remarkable shell form variation and convergence. Moreover, we discuss the suitability of these approaches to delimiting clades with recent divergence, which is not uncommon for limestone endemic fauna and flora. To improve management decisions in montane limestone endemics, our research highlights the need for increased molecular and ecological studies of these isolated and phenotypically variable species.

Keywords Limestone · Oreohelix · Species delimitation · Land snail · Phylogenetics

Introduction

Regions of high resource availability tend to be associated with high biodiversity (Storch et al. 2005; Cardinale et al. 2009; Cline et al. 2018) and are often sources for conflicts between extractive industries and environmental agencies over access to and protection of resources (Sonter et al. 2018). This conflict is particularly salient for the management of sensitive species restricted in distribution to areas rich in minerals targeted by industry for extraction (e.g., mining or quarrying). Such extractive activities can alter the distribution of mineralogical resources, which may in turn have detrimental effects on the composition of communities and persistence of resident species at multiple spatiotemporal scales (Miranda et al. 2003; Erskine et al. 2012; Che-Castaldo and Neel 2016; Murguía et al. 2016; Sonter et al. 2018).

Calcareous substrates derived from calcium carbonate ($CaCO_3$) bedrock (e.g., limestone, dolomite, and marble) are known to harbor endemic diversity and locally adapted populations (Kruckeberg 1986; Baskin and Baskin 1988; Schilthuizen 1994; Clements et al. 2006), and these same areas are some of the most at-risk sites from industrial mineral development for aggregate, cement, and agricultural applications (Tropek et al. 2010; Che-Castaldo and Neel

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2016). Conservation plans for these areas seek to balance societal needs for carbonate rock and the habitat requirements of endemic species occupying calcareous outcrops, but developing management plans for these communities is non-trivial (Clements 2008). One major hindrance in the development of management plans for these areas is that many limestone endemic fauna (snails: Alonso et al. 1985; Gittenberger 1991; Frest and Johannes 1997; Teshima et al. 2003; Haskell and Pan 2013; arthropods: Bauer 1989) and flora (Baskin and Baskin 1988; Rajakaruna 2004, 2017; Wang et al. 2017) possess a high degree of phenotypic variation within and between limestone outcrops, which can make species classification difficult. As many regulatory agencies protect taxa at the species level (Mace 2004; Frankham et al. 2012), there is a need to describe species and interspecific relationships in these communities so that conservation plans can be developed. However, because morphology-based delimitations can be misleading in limestone endemics due to a high degree of homoplasic characters (Conti et al. 1999; Giokas 2000; Elejalde et al. 2008), molecular data are oftentimes needed to evaluate morphology-based systematics of poorly studied taxa.

The Mountainsnails (family: Oreohelicidae) are a calciphilous family of montane-endemic land snails that includes two genera: *Radiocentrum* and *Oreohelix*–purportedly the most diverse genus of land snails in North America (82 currently recognized taxonomic species and subspecies; Pilsbry 1939; Nekola 2014). Many members of Oreohelicidae are restricted to single mountains, canyons, or only a few limestone outcrops within a given mountain range (Pilsbry 1939; Frest and Johannes 1997; Weaver et al. 2008). The narrow range of many *Oreohelix* species and potential threat of industrial and road development have contributed to the listing of over half of the family as critically imperiled (G1 or S1 rank) or imperiled (G2 or S2 rank) by NatureServe and local state governments (Table 1; NatureServe 2019). However, conserving oreohelicid diversity is complicated by a lack of systematic knowledge of the group. Many of the current taxonomic units of Oreohelicidae were described based on shell characters that may be prone to homoplasy, phenotypic plasticity, or a high degree of intraspecific variation (Henderson 1918; Chak 2007). Given this, as well as a few molecular studies suggesting a lack of support for recognized taxonomic units (Chak 2007) and molecular-based evidence for cryptic species (Weaver et al. 2008), management officials are hesitant to develop conservation plans or federally list threatened Oreohelicid species without both morphological and molecular support for current taxonomic statuses (Federal Register 2005, 2006, 2011).

The primary morphological characters used for delimiting Oreohelicid species are genitalia and shell morphology (Pilsbry 1939; Ports 2004). Genitalia morphology is relatively conserved in *Oreohelix* (Pilsbry 1939) with only three genitalia types being recorded and named after the nominal species of each group (*O. strigosa*, *O. subrudis*, and *O. yavapai* types). These have served to define the major groups from which the genus has been organized (Fig. 1). Given the relatively conserved genital morphology of the group compared to other land snails (Pilsbry 1939), shell morphology has been primarily invoked for species or subspecies delineation in combination with geographical isolation. However, species delimitation based on shell morphology has resulted in the description of several species complexes within the major genitalia groups. Within these complexes, it is common to find gradients of intergrading shell forms with the extremes usually being ascribed as species or subspecies. One of the major shell characters used for species delimitation in *Oreohelix* is shell ornamentation (e.g., increased shell biomineralization such as spiral ribs, vertical ribs, prominent keel; Fig. 2) but it is also a character with a high degree of variation between populations (Fairbanks 1975; Weaver et al. 2008), which has made morphological species classification challenging for experts and conservation officials. Morphological identification is further complicated by ornamentation convergence within and between the major genitalia groups (e.g., the spiral ribs of *O. haydeni* and *O. pilsbryi*, Fig. 2; Pilsbry 1939). These parallelisms of shell form across the genus have led previous authors to suggest much of shell form variation in the group may be environmentally driven (Henderson 1918), but the exact environmental associations of these characters remains elusive (Weaver et al. 2008).

In this study, we present the first family-wide molecular phylogeny of Oreohelicidae to assess species status and determine the diversification branching pattern and timing among species. We combined previously published sequences with newly generated sequence data from hitherto unsampled species to produce both maximum-likelihood and Bayesian phylogenetic reconstructions, as well as employ coalescent-based delimitation and barcode gap detection methods. The results of our study improve on our understanding of oreohelicid systematics and provide an important resource for the management and conservation of this threatened group.

Material and methods

Sampling

Field personnel collected specimens from a total of 274 localities across the western United States between 1998 and 2019 (Fig. 1). Collected adult snails were preserved in 95% ethanol. Additional tissue samples were taken from collections at the Junius Henderson Museum, University of Colorado, Boulder and the Florida Museum of Natural History, University of Florida, Gainesville (Appendix

Table 1 Oreohelicidae species sampling and conservation status at federal and state levels

Scientific name	Global status	Federal status	State	State status	Sampled in current study
Oreohelix alpina	G2	NL	US: MT	S1	–
Oreohelix amariradix	G1G2	NL	US: MT	S1S2	1
Oreohelix anchana	GH	NL	US: AZ	SNR	–
Oreohelix barbata	G1	FS:S	US: AZ, NM	S1,S1	1
Oreohelix californica	G1	NL	US: CA	SNR	–
Oreohelix carinifera	G1	NL	US: MT	S1	–
Oreohelix concentrata	G2	NL	US: AZ; MX: CH	SNR; SNR	1
Oreohelix confragosa	G1	NL	US: AZ	S1	–
Oreohelix cooperi	G1Q	FS:S	US: SD, WY	S2, S1	1
Oreohelix elrodi	G2G3Q	NL	US: MT	S1	1
Oreohelix eurekensis	G2	NL	US: UT	S1	–
Oreohelix eurekensis uinta	G1	NL	US: UT	SNR	–
Oreohelix grahamensis	G2	FS:S	US: AZ	S2	1
Oreohelix hammeri	GX	NL	US: ID	S1	1
Oreohelix handi	G1	NL	US: CA, NV	SNR, S1	1
Oreohelix haydeni alta	G1	NL	US: CO	SNR	–
Oreohelix haydeni betheli	–	NL	US: CO	SNR	1
Oreohelix haydeni bruneri	–	NL	US: CO	SNR	1
Oreohelix haydeni corrugata	G2	NL	US: UT	S1	1
Oreohelix haydeni haydeni	–	NL	US: UT	S2	–
Oreohelix haydeni hesperia	G2T1	NL	US: ID	S1	1
Oreohelix haydeni hybrida	–	NL	US: UT	SNR	1
Oreohelix haydeni mixta	–	NL	US: CO	SNR	–
Oreohelix haydeni oquirrhensis	–	NL	US: UT	SNR	1
Oreohelix haydeni perplexa	G2T1T3	NL	US: ID	SNR	1
Oreohelix hemphilli	G2T1T3	NL	US: NV	S2	1
Oreohelix hendersoni	G1G3	NL	US: CO	SNR	1
Oreohelix houghi	G1	NL	US: AZ	SNR	1
Oreohelix howardi	G1	NL	US: UT	SNR	1
Oreohelix idahoensis idahoensis	G1	BLM:S	US: ID	S1	1
Oreohelix idahoensis baileyi	G1	NL	US: ID	S1	1
Oreohelix intersum	G1T1	NL	US: ID	S1	1
Oreohelix jaegeri	G1	NL	US: NV	S1	–
Oreohelix jugalis	G1	BLM:S	US: ID	S1	1
Oreohelix junii	G1G2	NL	US: WA	S2S3	1
Oreohelix litoralis	G2	NL	US: NM	S1	–
Oreohelix loisae	G1	NL	US: NV	S2	1
Oreohelix magdalenae	G1G3	NL	US: AZ	S1	1
Oreohelix metcalfei acutidiscus	G2	FS:S	US: NM	SNR	–
Oreohelix metcalfei concentrica	G2T1	FS:S	US: NM	SNR	1
Oreohelix metcalfei cuchillensis	G2T1	NL	US: NM	S1	–
Oreohelix metcalfei hermosensis	G2T1	FS:S	US: NM	SNR	–
Oreohelix metcalfei metcalfei	G2T1T2	FS:S	US: NM	SNR	1
Oreohelix metcalfei radiata	G2T1	FS:S	US: NM	SNR	1
Oreohelix neomexicana	G2T2	NL	US: NV, TX	S3, SNR	1
Oreohelix nevadensis	G3	NL	US: NV	S1	1
Oreohelix parawanensis	G1	NL	US: UT	S1	1
Oreohelix peripherica newcombi	G1	NL	US: UT	SNR	–

Table 1 (continued)

Scientific name	Global status	Federal status	State	State status	Sampled in current study
Oreohelix peripherica peripherica	G2	NL	US: UT	SNR	1
Oreohelix peripherica wasatchensis	G2T1T2	NL	US: UT	S1	1
Oreohelix peripherica weberiana	–	NL	US: UT	SNR	1
Oreohelix pilsbryi	G2T1	FS:S	US: NM	S1	–
Oreohelix pygmaea	G1	FS: S	US: MT, WY	S1, S1	1
Oreohelix pygmaea maculata	–	NL	US: WY	SNR	–
Oreohelix strigosa berryi	G5T2	NL	US: MT, WY	S1S2, SH	–
Oreohelix strigosa buttoni	–	NL	US: UT	SNR	–
Oreohelix strigosa capax	G5T2Q	NL	US: ID	SNR	–
Oreohelix strigosa delicata	G5T1	NL	US: OR, WA	S1, S1	1
Oreohelix strigosa depressa	G5T5	NL	US: MT, NM, NV, WY	SNR, S2S3, S2?, SNR	1
Oreohelix strigosa fragilis	–	NL	US: ID, UT	SNR	1
Oreohelix strigosa goniogyra	G5T1	BLM:S	US: ID	S1	1
Oreohelix strigosa nogalensis	G5T2	FS:S	US: NM	S1	1
Oreohelix strigosa strigosa	–	NL	US: WA	S5	1
Oreohelix subrudis	G5	NL	CAN: AB, BC, SK; US: AZ, CO, ID, MT, NM, NV, WA, WY	SNR, S3, SNR; SNR, SNR, S5, S3, S3, SNR, SNR	1
Oreohelix swopei	G1	FS:S	US: NM	S1	–
Oreohelix tenuistriata	GH	NL	US: ID, UT	SH, SNR	–
Oreohelix variabilis	G2Q	NL	US: OR	S2	1
Oreohelix vortex	G2?	BLM:S	US: ID	S1	1
Oreohelix waltoni	G1	BLM:S	US: ID	S1	1
Oreohelix yavapai clutei	–	NL	US: AZ	SNR	–
Oreohelix yavapai cummingsi	G5T3Q	NL	US: UT	S1	–
Oreohelix yavapai extremitatis	G5TNR	NL	US: AZ, MT, WY	SNR, SNR, SNR	–
Oreohelix yavapai fortis	–	NL	US: AZ	SNR	–
Oreohelix yavapai magnicornu	–	NL	US: WY	SNR	–
Oreohelix yavapai mariae	G5T1	NL	US: MT	S1	–
Oreohelix yavapai profundorum	–	NL	US: AZ	SNR	–
Oreohelix yavapai yavapai	G5	NL	US: AZ	S1	1
Radiocentrum avalonense	G1	NL	US: CA	S1	1
Radiocentrum chiricahuana	G2	NL	US: AZ	SNR	1
Radiocentrum clappi	G2	NL	US: AZ	SNR	1
Radiocentrum ferrissi	G1	NL	US: NM, TX	S1, S1	–
Radiocentrum hachetanum	G2	NL	US: NM	S1	–
					50

NatureServe ranks correspond to global (G) or state (S) on a scale of 1–5 with 5 being the least threatened. NR, U, Q, T correspond to not ranked, unrankable due to possible lack of information, questionable taxonomic status, and intraspecific status, respectively. Federal sensitive species status abbreviations BLM stands for Bureau of Land Management and FS for Federal Forest Service

Table 1). Samples were identified as either recognized or proposed (Frest and Johannes 1997) taxonomic units using a combination of geographic location, shell, and genitalia characters when available (Pilsbry 1939; Burke and Leonard 2013).

DNA sequencing, genotyping, and dataset composition

Genomic DNA was extracted from muscle tissue removed from the foot of each animal using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) per the manufacturer's

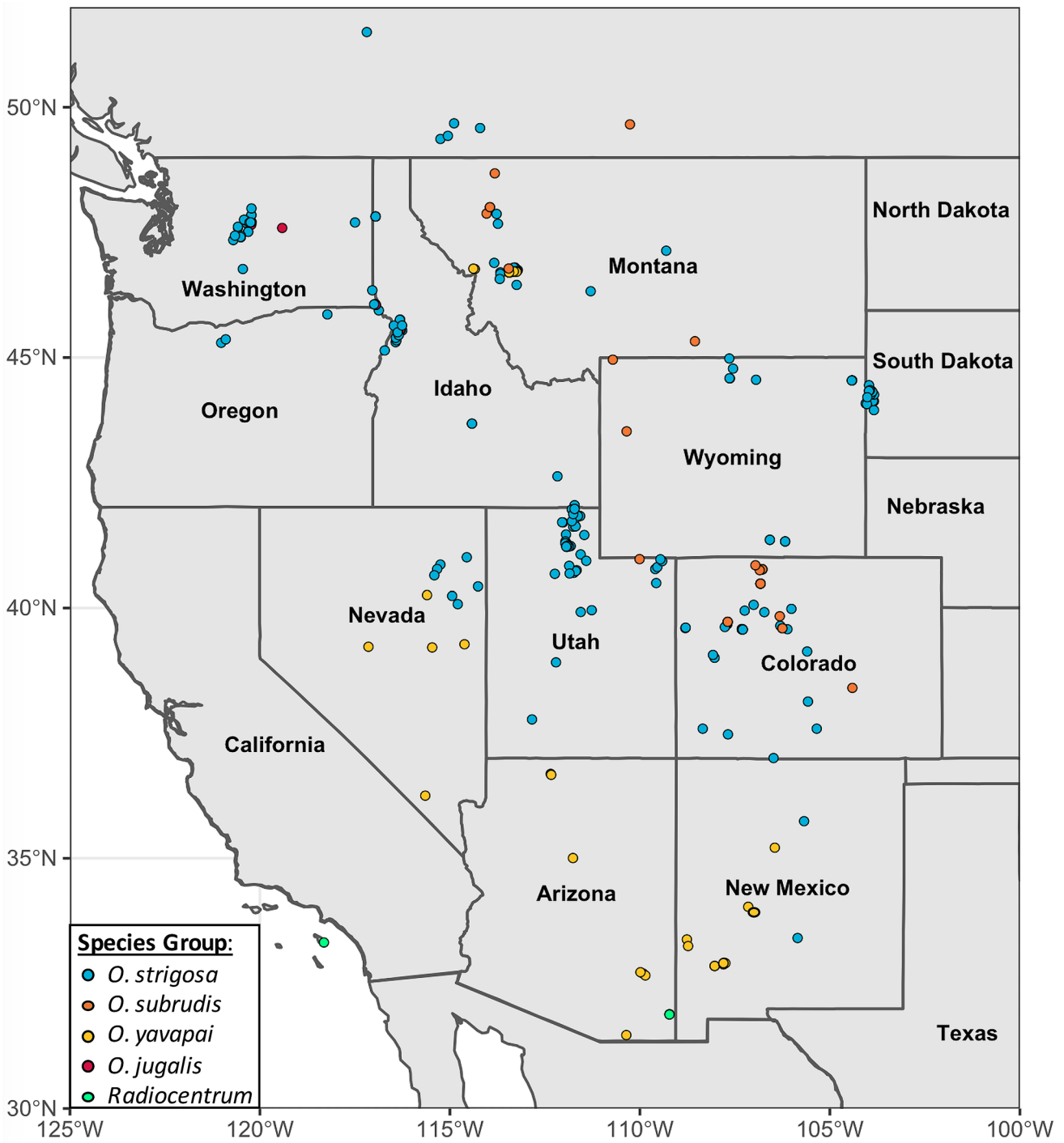

Fig. 1 Sampling localities color coded by species group. Blue corresponds to the *O. strigosa* group, orange to the *O. subrudis* group, yellow to the *O. yavapai* group, red to the *O. jugalis* group, and green to the *Radiocentrum* group

protocols. Partial sequences of the mitochondrial COI gene were amplified by PCR with primers LCO1490/HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ and 5′-GGTCAACAAATCATAAAGATATTGG-3′; Folmer 1994). All PCRs were performed in 25 µl reactions containing 2 µl DNA, 18 µl water, 2.5 µl buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 1 µl of 10 µM forward and reverse primer, and 0.25 µl of 5 U/µl of New England Biolabs Taq polymerase. The PCR conditions were as follows: an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 95 °C for 35 s, 52 °C for 60 s, 72 °C for 45 s, and finalized with a final extension step at 72 °C for 5 min.

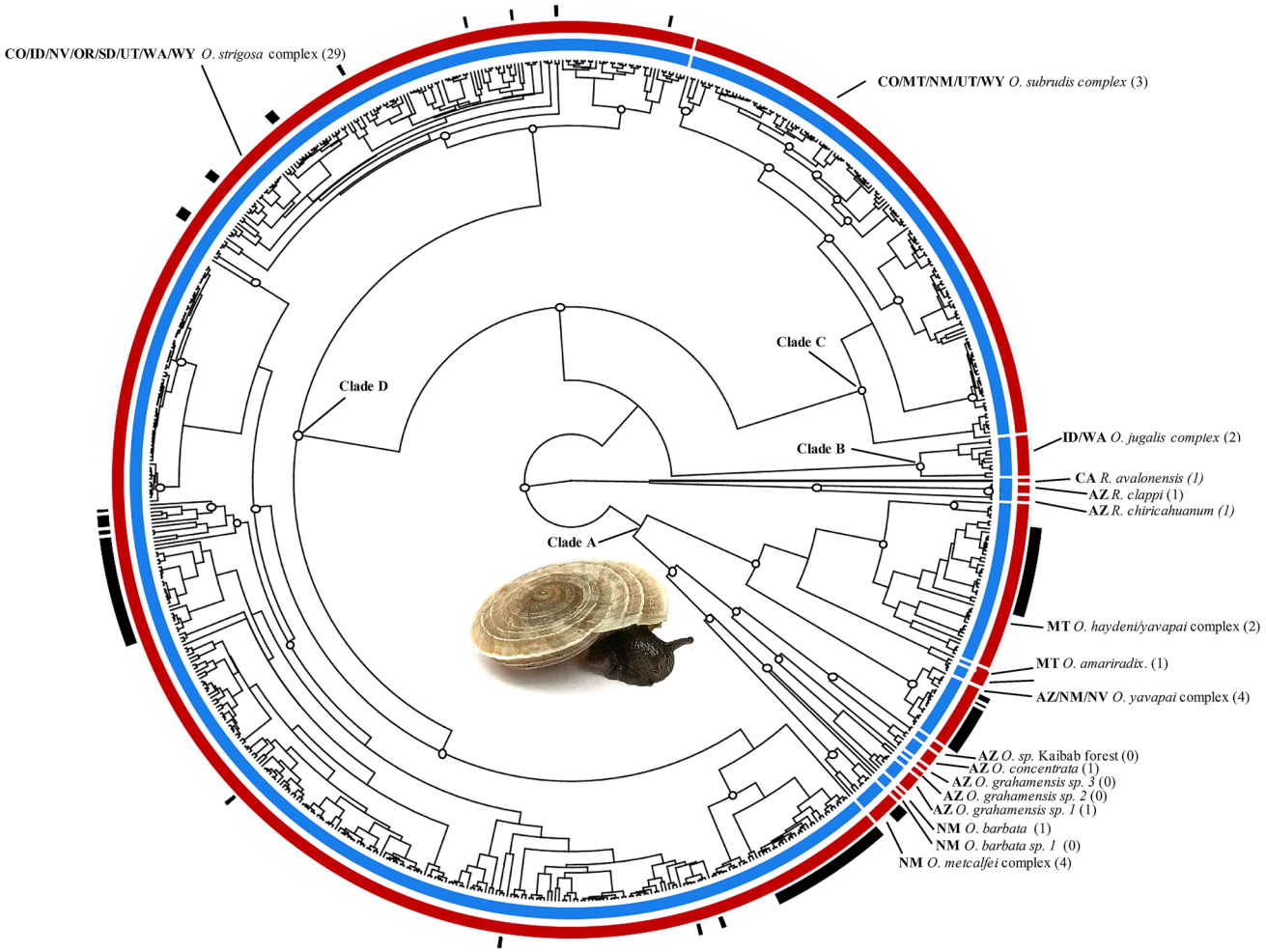

Fig. 2 Beast chronogram with delimited species according to species delimitation method. The blue circle depicts the 15 species delimited by mPTP and red circle the 16 by ABGD. The outer ring indicates ornamentation presence (black) and absence (white). Delimited species are annotated with taxonomic names and state location. Numbers in parentheses are the number of previously recognized taxonomic units included under the newly delimited species. Nodes with hollow circles represent clades with greater than 0.80 posterior probability

To verify amplifications, amplicons were electrophoresed in a 1% agarose gel. PCR products were then purified using the Qiaquick PCR cleanup kit (Qiagen). Bi-directional DNA Sanger sequencing was outsourced to Eurofins.

MWG Operon, Louisvillle, KY, USA (https://www.eurofins.fr). Chromatograms in both directions were compared and consensus sequences were assembled using Chromas v.2.6.2 (Technelysium, https://www.technelysium.com.au/chromas.html).

We added to these data a set of 261 homologous *Oreohelix* sequences from GenBank from previous molecular studies of the group (Weaver 2006; Chak 2007; Weaver et al. 2008; van Paridon et al. 2017; Dempsey et al. 2020). In addition, we added a single individual of *Megomphix* to serve as an outgroup as previous morphological studies have indicated Oreohelicidae and Megomphicidae may be sister families (Emberton 1991). The combined 861 sequence dataset contained representatives of 60.9% (52 species) of all currently recognized species and subspecies in Oreohelicidae. Multiple sequence alignments were constructed using the MAFFT online webserver (https://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2019) specifying a gap opening penalty of 5 and using the remaining default values. The initial 572 bp alignment contained COI fragments ranging in length from 296 to 572 bp with a mean sequence length of 565 bp. No indels or premature stop codons were observed. Identical sequences (357 sequences) matched another from the same or nearby locality and were removed prior to phylogenetic analysis for a final alignment of 504 sequences.

Phylogenetic analyses

We first selected a model of nucleotide sequence evolution using the corrected Akaike information criterion (AICc)

and decision theory (Minin et al. 2003), implemented by the automodel command in PAUP* v4.0a152 (preview release; Swofford and Sullivan 2003). The JC + Γ model was chosen for our dataset. Phylogenetic relationships were then inferred using maximum-likelihood performed in RAxML (Stamatakis 2006), specifying the JC + Γ model and conducting ten replicate runs. Nodal support was assessed using 100 bootstrap replicates with two tree searches per bootstrap. We used the resulting ML phylogeny to test the assumption that the data set has evolved in a clock-like fashion by testing for a global molecular clock in PAUP* using the likelihood-ratio test (LRT) of Felsenstein (1988). As the strict clock model was rejected, the relaxed clock model was used for subsequent analyses. Additionally, we tested the level of genetic saturation at the COI gene using DAMBE7 (Xia 2018) using the 'Xia method' (Xia et al. 2003) and visually assessed saturation using the R package 'ape' (Paradis and Schliep 2018). Visual inspection was accomplished by plotting uncorrected genetic distances vs corrected genetic distances using the Gamma shape parameter value from the PAUP* automodel command (JC + Γ; Gamma shape parameter = 0.666; Supplemental Fig. 3). The saturation test in DAMBE using the default parameters indicated little saturation (P values < 0.001; proportion of invariant sites: 0.407). The slope of uncorrected to corrected genetic distances indicates weak to moderate saturation at 25–30% sequence divergence (Supplemental Fig. 3). These analyses indicate weak-to-moderate saturation, which may cause relationships and divergence time estimates at the deeper nodes to be more uncertain (Xia et al. 2003) (Fig. 3).

We estimated the timing of *Oreohelix* divergence events by inferring an absolute evolutionary timescale using a fossil calibration point implemented in BEAST v1.8.4 (Heled and Drummond 2009). Past systematic revisions have placed nearly all previously ascribed *Oreohelix* fossils into the genus *Radiocentrum* (Roth 1986; Pierce and Constenius 2001). Of the few *Oreohelix* fossils remaining, most are from the Quaternary with only a single Oreohelicid in the early Miocene (20.8 MYA) from the Deep River Formation (Roth and Emburton 1994), though the validity of the assignment of this fossil to the genus *Oreohelix* is not certain (Roth 2019, personal communication). As it appears *Radiocentrum* has been present since at least the late Cretaceous and *Oreohelix* possibly since the early Miocene, we chose to fossil calibrate using the earliest date for the Deep River formation to allow for the possibility that *Oreohelix* is a relatively recent emergence from recent *Radiocentrum* as conjectured by Pierce and Constenius (2001). To have another suitable calibration for comparison, we used fossil records of *Oreohelix* from the mid-Blancan age Shooting Iron Formation as a calibration point (Love 1989). For the Deep River fossil calibration, we used a log-normal prior distribution with an offset of 20.8 MYA, mean of 3.0 MYA, and standard deviation of 1.5 MYA for estimating the split between *Oreohelix* and *Radiocentrum*. We used a log-normal distribution with an offset of 3.8 MYA, mean of 3.0 MYA, and standard deviation of 1.5 MYA for the Shooting Iron Calibration. We ran and subsequently combined four independent MCMC chains each of 100 million generations, sampling every 5000 generations, and discarding the first 20% as burn-in using LogCombiner (Drummond and Rambaut 2007) for both calibration points. Convergence was assessed visually using TRACER v. 1.7.1 (Drummond and Rambaut 2007; Rambaut et al. 2018) and by verifying greater than 200 effective sample size for all parameters estimated.

Species delimitation

To delimit species, we first used the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al. 2012) through the online server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with the default settings. The ABGD method compares pairwise genetic distances from gene fragments to differentiate smaller intraspecific divergence from greater interspecific divergence. ABGD then delimits sequences into groups over a user specified range of maximal intraspecific divergence values and reports the number of groups for each recursively determined maximal intraspecific divergence value (Puillandre et al. 2012).

We then implemented the multi-threshold Generalized Mixed Yule Coalescent (GMYC) method (Pons et al. 2006; Fujisawa and Barraclough 2013) with our ultrametric BEAST chronogram in R (R Core Team 2019). The single threshold GMYC model seeks a global threshold that delimits between species-level to population-level processes by separately modelling the fit of within- vs. between-species branching models resulting in a given ultrametric tree. The method operates by finding the maximum likelihood (ML) solution of a model incorporating diversification between species using a Yule speciation process and branching within species using a neutral coalescent (Pons et al. 2006). The multi-model approach relaxes the assumption that all speciation events are older than all coalescent events in the tree (Fujisawa and Barraclough 2013) and allows for the fitting of multiple thresholds across individual clades of the tree.

Finally, we used the multi-rate Poisson Tree Process (mPTP) model (Kapli et al. 2017) with our RAxML tree operated through the online server (https://mcmc-mptp.h-its.org/mcmc/) to delimit species. The PTP model group seeks to delimit species by modelling branching processes based on the number of accumulated expected substitutions between subsequent speciation events. The underlying assumption is that each substitution has a chance to generate a branching event with branching events being more probable within than between species. The original PTP is a two-parameter model that assumes models within and between

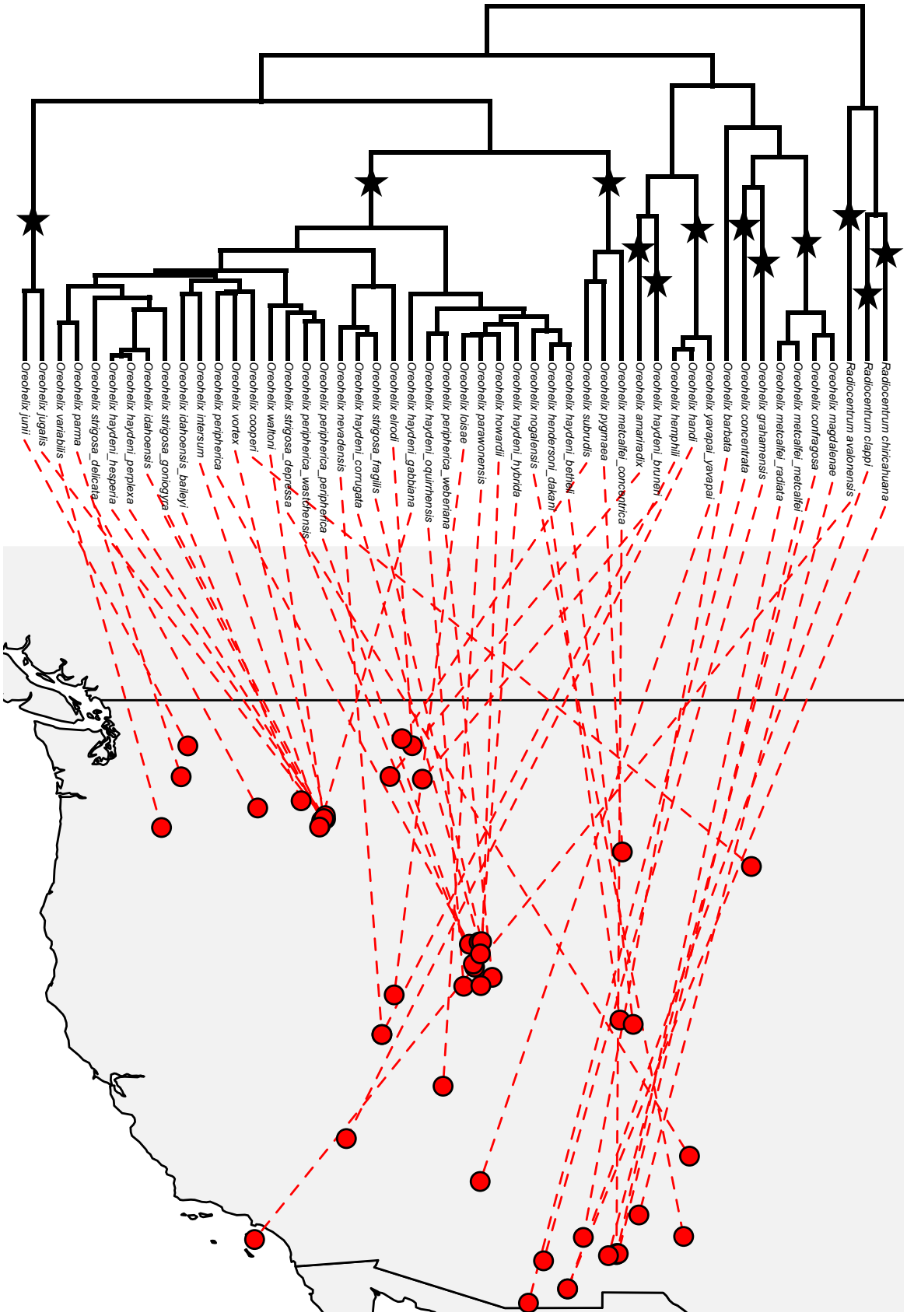

Fig. 3 Geophylogeny of recognized taxonomic units. Stars on the phylogeny indicate delimited species. Undescribed or cryptic species are not presented

species branching using a single coalescent and speciation parameter, respectively (Kapli et al. 2017). In contrast to the original PTP (Zhang et al. 2013), mPTP is more robust to sampling- and population-specific biases in empirical datasets by assigning each delimited species a distinct intraspecific coalescent distribution instead of assuming a single global distribution for all delimited species. We used default parameters for the mPTP analyses.

Results

Phylogenetic analyses

All Bayesian and maximum-likelihood approaches were concordant in topology for major clades (Fig. 2, Supplementary Fig. 1), so we chose to focus our discussion of the results to the time-calibrated BEAST tree. The 95% highest posterior density (HPD) of our mean substitution rate for the early Miocene Deep River fossil *Oreohelix* was 0.00442–0.0131 substitutions/site/MYA (mean 0.00889), which is outside the range of substitution reported for other terrestrial gastropods at the COI gene (0.028–0.130 substitutions/site/MYA; Van Riel et al., 2005). Using the Shooting Iron mid-Blancan age fossil as a calibration point, we recovered a 95% HPD of 0.0261–0.068 substitutions/site/MYA (mean 0.0449) for our mean substitution rate which fits well within the range of mean substitution rates recovered from other terrestrial gastropods. Given the previously discussed concerns regarding the generic assignment of the Deep River formation fossil specimen used for calibration, and that it resulted in an abnormally low mean substitution rate, we focus the remaining sections of the paper on the Shooting Iron formation calibration results.

Radiocentrum and *Oreohelix* were recovered as reciprocally monophyletic with a high degree of posterior probability (PP) for all Bayesian analyses (Fig. 2; 1.00 PP). The 95% highest posterior density (HPD) of the split between *Oreohelix* and *Radiocentrum* encompassed the estimated earliest split between them using fossils (mean divergence date: 6.37 MYA; 95% HPD: 4.08–10.46 MYA). The *Oreohelix* genitalia groups proposed by Pilsbry (1939) were recovered as monophyletic and correspond to major deep splits in the tree, with the exception of the *O. jugalis/junii* species group which was placed within the *O. subrudis* group by Solem (1975) (Fig. 2): Clade A is a weakly supported group (0.55 PP; mean divergence date: 4.99 MYA; 95% HPD: 3.00–8.28 MYA) that includes all members of the *O. yavapai* species group from Arizona, Montana, Nevada, and New Mexico; Clade B unites samples from Idaho and Washington (1.00 PP; mean divergence date: 1.19 MYA; 95% HPD: 0.37–2.34 MYA) as part of the *O. jugalis/junii* species group; Clade C contains all samples of the O. *subrudis* genitalia group (1.0 PP; mean divergence date: 1.90 MYA; 95% HPD: 0.84–3.29 MYA), which includes samples from Colorado, Montana, New Mexico, Nevada, Utah, and Wyoming. Clade D comprises approximately two-thirds of the samples, including all of the samples of the *O. strigosa* genitalia group from Colorado, Idaho, Nevada, New Mexico, Oregon, Utah, Canada, and Washington (1.0 PP; mean divergence date: 2.25 MYA; 95% HPD: 1.06–3.78 MYA).

Species delimitation

ABGD resulted in a narrow range of delimited species (15–16) across the specified default prior range of maximal distance (0.001–0.1) but with relatively stable estimates of 16 *Oreohelix* species between prior maximal distances of 0.0129 to 0.03594. These results were largely concordant with the 15 species delimited by mPTP (Fig. 2). The only differences between the approaches were whether *O. barbata* was delimited into one or two species and whether *Radiocentrum* were delimited into one or three species. Both approaches delimited one new species from the Kaibab National Forest. Many of the delimited species for these approaches were located in the southwest U.S., with both approaches splitting many previously taxonomically recognized southwestern species into multiple species. *O. grahamensis* and *O. barbata* were delimited into three and two species, respectively. Clade D with the largest number of recognized taxonomic species (29 species) was lumped into a single delimited species unit in all analyses excluding GMYC. The *O. metcalfei* complex (Clade A) from New Mexico (four species) and *O. haydeni/yavapai* Montana complex (three species) were also delimited as a single species. Using the criteria that a delimited species is threatened if all the previous taxonomic units that constitute the new delimited species are listed as NatureServe rank G2 or higher, we found 11 threatened species using these two approaches (Supplemental Table 1). The multi-model GMYC delimited a mean of 264 species ($P < 0.00001$ CI: 260–269 species), which is well beyond any previous estimate of species number in Oreohelicidae.

Discussion

Patterns of molecular divergence and morphological convergence

Accurate divergence time estimates between clades can provide key insights into historical demographic processes or ecological factors associated with diversification between species, either of these may in turn provide crucial information for the management of threatened species (Crandall 2009). Here, we employ a single mitochondrial gene (COI)

to reconstruct the evolutionary history of Oreohelicidae, which limits (1) our perspective of the evolutionary history of the group, by only having one gene history for comparison, and (2) our power to estimate accurate divergence times and determining species relationships, due to the size of the gene fragment (Heled and Drummond 2009). Many different gene histories are possible, which may not accurately represent the true 'species tree', and this realization should temper the reader's interpretation and application of the single gene history results outlined herein (Rannala and Yang 2017). However, there is often substantial phylogenetic information contained in a single gene, which can be utilized for understanding the processes that have shaped the diversity of extant endangered taxa and can also provide a foundation for future conservation genetic work. Previous authors have considered *Oreohelix* an ancient genus in a 'stage of prolific speciation' (Henderson 1918; Pilsbry 1939). Our finding that *Oreohelix* and *Radiocentrum* split in the early Pliocene to late Miocene (Supplemental Fig. 1), with a mean date in the late Miocene (6.37 MYA), confirms that *Oreohelix* is a relatively recent split from *Radiocentrum* as opposed to an ancient Cretaceous split proposed by Pilsbry (1939). We also find that many currently recognized taxonomic species are polyphyletic, yet morphologically distinct. Together, these results indicate that extant Oreohelicidae are relatively younger than previously thought and that several forms have arisen convergently in geographically separated localities.

Many recognized species of conservation concern were found to be polyphyletic, indicating that the characters used for species delimitation may not be suitable for diagnosing species (Fig. 2). Most of the polyphyletic species fall within the *O. strigosa* species complex (Clade D) but also include members of the Montana *O. haydeni/yavapai* complex (Clade A). Four of the polyphyletic species of conservation concern appear to be the result of distinct shell ornamentation morphologies used for species classification evolving multiple times separately (e.g., *O. haydeni, O. peripherica, O. idahoensis, O. hemphilli*). For example, the homoplasic shell character of spiral ribs used for assigning specimens to *O. haydeni* must have evolved separately no fewer than 11 times across widely geographically separated areas (Fig. 2). In contrast, there are examples of recently diverged, sympatric, and possibly cryptic lineages of *Oreohelix* displaying the same shell morphology and co-occurring at the same site (Weaver et al. 2008). Indeed, the rapid evolution of ornamented shell characters across distinct, phylogenetically separated clades (e.g., *O. haydeni;* this study) and convergence of ornamentation in the same locality in separate lineages (e.g., *O. peripherica* Weaver et al. 2008) indicates ornamentation can evolve quickly, and possibly in response to environmental conditions.

What are the possible factors that may be driving parallelisms of shell form and resulting polyphyletic taxonomic units in Oreohelicidae? Previous studies have hypothesized that ornamentation results in increased mechanical strength, increased surface water adhesion, decreased evaporation, and decreased insolation (Giokas 2008). Any of these proposed benefits could promote ornamentation evolution for the *Oreohelix* species that occupy arid environments or localities with a high density of predators. However, the occurrence of ornamentation across geographically separate areas that differ substantially in climatic and biotic conditions may indicate that no single axis of selection may be driving ornamentation evolution across Oreohelicidae. Ornamentation may have multiple functions in different environmental contexts, which may make disentangling any functional benefit of ornamentation difficult without a more comprehensive assessment of the climactic and biotic conditions across the geographical distribution of a given lineage.

While the function of terrestrial mollusk shell ornamentation may be elusive, it has been proposed that many forms of molluscan ornamentation are associated with regions rich in calcium carbonate (Alonso et al. 1985; Teshima et al. 2003; Watson et al. 2012). The greater availability of calcium carbonate in these regions may allow for increased shell biomineralization and ornamentation expression. In *Oreohelix*, all ornamented species save one (*O. waltoni)* are restricted to limestone, marble, or dolomite outcrops (Linscott et al. in prep). Conversely, many thin shelled 'hairy' forms of *Oreohelix* are solely found on volcanic rock (Frest and Johannes 1997). Further, *Oreohelix* ornamentation expression decreasing along transects crossing geologic boundaries where the rock type shifts from predominantly calcium carbonate to another composition (e.g., *O. idahoensis and O. waltoni*; Pilsbry 1939; Linscott and Parent in prep.) may indicate that ornamentation expression is plastic or locally selected for in calcareous environments. Local selection according to edaphic or geological factors can promote adaptive divergence and possible speciation (Clements et al. 2006; reviewed in Rajakaruna 2017). However, many smooth or unadorned *Oreohelix* species occupy similar calcareous bedrock habitat as ornamented forms, which may indicate a degree of standing genetic variation is necessary for ornamentation to evolve. Ornamentation expression is predominantly expressed in the large *O. strigosa* species complex (Clade D) as well as in the *O. yavapai* complex (Clade A). Within these clades, ornamented types (e.g., keel, horizontal ribs, vertical ribs) are very recently diverged from a smooth or another ornamented phenotype (Fig. 2). If local adaptation to edaphic or geologic factors is occurring in *Oreohelix,* our estimates of species number may be underestimated given that divergence may be recent and lineage sorting has not fully occurred (Rajakaruna 2007).

Species number and conservation implications

Delimiting the boundaries between species is a challenging and necessary task for informed management of threatened groups. Generally, it is expected that regions of long temporal stability and isolation will possess a high degree of phylogenetic diversity and well-demarcated species boundaries (Moritz 2002). In such regions, it is expected that single gene phylogenetic reconstructions and species estimation approaches will reasonably capture the evolutionary history of a group (Reid and Carstens 2012). However, secondary contact or recent divergence can make the delimitation of species and conservation units challenging, particularly when there is limited molecular data (Leaché et al. 2014; Jackson et al. 2017). Resolving species relationships in these situations require multiple-genes or genomic sampling to understand the extent of admixture and/or genome-wide adaptive divergence. Scenarios of secondary contact or recent divergence are rarely detected before a first-pass molecular delimitation has taken place; single gene reconstructions are suitable for determining the clades that need greater molecular sampling and for delimiting moderate to highly diverged lineages.

There was substantial conflict in the number of species between GMYC and the non-ultrametric tree based approaches. GMYC delimited a mean of 264 species, which is more than five-fold increase in species number given our taxonomic sampling. The unrealistic number of species generated by GMYC is possibly due to a combination of possible model violations including our choice of priors for our divergence time analysis (Birth–Death tree prior over a coalescent tree prior; Monaghan et al. 2009), the proportion of singletons in the data, and/or the predominant composition of the rapidly splitting *O. strigosa* clade in our dataset (Reid and Carstens 2012; Talavera et al. 2013). However, even when we used a coalescent tree prior, pruned singletons from our tree, and removed the *O. strigosa* clade we still recovered extremely high estimates of species number (Supplemental Table 3). Given the unrealistic numbers of species delimited by GMYC, we choose to omit this analysis from further discussion.

ABGD and mPTP produced a concordant set of 16 species of *Oreohelix* representing a close to three-fold reduction in species given our taxonomic sampling. Eight of the delimited species are from the *O. yavapai* species group, which is distributed throughout the sky-islands of the southwestern United States and mountainous regions of western Montana (Fig. 2). Several delimited species from these regions were previously considered populations of existing taxonomic units (*O. grahamensis* sp. 1, *O. grahamensis* sp. 2, *O. barbata* sp. 1) and occupy the same mountain range as their sister taxon. In addition to this cryptic diversity, a single undescribed species was also found to be distinct from the Kaibab National Forest (Fig. 2).

A description of new species is beyond the scope of this study and will have to await future work. However, that we detected several cryptic and undescribed species in the *O. yavapai* species group with mPTP and ABGD, two approaches that are considered relatively conservative with respect to estimates of species diversity (Reid and Carstens 2012), indicates that there may be significant cryptic diversity within this group that remains undiscovered. Indeed, that we find several cryptic and undescribed species from the Pinaleño (e.g., *O. grahamensis* sp. 1–2) and Mogollon Mountains (*O. barbata* sp. 1) is consistent with systematic studies of other resident taxa that possess substantial genetic diversity without corresponding external morphological differences (Pinaleño Mountains: Weaver et al. 2010; Mogollon Mountains: Burbrink et al. 2011). Given the isolation and long-term stability of these regions, greater species diversity may be discovered with further sampling, and we suggest should be an aim of future exploratory conservation work.

In contrast with the aforementioned splits, our delimitation approach lumped together many recognized taxonomic species and subspecies (37 species total) into three species with the lion's share of diversity being placed in a single delimited species, the *O. strigosa* complex (29 species). It has long been recognized that this complex has the greatest degree of shell form variation, convergence, and intergradation compared to other *Oreohelix* species complexes and among North American land snails in general (Pilsbry 1939). Given the high degree of morphological diversity associated with limestone habitats and the relatively frequent branching of the *O. strigosa* group compared to other *Oreohelix* groups (Figs. 2, 3), this complex is in a stage of recent divergence as put forward by Henderson (1918) and Pilsbry (1939) which may make detecting species boundaries difficult with our current methods and data. Reid and Carstens (2012) evaluated the ability of the PTP model family to delimit species across a wide range of simulated scenarios and found that rapid, recent radiations can lead to inaccurate results as coalescent and speciation events become indistinguishable. Similarly, any observable barcode gap should be smaller or more difficult to detect in recently diverged species, which may make it difficult for genetic distance based methods like ABGD to delimit species accurately (Kapli et al. 2017). While the current methods used in this study identified several threatened moderate—to highly diverged lineages, additional work is needed to definitively evaluate species boundaries and address the taxonomic discrepancies in the recently diverged *Oreohelix* clades (e.g., *O. strigosa* group) with richer genomic datasets and more robust genomic methods.

Moving forward, this study leaves the species status of many recently diverged *Oreohelix* unchanged while

suggesting that undescribed and cryptic diversity exists in the arid southwestern United States and Western Montana. Many of the delimited 'species' identified in this study are composed of many morphologically distinct and geographically isolated taxonomic units, thus these delimited 'species' in our study possess remarkable shell form variation and population structure, which may be revealed to be species or subspecies with further investigation. If we take the results of our analyses at their face value and ignore the aforementioned possibilities—there still exists substantial localized and distinct shell form variation that may facilitate persistence in many of the threatened, delimited species we identify in this study which may warrant protection. However, approximately 35 species and subspecies would be synonymized and potentially down-listed at federal and state levels. Conversely, if we were to treat each occurrence of ornamentation evolution in the *O. strigosa* group as a species or conservation unit, we would need to develop conservation plans for 12 new units (Fig. 2). Similar but smaller increases in species number would occur in the other species groups. As either extreme appears unreasonable, genomic and ecological criteria need to be developed from future studies to apply an appropriate threshold for determining species status and resulting conservation priorities for many of the recently diverged clades identified in this study.

Devising conservation priorities for the threatened oreohelicid diversity identified in this study will require addressing three issues: (1) discerning areas where substantial genetic diversity exists when phenotype and habitat preference appears to be conserved (e.g., *O. grahamensis* and its associated delimited cryptic species); (2) identifying populations where putative local adaptation is present (e.g., *O. strigosa* group); (3) determining a threshold of morphological or genetic distinctiveness to qualify for protection and then maintaining that diversity to increase overall species viability and adaptive variation (Crandall et al. 2000). Surveying the genetic diversity of populations spanning a species' range can address the first issue (1) but demarcating and maintaining conservation units within species to help ensure species persistence (2, 3) can be challenging (e.g., Mexican wolf, Geffen et al. 2004). For *Oreohelix*, this will require determining the major factors responsible for shell form variation, understanding the possible adaptive roles of such variation, and evaluating whether, if any, genomic divergence is substantial enough to warrant species/subspecies recognition. However, this task is complicated by a lack of systematic knowledge of *Oreohelix* species habitat requirements and factors responsible for shell form variation. Edaphic specialization to calcareous rock/soils may be occurring in *Oreohelix*, given the association of ornamented shell morphologies with carbonate rock/soils, but whether these morphological-geological associations reflect substantial genomic divergence that qualifies for species or subspecies recognition has yet to be determined. The methods we utilize in this study do not perform well for delimiting species in recently diverged groups (Reid and Carstens 2012), so our analyses offer limited insight for addressing this topic except to expose its relevance for the conservation of *Oreohelix* species.

The areas of greatest shell form variation and taxonomic diversity are concentrated in geologically diverse regions (Frest and Johannes 1997; Linscott and Parent in prep.). If edaphic specialization is occurring and results in substantial genomic divergence, conservation priorities should focus on protecting the soil and rock habitat requirements for edaphic specialized species, and hence, geologic diversity. It may be that future conservation plans for some *Oreohelix* species will resemble that of edaphically specialized plant species where the focus is on protecting the underlying geologic resource (Sonter et al. 2018; Corlett and Tomlinson 2020). Indeed, disturbances to limestone outcrops from commercial industries (e.g., quarrying, Clements et al. 2006; road building, Frest and Johannes 1997; or grazing, Labaune and Magnin 2002) may have effects on the distribution of phenotypes by altering the biotic, geologic, or edaphic factors influencing shell form expression. Future studies should investigate the effects that these factors have on *Oreohelix* distribution and shell form expression so that conservation plans can be developed balancing the habitat requirements of limestone endemic *Oreohelix* and societal needs for carbonate rock.

Together, our findings indicate substantial discordance between morphology-based taxonomy and genetic diversity in Oreohelicidae. We identify several possible cryptic species within existing taxonomic units and provide molecular support for the distinctiveness of 13 ecologically sensitive or threatened species. We propose that much of the phenotypic diversity within Oreohelicidae may be environmentally associated and related to calcium carbonate availability. Conserving the phenotypic and genetic variation of these calcareous rock endemic populations will require future studies on the genomic distinctiveness and habitat requirements of these taxa. The present study emphasizes the need for additional empirical studies on the genetic diversity of limestone endemic fauna in montane environments and sheds valuable light onto the management of limestone outcrops and their biodiversity conservation strategies.

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