



Evolutionary history of Ridge-nosed Rattlesnakes (*Crotalus willardi*): A specialized and diverse montane species

Michael J. Buontempo^a, Philip Lavretsky^a, Rhet M. Rautsaw^{b,c}, Lauren M. McFarland^a, Ramses Alejandro Rosales-García^b, Jason L. Strickland^d, Miguel Borja^e, Jason Jones^f, Ricardo Ramírez Chaparro^f, Robert W. Bryson Jr.^g, Christopher L. Parkinson^b, Michael G. Harvey^{a,*}

^a Department of Biological Sciences, The University of Texas at El Paso, El Paso, TX, USA

^b Department of Biological Sciences, Clemson University, Clemson, SC, USA

^c Pacific Biosciences, 305 O'Brien Drive, Menlo Park, CA, USA

^d Department of Biology, University of South Alabama, Mobile, AL, USA

^e Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango, Gómez Palacio, Dgo., Mexico

^f Herp.mx A.C. C. P. 28989, Villa de Álvarez, Colima, Mexico

^g Moore Laboratory of Zoology, Occidental College, Los Angeles, CA 90041, USA

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ABSTRACT

Mountain organisms often exhibit complex distributions, patterns of variation, and evolutionary histories due to the topographical, climatic, and orogenic complexity of the areas in which they occur. Many montane taxa, however, lack the detailed sampling across space and the genome required to characterize diversity or estimate key evolutionary parameters. Here, we leverage comprehensive sampling, genome-wide data, and a draft genome assembly to investigate patterns of diversity and evolutionary history within a widespread but poorly studied snake, the Ridge-nosed Rattlesnake (*Crotalus willardi*), that inhabits the montane pine-oak woodlands in the Sierra Madre Occidental and Madrean Archipelago. We test for population genetic structure, measure gene flow and admixture between populations, and estimate phylogenetic history and divergence times among groups. We find substantial genetic structure, with up to seven differentiated populations, only three of which correspond with existing subspecific taxonomy. We identify one putative recent admixture event as well as evidence of historical gene flow among populations. We find that southern populations are generally older than those currently occurring in the Madrean Archipelago, which diverged within the last 0.6 million years. Together, our results reveal that current taxonomy fails to capture the substantial diversity and complex evolutionary processes at work in this species of montane snake and suggest that high-resolution sampling is crucial to understanding the biotic history of complex montane regions.

1. Introduction

Montane regions are some of the most diverse ecosystems in the world (Janzen 1967, Fjeldså et al., 2012). This diversity is due to the highly heterogeneous habitat distributions and topographic complexity of these regions, which produce extreme elevational gradients and fragmentation of high-elevation habitats by lower elevation valleys and intervening plains (Rahbek et al. 2019). This complexity can be further amplified by differing geology of regions within the same mountain range causing edaphic variation that shapes floral diversity (Antonelli

et al. 2018). The complexity of montane environments drives their diversity but also creates unique challenges for montane organisms with changing climates and environments (Urban 2018).

The Madrean Archipelago and the larger Sierra Madre Occidental to the south exemplify mountain diversity and the associated conservation challenges. The Madrean Archipelago is the network of isolated mountain ranges – referred to as Sky Islands – in the states of Arizona (USA), New Mexico (USA), Sonora (MX), and Chihuahua (MX) between the Sierra Madre in Mexico to the south and the Rocky Mountains to the north (Warshall 1995). It is a highly diverse region with a unique

* Corresponding author at: Department of Biological Sciences, The University of Texas at El Paso, 500 West University Ave., El Paso, Texas, USA 79968.
E-mail address: mgharvey@utep.edu (M.G. Harvey).

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combination of flora and fauna both from the ranges to the north and south and endemic taxa restricted to the region. This diversity is largely attributed to the separation between the mountains making up the Madrean Archipelago and the wide range of habitats across elevations, which resembles the latitudinal gradient in habitats from Mexico to Canada (Warshall 1986). Moving south, the larger and more continuous Sierra Madre Occidental reflects similar elevational gradients while encompassing even larger areas of both continuous and fragmented habitats. This is particularly true for the high-elevation pine-oak woodlands that characterize the upper reaches of both regions (Marshall, 1957).

The complexity of topography and habitats in the Madrean Archipelago and Sierra Madre Occidental appears to be a major driver of diversity in many groups of organisms. Isolation is prevalent, as evidenced both by endemic taxa and, with the advent of molecular data, population genetic structure. Plants (McLaughlin, 1995), arthropods (Bryson et al. 2013a,b; Smith and Farrell 2005; Mitchell and Ober 2013), birds (Manthey and Moyle 2015, Manthey et al. 2016, McCormack et al. 2008), reptiles (Tennesen and Zamudio 2008, Middendorf et al 2005), amphibians (Bryson et al. 2014), and mammals (Atwood et al. 2011, Gould et al. 2022) all show varying but often high degrees of isolation and differentiation. Connectivity and gene flow also vary among taxa but are often quite low across the Madrean Archipelago, in particular (Atwood et al. 2011, Holycross and Douglas 2007, McCormack et al. 2008). Finally, although much of the diversity in the Madrean Archipelago predates the most recent glaciation (Bryson et al. 2013a,b; Smith and Farrell 2005; Masta 2000), it has accrued quite rapidly (McCormack et al. 2008). However, major knowledge gaps remain. Relatively few studies have well-sampled genetic data from across both the Madrean Archipelago and Sierra Madre Occidental. Also, certain taxonomic

groups remain poorly studied, including some of the least dispersive terrestrial taxa that are likely to be most impacted by changes to the region.

Rattlesnakes (Viperidae: Crotalinae: *Crotalus* and *Sistrurus*) are a radiation of vipers that span the majority of North America, south through central America and into northern South America (Campbell and Lamar, 2004; Gloyd, 1940; Klauber, 1956, 1972; Blair and Sánchez-Ramírez, 2016; Myers et al. 2024). Recent studies have suggested that the group rapidly diversified in the Neogene period with speciation rates declining thereafter (Blair et al., 2019; Blair and Sánchez-Ramírez, 2016; Holding et al. 2021; Myers et al. 2024). Studies on *Crotalus* phylogeography suggest that the pine-oak woodlands of the Mexican Highlands served as a major species pump for many lineages (Blair et al., 2019; Blair and Sánchez-Ramírez, 2016; Bryson et al. 2011a,b). The high diversity of the genus in these regions, including several species complexes, supports the idea that the Mexican Highlands have been a major catalyst of rattlesnake speciation. Broadly, diversity at and below the species level has been ascribed to isolation in refugia during recent glacial cycles (Betancourt et al. 1990), but detailed mechanisms responsible for speciation remain unknown.

One *Crotalus* species tightly associated with high elevations and pine-oak woodlands is the Ridge-nosed Rattlesnake (*Crotalus willardi*). This species is specialized to this habitat and has a distinct morphology including unique reddish-brown patterning, distinct white facial stripes, and an upturned rostral scale for which they are named (Barker 1992, Campbell and Lamar, 2004). They are, however, poorly understood – even their phylogenetic placement in the rattlesnake tree is uncertain (Blair and Sánchez-Ramírez, 2016; Holding et al., 2021). Within *C. willardi*, there are currently five described subspecies (Fig. 1; Campbell and Lamar, 2004): nominate *C. w. willardi* of southeastern Arizona

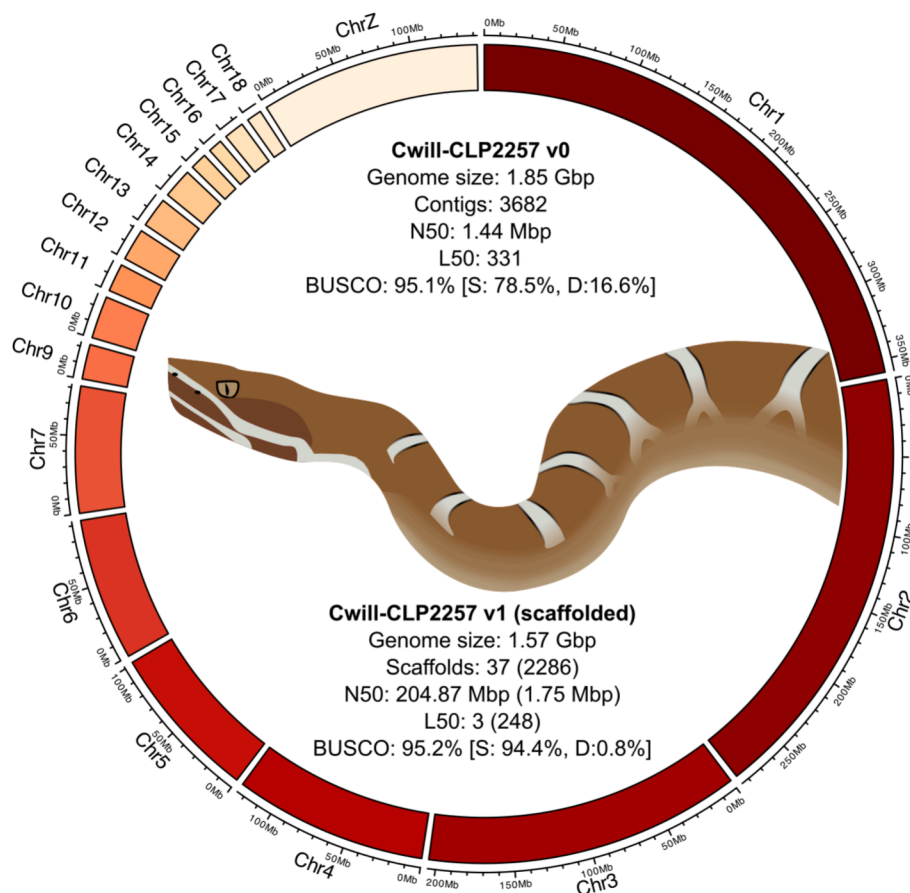


Fig. 1. Genome assembly visualization for CLP2257; *Crotalus willardi silus* including the genome size, number of contigs, N50/L50, and BUSCO completeness. Statistics for two genome assemblies are shown – the original v0 assembly and the *C. adamanteus* scaffolded version (v1).

and northern Sonora, *C. w. obscurus* of southwestern New Mexico, southeastern Arizona, and adjacent northern Mexico, *C. w. silus* from Sonora and Chihuahua south to Durango, *C. w. amabilis* of the Sierra del Nido in Chihuahua, and *C. w. meridionalis* from Durango south to Jalisco (Rautsaw et al. 2022). Only one of the subspecies, the New Mexico Ridge-nosed Rattlesnake (*C. w. obscurus*), has received focused study (Holycross and Douglas 2007, Holycross et al. 2002, Smith et al. 2001). Additionally, the only phylogenetic study on *C. willardi* was completed in 1992 utilizing morphological and biochemical (allozymes) data (Barker 1992), which supported the five currently described subspecies. Although these five subspecies were, at the time, separated due to morphological differences, statistical reevaluation of phenotypes were found to be consistent with the allozyme diversity (Barker 1992). Since then, mitochondrial DNA (mtDNA) and phylo-transcriptomic data sets have been captured from *C. willardi* for inclusion in large-scale phylogenies, but these studies have been limited in number and geographic spread of sample collection (Murphy et al. 2002; Holding et al. 2021; Myers et al., 2024).

Here, we leverage comprehensive geographic sampling, a genomic approach (ddRAD-Seq), and a draft genome assembly to examine the diversity and evolutionary history of *C. willardi* across its range in the Madrean Archipelago and Sierra Madre Occidental. The overarching goals are to (1) generate the first draft chromosomal-level genome, (2) identify the level of genetic diversity and differentiation among groups within the complex, (3) test for evidence of recent or historical gene flow, and (4) estimate relationships and divergence times of major groups. We discuss the biogeographic and demographic history of the complex and potential implications for future conservation both of the diversity within this complex and of natural corridors of population connectivity in the imperiled habitats of the Madrean mountains.

2. Methods

2.1. Sampling and DNA Extraction

A total of 56 samples were collected from across the range of *C. willardi* in the Sierra Madre Occidental (Table S1), encompassing the Mexican states of Sonora, Chihuahua, and Durango, and the Madrean Archipelago, including SW New Mexico and SE Arizona. Sampling contained multiple representatives of each currently recognized subspecies (Barker 1992, Rautsaw et al. 2022). In addition, four outgroup samples were included from *C. pricei*, a close relative of *C. willardi* (Holding et al. 2021; Myers et al. 2024). Genomic DNA was extracted using DNeasy Blood and Tissue kit following the protocols provided by the manufacturer (Qiagen, Valencia, CA, USA). We checked for DNA quality on a 1 % agarose gel and ensured a minimum quantity of 20 ng/ μ L using a Nanodrop 2000 (Thermo Scientific, Waltham MA). Genomic DNA for genome sequencing was extracted using a Monarch HMW DNA Extraction Kit (NEB, Ipswich, MA, USA).

2.2. Genome assembly

A single sample from the center of the *C. willardi* distribution was chosen as the reference genome individual (CLP2257; *C. willardi silus*). The University of Delaware Sequencing and Genotyping Center performed PacBio HiFi Sequencing on a single Sequel IIE 8 M SMRT Cell. HiFi reads were assembled with hifiasm v0.18.9 (Cheng et al. 2021). This initial draft assembly (v0) was used to map the ddRAD-Seq reads and call variants. We later noticed some misassembled toxin gene complexes (Rosales-García unpublished) and generated a second draft assembly (v1) by running the original assembly through `purge_dups` (Guan et al. 2020) and scaffolding the genome to the chromosome-level *Crotalus adamanteus* genome assembly (Hogan et al. 2024) using RagTag v2.1.0 (Alonge et al. 2022). BUSCO v5.2.1 (Manni et al. 2021) was run using the tetrapoda_odb10 database to assess completeness.

2.3. ddRAD-Seq library preparation

We conducted double digest restriction-site associated DNA sequencing (ddRAD-seq) library preparation (Peterson et al. 2012) following protocols outlined in DaCosta & Sorenson (2014; also see Lavretsky et al. 2015), but with fragment size selection following Hernández et al. (2021). Briefly, genomic DNA was enzymatically fragmented using SbfI and EcoRI restriction enzymes, and Illumina TruSeq compatible barcodes were ligated to the sticky ends generated for demultiplexing purposes. The libraries were quantified, pooled in equimolar concentrations, and the multiplexed library was sequenced on Illumina HiSeq X using single-end 150 bp chemistry at Novogene (Novogene CO., Ltd., Sacramento, CA, USA).

We processed raw Illumina reads using a custom in-house computational pipeline (Python scripts available at <https://github.com/jon-mohl/PopGen>) described by Lavretsky et al. (2020) which automated sequence filtering, alignment, and genotyping using a combination of trimmomatic (Bolger et al. 2014), Burrows-Wheeler aligner v07.15 (Li and Durbin, 2011), and samtools v1.7 (Li and Durbin, 2009; Li H, Durbin R 2009). We aligned quality reads to the *C. willardi* v0 draft genome (see above).

2.4. Population structure analyses

Nuclear population structure was assessed across samples using an independent set of bi-allelic SNPs filtered in PLINK v.1.90 (Purcell et al., 2007) for singletons (i.e., minimum allele frequency (`--maf` 0.017) and any SNP missing ≥ 20 % of data across samples (`--geno` 0.2), as well as any SNPs in linkage disequilibrium (LD) (`--indep-pairwise` 2 1 0.5).

First, we estimated shared ancestry across all individuals using fineRADstructure (Malinsky et al. 2018), which includes the programs RADpainter version 0.1 and finestructure (Lawson et al. 2012). fineRADstructure infers a matrix of co-ancestry coefficients based on the distribution of identical or nearest neighbor haplotypes among samples. When individuals share haplotypes with multiple other individuals at a locus, their co-ancestry is equally divided among those individuals. The co-ancestry index is therefore more heavily influenced by rare alleles, which tend to reflect recent alleles and capture recent shared ancestry. We used the R scripts `fineradstructureplot.r` and `finestructurelibrary.r` to visualize results.

Next, we inferred population assignments across individuals using the ADMIXTURE v1.3 (Alexander et al. 2009, Alexander and Lange 2011) program. ADMIXTURE analyses were across K populations between 1 and 10. The optimum K was selected based on the average of cross-validation errors across the iterations per K value, but other values of K were evaluated to assess whether additional structure exists that makes sense geographically. Standard errors were obtained across analyses based on a 100 bootstrap values.

2.5. Phylogenetic tree Estimation and divergence history

We estimated phylogenetic trees using both concatenated and coalescent approaches. We first estimated a tree from the concatenated, independent SNPs in a Maximum Likelihood (ML) framework using RAxML (Stamatakis, 2014) through the Cipres portal (RAxML-HPC BlackBox 8.2.12 on XSEDE, Miller et al. 2010). We used a fast ML algorithm (`-f d`), GTR + G substitution model, and 100 bootstrap replicates, with 20 starting trees (10 random, 10 parsimony), and using the Lewis method for ascertainment bias correction. We also inferred a tree of all individuals using SVDquartets (Chifman and Kubatko 2014) with a random sample of 2,000,000 quartets, 100 bootstrap replicates, and *C. pricei* set as the outgroup. Specifically, we converted SNPs to a nexus alignment with two alleles per individual. The tree was inferred by partitioning based on the individuals (coalescing alleles from the same individual together). A majority rule consensus tree was inferred from the bootstrap replicates.

To infer a “species tree” of the ADMIXTURE-identified populations, we used SNAPP (Bryant et al 2012) and SNAPPER (Stoltz et al., 2020) in the Beast2 package (Bouckaert et al. 2019). Input files for SNAPP and SNAPPER were prepared with the ruby script `snapp_prep.rb` (Stange et al., 2018). We conducted short initial runs to benchmark the computational tractability of our dataset with both methods. We found that our full dataset containing all SNPs and individuals was not computationally tractable with either method. SNAPP was too slow with all individuals but could handle the full set of SNPs when each genetic group (from ADMIXTURE) was reduced to one individual (Table S2). The individual with the most complete set of SNP genotypes per population was selected. After removing invariant sites from the reduced matrix, 7,621 biallelic sites remained. SNAPPER more effectively scaled to larger numbers of samples but only ran reasonably efficiently if the set of loci was reduced. We ran SNAPPER with all individuals but with 1000 randomly selected SNPs. We used DensiTree to visualize the posterior distribution of inferred trees (Bouckaert and Heled 2014). We time-calibrated the SNAPP and SNAPPER trees using the divergence time between *C. pricei* and *C. willardi* from Myers et al. (2024). We placed an age constraint on that node using a lognormal distribution with a mean of 6.9756 Mya and sd of 0.05 (approximating the range of uncertainty based on the CI from that study).

Treemix version 1.2 (Pickrell et al. 2012) was used for an additional estimate of phylogenetic topology as well as historical gene flow. Treemix jointly estimates a Maximum Likelihood species tree with gene flow vertices, measured by direction and weight (w), that best explains the allele frequencies among groups. We evaluated uncertainty in the Treemix topology by running 100 bootstrap replicates using code from <https://github.com/mgharvey/misc.python>.

To better contextualize the divergence history of the group, we used an existing species distribution model from VenomMaps (Rautsaw et al. 2022) as a cost surface in a least-cost path (LCP) analysis. We modeled low-suitability environments as higher traversal costs. To model LCPs over the divergence history of the group, we followed Flannery-Sutherland et al. (2025) in generating sequential LCPs for each divergence event in the phylogeny – first between pairs of sister taxa (e.g., *C. w. obscurus* → *C. w. willardi*) and then subsequently between this LCP and the next most closely related taxon (e.g., [*C. w. obscurus* – *C. w. willardi*] → *C. w. silus 2*).

3. Results

3.1. Genome Statistics

The initial genome assembly (v0) had a size of ~ 1.8 Gbp with 3,682 contigs and a N50 of 1.44 Mb. BUSCO found the genome was 95.1 % complete with 78.5 % single-copy and 16.6 % duplicated (Fig. 1). This assembly was used to call ddRAD-Seq variants. The subsequent v1 genome assembly – which was run through `purge_dups` and `RagTag` scaffolding – had a size of 1.57 Gbp with 37 scaffolds (2,286 contigs with 0.014 % gaps) and an N50 of 204.87 Mbp. BUSCO found this genome maintained high completeness at 95.2 %, but had much higher single-copy (94.4 %) and lower duplicate (0.8 %) orthologs (Fig. 1).

3.2. Sampling and sequencing

We recovered a total of 5,621 ddRAD-seq loci (743,533 base-pairs (bp)), including 5,253 autosomal (693,854 bp) and 305 Z-linked (41,836 bp) loci, that met our criteria for sequencing coverage and missing data (Supplementary Materials Fig. S1). We attained a median depth of 145x per locus (range: 41–320x across samples). In addition, we were able to recover 63 W-linked (7843 bp) loci and used these to confirm sex by the presence (ZW female) or absence (ZZ male) of data from these loci. After filtering for minimum allele frequency and linkage, the final dataset used for inference contained 10,670 SNP sites.

3.3. Population structure

The coancestry matrix revealed five major genetic clusters, two of which contained additional sets of individuals with high coancestry (Fig. 2). The optimum number of populations (K) from ADMIXTURE analysis was seven (Fig. 2, Table S3). These seven groups show strong correspondence with the sets of related individuals observed in the coancestry matrix. *Crotalus willardi willardi*, *C. w. obscurus*, and *C. w. amabilis* formed distinct populations largely aligning with current subspecies distributions (Fig. 2, Table S3). Meanwhile, *C. w. silus* and *C. w. meridionalis* were split into two populations each. For ease of description, each population for *C. w. silus* and *C. w. meridionalis* can be identified latitudinally (Fig. 3). We will define southernmost populations of each as *meridionalis 1* and *silus 1* and the northernmost populations as *meridionalis 2* and *silus 2* based on a hypothesized south-to-north expansion from the central Mexican highlands (Fig. 3). The *C. w. silus 2* population shared relatively higher co-ancestry with the *C. w. willardi* and *C. w. obscurus* populations compared to the remaining populations (Fig. 2, Table S3). Interestingly, the *C. w. meridionalis 1* and *C. w. silus 1* populations shared higher coancestry to each other than with other populations of their respective subspecies (Fig. 2, Table S3). Additionally, despite large geographic distance, the *C. w. meridionalis 2* populations shared high coancestry with the *C. w. amabilis* population (Figs. 2 and 3). Overall, the southern part of the distribution showed deeper and more complex divergences.

3.4. Phylogenetic trees and divergence times

Phylogenetic analysis of concatenated SNPs using RAxML and of random quartets using SVDquartets were largely concordant (Fig. 3 & Fig. S1). Both methods produced a well-supported topology in which three of the five *C. willardi* subspecies are monophyletic: *C. w. amabilis*, *C. w. obscurus*, and *C. w. willardi*. The remaining two subspecies, *C. w. meridionalis* and *C. w. silus*, were polyphyletic matching the population structure identified by ADMIXTURE and coancestry analysis. The primary difference between the RAxML and SVDquartets phylogeny occur at the first diverging lineages. RAxML places the *C. w. meridionalis 1* and *C. w. silus 1* populations as sister taxa with low support (28 % BS; Fig. S1) and both sister to the remaining populations while SVDquartets places these as independent branches with *C. w. meridionalis 1* branching first and *C. w. silus 1* branching after with high support (100 % BS; Fig. 3). The next branch consists of sister populations *C. w. amabilis* and *C. w. meridionalis 2* which together form the sister group to *C. w. silus 2*, *C. w. willardi*, and *C. w. obscurus*. The only other major difference between RAxML and SVDquartets inferred phylogenies is that RAxML found *C. w. silus 2* to be monophyletic while SVDquartets found this population to be paraphyletic with the two northernmost individuals assigned to this population branching independently. The final group consisted of *C. w. willardi* and *C. w. obscurus* as sister to each other, which together were sister to *C. w. silus 2* (Fig. 3 & Fig. S1).

Both Bayesian analyses in SNAPP (5.5 million generations) and SNAPPER (4.4 million generations) converged based on ESS values > 300 and visual inspection of the traces. Both SNAPP and SNAPPER analyses recovered step-wise divergences at the tree's base with *C. w. meridionalis 1* and then *C. w. silus 1* diverging from the lineage leading to all other groups. The species tree from SNAPP (Fig. 4) with only one sample per genetic group estimated a similar topology to the RAxML and SVDquartets tree.

However, support was low for the sister relationship between *C. w. obscurus* and nominate *C. w. willardi* (PP = 60.0 %). The species tree from SNAPPER with all individuals but a reduced set of 1000 SNPs instead placed *C. w. obscurus* as sister to *C. w. silus 2* individuals with full support (Fig. 4). DensiTree (Bouckaert and Heled 2014) plots reveal support in the posterior distribution of SNAPP trees for both topologies, whereas the SNAPPER trees support only the latter topology (Fig. 4). Treemix inferred the same topology as SNAPP, RAxML, and SVDquartets

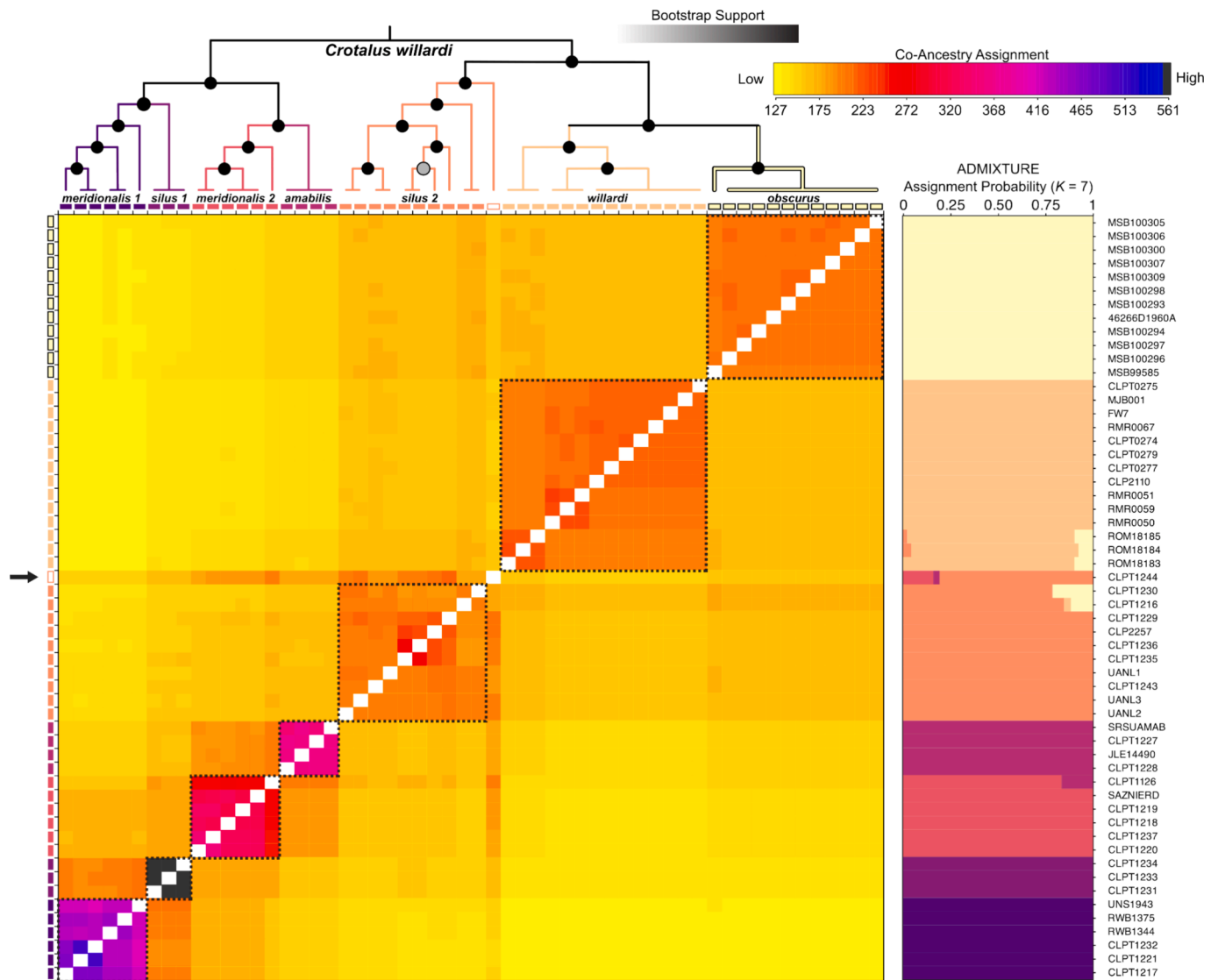


Fig. 2. (Left) FineRADstructure coancestry matrix with yellow representing the lowest amount of coancestry and black representing the highest. Dotted boxes highlight the ADMIXTURE-identified populations. (Top) Coancestry-based dendrogram with nodes labeled with their respective bootstrap support. (Right) The results of ADMIXTURE ($K = 7$) for each individual. Colors in the ADMIXTURE and dendrogram represent the seven populations identified and are consistent throughout. The black arrow to the left of the coancestry matrix highlights the hybrid found via genetic analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. S2). The Treemix model indicated that 98 % of the variation in gene trees can be explained by vertical transmission without gene flow. Given that SNAPPER only used a subset of SNPs and the results of RAXML, SVDquartets, SNAPP, and TreeMix were concordant, we refer to this topology as the “consensus topology.”

Divergence times in the time-calibrated SNAPP and SNAPPER trees ranged from 0.5 to 2.5 mya. The oldest estimated divergences occurred with *C. w. meridionalis 1* and *C. w. silus 1* splitting approximately 2.5 and 2 mya respectively. The rest of the clade has stepwise splits with *C. w. meridionalis 1* and *C. w. amabilis* branching at 1.4 mya, then diverging from each other around 1.1 mya. The final three groups, which were the source of some topological incongruence between SNAPP and SNAPPER, split at roughly the same time between 0.6 and 0.5 mya.

Phylogeographic LCP analysis indicated that sister taxa were generally separated by intervening habitat that provides potential colonization routes (Fig. S3). *C. w. meridionalis 1* and the taxa in its sister clade are linked by a least-cost path along the western front of the Sierra Madre Occidental, with subsequent divergences separated by paths leading northeastward across Barranca del Cobre. This central region is

then linked to more recently diverged lineages by least-cost paths to both the south (*C. w. meridionalis 2*) and north (remaining taxa).

4. Discussion

4.1. Population differentiation and distribution of genetic groups

Our results reveal evidence of a high level of intraspecific diversity within *C. willardi* that does not correspond closely with established subspecific taxonomy. Of the five currently recognized subspecies, only three were monophyletic. The other two occurred polyphyletically in our trees, with the groups separated by deep divergences involving well-supported nodes. This contrasts with current taxonomic treatment and prior work that supported the monophyly of the existing subspecies (Barker 1992). The split in populations previously treated as *C. w. meridionalis* is most striking as these lineages seemingly diverged ~ 2.5 mya. In particular, *C. w. meridionalis 1* individuals are found at the southernmost part of the distribution and appear as an early branch in the inferred phylogenies, while the remaining *C. w. meridionalis 2*

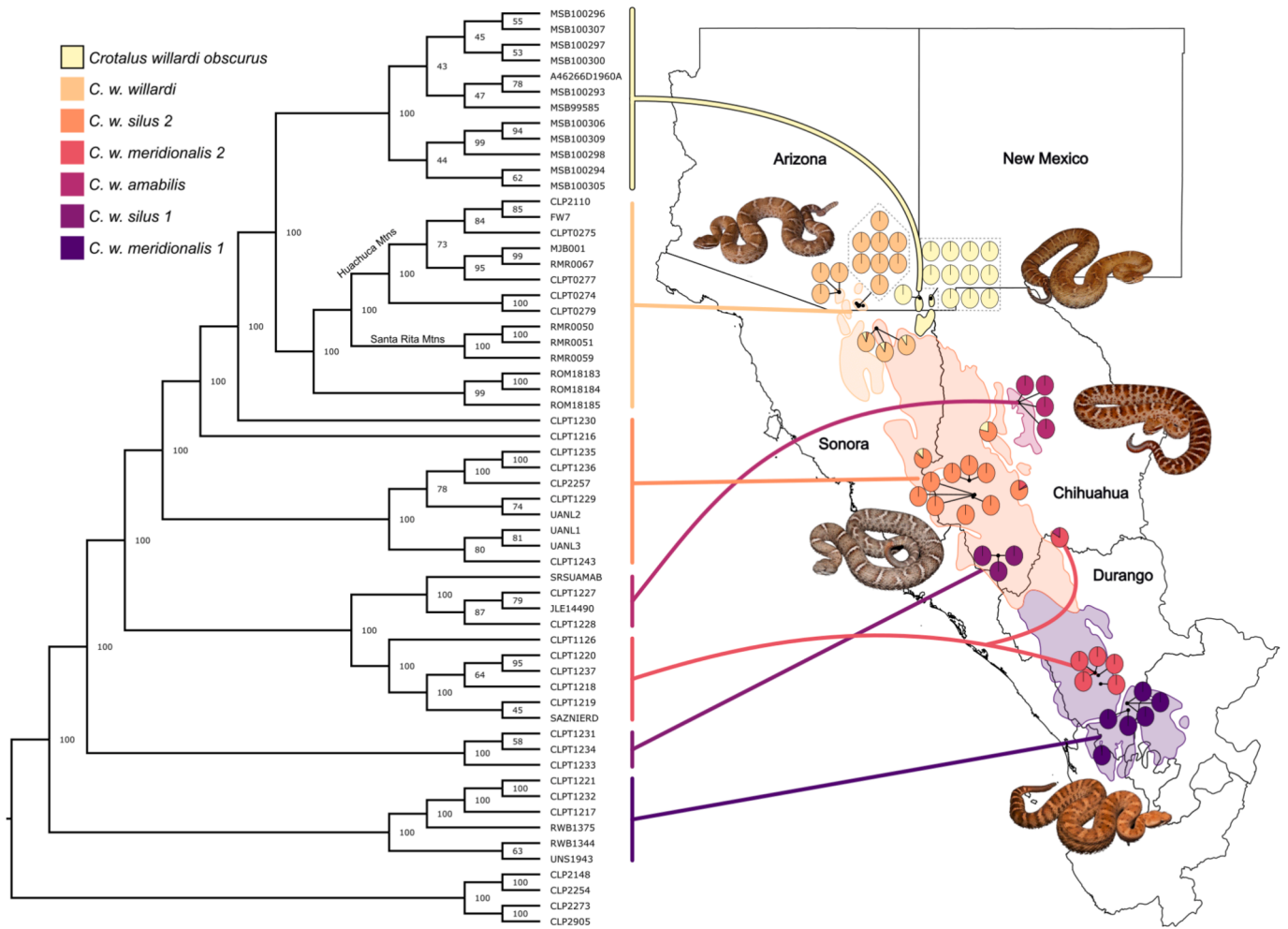


Fig. 3. SVDquartets cladogram depicting the relationships of individuals and ADMIXTURE-identified populations. *C. w. willardi*, *C. w. obscurus*, and *C. w. amabilis* form monophyletic clades, while *C. w. silus* and *C. w. meridionalis* are polyphyletic. The phylogeny is rooted using *C. pricei* as an outgroup and nodes are labeled with bootstrap values. Pie charts at each locality depict the proportion of ancestry assigned to each of the seven groups.

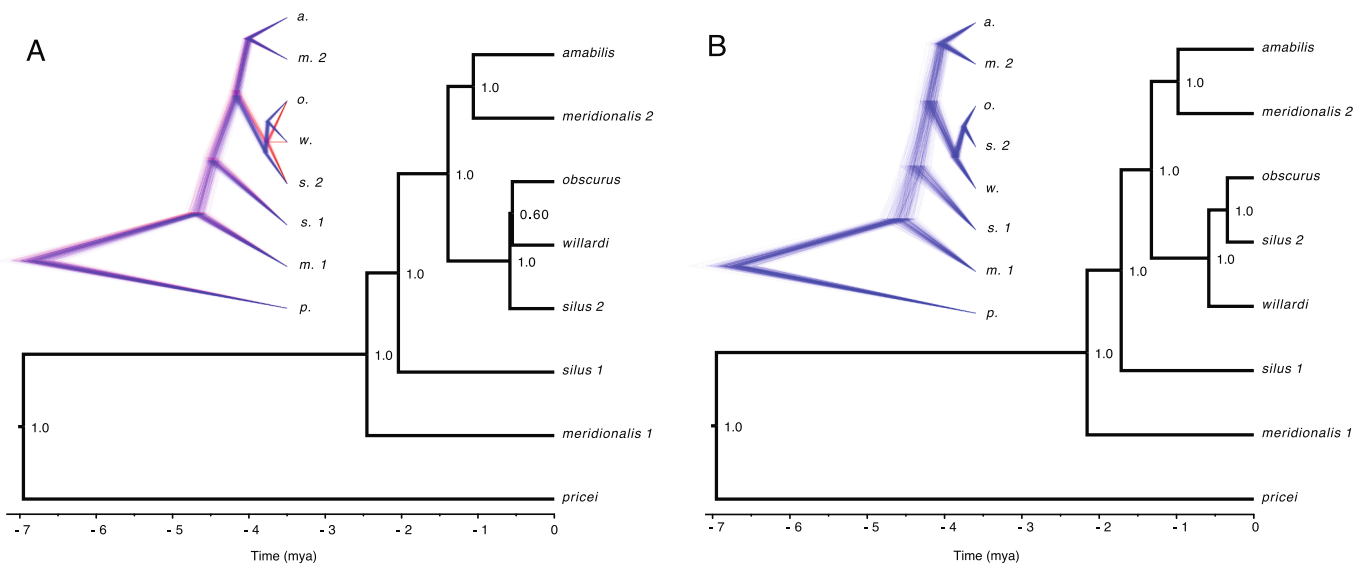


Fig. 4. Trees based on Bayesian analysis of SNP data accounting for coalescent stochasticity: (A) SNAPP tree based on all SNPs but one individual per genetic group (from Admixture), (B) SNAPPER tree based on a random subset of 1000 SNPs but including all individuals. Inset DensiTree plots depict the posterior distribution of SNAPP/SNAPPER trees with red representing alternate but less supported topology. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

individuals fall out sister to, although deeply diverged from, *C. w. amabilis*. These two groups – found in the southernmost part of *C. willardi* range – are separated by the Rio San Pedro Mezquital river valley. Clearly, these two groups are not closely related despite prior monotypic treatment due to perceived phenotypic similarities (Barker 1992). Similarly striking, the split of populations previously treated as *C. w. silus* (by range), is only slightly less deep at ~ 2 mya. Here, the *C. w. silus 1* is inferred as another long branch that diverged shortly after the basal split of *C. w. meridionalis 1* while *C. w. silus 2* population is found sister to *willardi + obscurus*. These two *C. w. silus* populations occur in the Sierra Madre Occidental proper and are separated by breaks in suitable habitat and elevation (Fig. 5). Their affinity is based on range alone (Barker 1992, Rautsaw et al. 2022); they do, however, closely resemble the *meridionalis/amabilis* phenotypes (anecdotally, morphological analysis is needed). This cryptic diversity highlights the need for additional taxonomic and genetic work in the region and the importance of conservation in the Sierra Madre Occidental.

There are also regions where the most recent mapping of subspecies distributions does not align with our genetic classifications. Of note, *C. w. meridionalis 2* extends much further north than previous estimations (Fig. 3, Rautsaw et al. 2022). Additionally, we see part of the Madrean Archipelago at the northernmost part of the range is assigned to *C. w. willardi* and not *C. w. silus* as previously predicted. These insights highlight the improvements that can be made to knowledge of reptile distributions based on spotty occurrence data and phenotypes using well-sampled genetic analyses.

4.2. Evidence of gene flow and hybridization

The Treemix model indicated that most of the genetic variation could

be explained without gene flow, suggesting that recent gene flow has been limited. The coancestry analysis further supports this, as there is limited recent coancestry between groups. However, one putative intergrade individual was identified which can be most clearly seen in the coancestry plot (Fig. 2, black arrow). While assigned to *C. w. silus 2* by ADMIXTURE, this individual is a putative intergrade between *C. w. silus 2* and *C. w. meridionalis 2* – collected from the eastern edge of the *C. willardi* distribution in Chihuahua – as well as the southern edge of *C. w. silus 2* and northern edge of *C. w. meridionalis 2*. This is the only evidence we found of recent introgression, but there is evidence of ancient hybridization among subspecies from several sources. In particular, the ADMIXTURE analysis shows that *C. w. silus 2* has low levels of admixture in many individuals. As *C. w. silus 2* shares borders with several of the other genetic groups seen within the continuous Sierra Madre Occidental, this fits with the idea that present-day biogeography is a strong predictor of even historical gene flow (Singhal et al. 2021). Additionally, there may have been ancient hybridization between the three northernmost subspecies resulting in conflicting support for two topologies in that group in our phylogenetic analyses. Further sampling in the Madrean Archipelago within northern Chihuahua and Sonora is needed to evaluate support for ancient and perhaps even recent admixture.

4.3. Divergence times and hypothetical biogeographic history

The deepest divergences within *C. willardi* primarily involve the southern subspecies. The northern clade including *C. w. obscurus*, *C. w. silus*, and *C. w. willardi* diverged relatively recently (< 0.6 mya). This supports a general historical scenario in which *C. willardi* lineage originated towards the southern end of its range and migrated northward

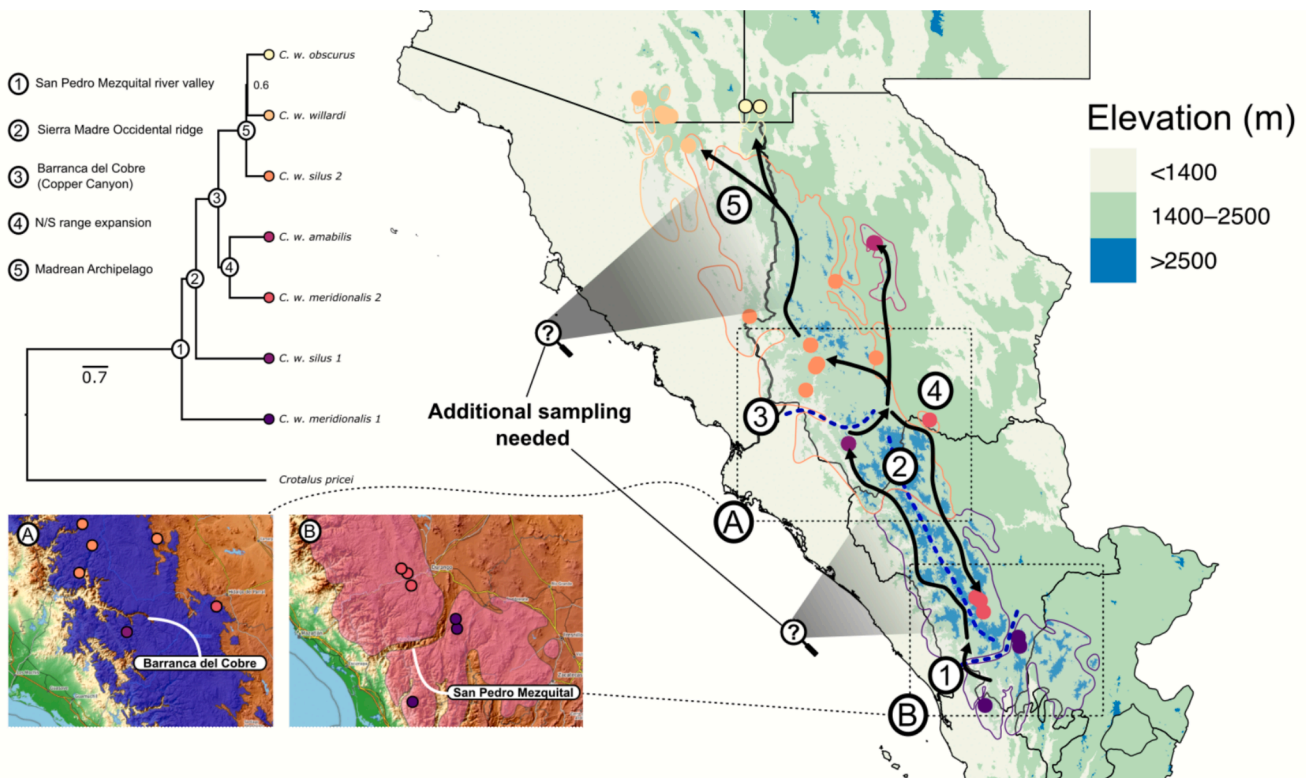


Fig. 5. Summary of a biogeographic hypothesis for *C. willardi* based on phylogeographic least-cost path analysis. SNAPP phylogeny annotated with hypothesized biogeographic events are also shown on the map to the right. Optimal elevation for *C. willardi* occurs between 1400–2500 m – suggesting the Sierra Madre Occidental ridge forms an east–west biogeographic break south of the Barranca del Cobre valley. The San Pedro Mezquital and Barranca del Cobre valleys form additional biogeographic breaks that can explain the phylogenetic divergence in this species (inset maps display zoomed in regions). Finally, the Madrean Archipelago likely facilitates additional divergence in the north. Additional sampling is needed along the southwestern Sierra Madre Occidental and northern Chihuahua and Sonoran regions.

with the most recent groups occupying the northern parts of the Madrean Archipelago. Other studies on reptiles in the region have found similar patterns in which older diversity is concentrated in the southern portion of species distributions (e.g., Bryson et al. 2011a,b; Bryson et al. 2012a; Wood et al. 2011).

However, our results also add complexity to this general biogeographic model. Some groups are separated on an east–west axis, with *C. w. meridionalis* 2 occurring to the east of *C. w. silus* 1. This break may be due to the presence of higher elevation habitat at the crest of the Sierra Madre Occidental ridge, subdividing appropriate habitat for *willardi* on either slope (Fig. 5). Similar east–west breaks along the Sierra Madre Occidental have been seen in other taxa (Smith & Lemos-Espinal 2025, Kobelkowsky-Vidrio et al. 2014). Phylogenetic relationships reveal further complexity, with taxa in close proximity sometimes distantly related from one another. For example, at the southern end of the range, *C. w. meridionalis* 1 is in close geographic proximity to the relatively distantly related *C. w. meridionalis* 2. They appear to be separated by the San Pedro Mezquital River, a putative biogeographic break hypothesized for several other codistributed montane taxa (Azzinnari et al. 2021; Bryson et al. 2011a, 2012b; Fig. 5). Further north, much of the intervening areas between sister taxa *C. w. meridionalis* 2 and *C. w. amabilis* are occupied by more distantly related *C. w. silus* 2, which now occurs in close proximity to early diverging *C. w. silus* 1, albeit separated by the Barranca del Cobre system (Fig. 5). Similar patterns involving interwoven early- and late-diverging populations been found in reptiles and amphibians occurring in other, similarly (e.g., Warwick et al. 2021, Joger et al. 2009).

Modeling biogeographic history and identifying ancestral areas is challenging, particularly with small clades in complex environments (Ree & Smith, 2008; Sanmartín & Ronquist, 2004; Lamm et al., 2016; Matzke, 2013, 2014). Beyond the hypothetical general northward expansion and potential complex history in the central Sierra Madre Occidental described above, we are reluctant to speculate on the details of the biogeographic history of *C. willardi*. However, the combination of divergence history and the sequential least-cost path analysis (Fig. S3) provides some substantiation of a general set of potential colonization paths across the species' range (Fig. 5). They indicate a potential path for an early northward expansion across the San Pedro Mezquital river valley and along the western Sierra Madre Occidental, with the ridge restricting eastward expansion. The Barranca del Cobre canyon system may have prevented further expansion northward, whereas suitable elevations to the east may have enabled eastward expansion and subsequent expansion to both the north and back south along the eastern edge of the Sierra Madre Occidental. These putative paths around the upper elevations of the Sierra Madre and the Barranca del Cobre could explain the current proximity of deeply diverged populations. Finally, once north of Barranca del Cobre, we hypothesize *C. willardi* likely expanded westward and northward in a broad front through the northern Sierra Madre Occidental to the Madrean Archipelago, the site of recent expansion and divergence. Additional sampling, particularly surrounding putative biogeographic breaks involving the San Pedro Mezquital river, Sierra Madre Occidental ridge, Barranca del Cobre, and Madrean Archipelago, may help to further substantiate and add detail to the hypothetical scenario outlined above.

4.4. Conservation implications and Conclusions

Together, our results show a much more complex evolutionary history and population structure than previously thought. This includes genetically unique populations that were previously unrecognized or that are narrower in distribution than expected, as we found in *C. w. meridionalis* and *C. w. silus*. The relatively low levels of gene flow observed highlight the isolation of populations in this group and their potential susceptibility to demographic challenges. Some of the populations identified here may face threats and outlooks as severe as those facing the better-studied and ESA-listed (Threatened) *C. w. obscurus*

(Davis et al. 2015, Holycross and Douglas, 2007). Moreover, alterations to habitat, environmental change, or even human-mediated movement may alter distributions and patterns of gene flow in unpredictable ways. Given the elevated risk of extinction to montane species across the globe associated with climate change (Urban 2018, La Sorte and Jetz 2010), better understanding of all groups within *C. willardi* is an urgent need.

We did not perform a formal species delimitation analysis or assessment of the taxonomic status of *C. willardi*. Recent herpetological work has highlighted the challenges associated with disentangling species from intraspecific geographic structure (e.g., Chambers et al. 2025; Prates et al. 2024; Pyron et al. 2024; Chaitanya et al. 2025). Cryptic diversity, deep and varying genetic structure, and limited gene flow certainly make *C. willardi* a prime candidate for contribution to this debate. That said, we echo the concern (Stanton et al. 2019) that splitting charismatic taxa like some of those within *C. willardi* should be done carefully to avoid taxonomic inflation, which could lead to inadequate conservation of other, similarly precarious montane populations. Future efforts should attempt to fill gaps in our sampling, particularly in potential contact zones and in sampling gaps in the southern portion of the range. Regardless of their status, we suggest that the evolutionary diversity, both phenotypic and genetic, within *C. willardi* merits additional work and conservation consideration.

In conclusion, we were able to assemble a new genome, identify genomic differentiation among populations, assess levels of gene flow, and estimate the relationships and divergence times among groups in an enigmatic montane snake species. Our results revealed up to seven genetically differentiated populations with mixed correspondence to previous taxonomic treatments, particularly in the subspecies *meridionalis* and *silus*. We found support for limited but potentially important recent introgression and ancient gene flow between certain lineages. Divergence dating suggests the deepest diversity is present in the central and southern part of the range of *C. willardi*. However, the complexity of the distributions of genetic groups and their relationships highlight the biogeographic complexity of the Sierra Madre Occidental and Madrean Archipelago and the need for a comprehensive consideration of diversity in conservation efforts.

CRedit authorship contribution statement

Michael J. Buontempo: Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Philip Lavretsky:** Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Rhett M. Rautsaw:** Writing – review & editing, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lauren M. McFarland:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Ramses Alejandro Rosales-García:** Writing – review & editing, Resources, Investigation, Formal analysis. **Jason L. Strickland:** Writing – review & editing, Resources, Funding acquisition. **Miguel Borja:** Writing – review & editing, Resources. **Jason Jones:** Resources, Methodology. **Ricardo Ramírez Chaparro:** Resources, Methodology. **Robert W. Bryson:** Writing – review & editing, Resources, Methodology. **Christopher L. Parkinson:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Michael G. Harvey:** Writing – original draft, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymp.2025.108522>.

Data availability

Data submissions to NCBI are pending (PRJNA1304146).

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