Phylogenomic Discordance is Driven by Wide-Spread Introgression and Incomplete Lineage Sorting During Rapid Species Diversification Within Rattlesnakes (Viperidae: *Crotalus* and *Sistrurus*)

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Abstract.—Phylogenomics allows us to uncover the historical signal of evolutionary processes through time and estimate phylogenetic networks accounting for these signals. Insight from genome-wide data further allows us to pinpoint the contributions to phylogenetic signal from hybridization, introgression, and ancestral polymorphism across the genome. Here, we focus on how these processes have contributed to phylogenetic discordance among rattlesnakes (genera *Crotalus* and *Sistrurus*), a group for which there are numerous conflicting phylogenetic hypotheses based on a diverse array of molecular datasets and analytical methods. We address the instability of the rattlesnake phylogeny using genomic data generated from transcriptomes sampled from nearly all known species. These genomic data, analyzed with coalescent and network-based approaches, reveal numerous instances of rapid speciation where individual gene trees conflict with the species tree. Moreover, the evolutionary history of rattlesnakes is dominated by incomplete speciation and frequent hybridization, both of which have likely influenced past interpretations of phylogeny. We present a new framework in which the evolutionary relationships of this group can only be understood in light of genome-wide data and network-based analytical methods. Our data suggest that network radiations, like those seen within the rattlesnakes, can only be understood in a phylogenomic context, necessitating similar approaches in our attempts to understand evolutionary history in other rapidly radiating species. [Anomaly zone; crotalinae; diversification; hemiplasy; introgression; phylogenomics; recombination.]

La filogenómica nos permite descubrir la señal histórica de los procesos evolutivos a través del tiempo y estimar redes filogenéticas tomando en cuenta estas señales. El conocimiento de datos genómicos incluso permiten distinguir la contribución de la señal filogenética de la hibridación, introgresión, y de polimorfismos ancestrales a lo largo del genoma. En este trabajo nos enfocamos en como estos procesos han contribuido a la discordancia filogenética entre las serpientes de cascabel, un grupo en el que hay numerosos conflictos en las hipótesis filogenéticas obtenidas de un grupo variado de datos moleculares y métodos analíticos. Nosotros abordamos la inestabilidad de la filogenia de las serpientes de cascabel (generos *Crotalus* y *Sistrurus*) usando datos genómicos generados de transcriptomas muestreados en la mayoría de las especies conocidas. Estos datos genómicos, analizados con métodos basados en coalescencia y redes filogenéticas, revelaron numerosos casos de especiación rápida donde los arboles de genes individuales conflictúan con el árbol de especies. Además, la historia evolutiva de las serpientes de cascabel esta dominada por una especiación incompleta y una frecuente hibridación, las cuales probablemente han influenciado interpretaciones pasadas de las filogenias. Nosotros presentamos un nuevo marco en el que las relaciones evolutivas de este grupo solo pueden ser entendidas en base a datos de genomicos y métodos analíticos basados en redes filogenéticas. Nuestros datos sugieren que la radiación en redes filogenéticas, como se ha visto dentro de las serpientes de cascabel, solo puede ser entendida en un contexto filogenómico, necesitando aproximaciones similares en nuestro intento de entender la historia evolutiva en otras especies con radiaciones rápidas. [Palabras claves: Introgresión; zona anómala; diversificación; filogenómica; hemiplacia; recombinación; Crotalinae.]

Robust phylogenies are fundamental for understanding processes such as trait evolution, speciation, and biogeography (Barraclough and Nee 2001; Ronquist and Sanmartín 2011). However, as genomic-scale datasets have accumulated, a major theme has been phylogenetic conflict between studies, often with these conflicting results being highly supported (Hackett et al. 2008; Jarvis et al. 2014; Shen et al. 2017). Even reanalysis of published datasets can produce conflicting topologies (Song et al. 2012; Gatesy and Springer 2014), highlighting the importance of the methodological approach employed and the underlying model assumptions (Linkem et al. 2016). Because of these observed discrepancies, exploring the causes of gene tree discordance is often necessary and has become common (Smith et al. 2015; Huang et al. 2017).

Discordance among gene trees can result from multiple sources stemming from both biological processes and methodological issues. For example, error or noise can be introduced during data assembly and filtering or via misspecified model parameters of molecular evolution (Cooper 2014; Liu et al. 2014). The key biological processes that can lead to incongruences among gene trees and conflict between gene trees and the species tree include gene duplication and/or loss, incomplete lineage sorting (ILS), and introgression (Pamilo and Nei 1988; Doyle 1992; Maddison 1997; Galtier and Daubin 2008). Of these biological processes, ILS as a source of discordance has been the focus of numerous phylogenomic studies since the introduction of multispecies coalescent methods (Edwards 2009). However, it is possible that multiple processes are responsible for gene tree heterogeneity in empirical studies (Cai et al. 2021; Owen and Miller 2022).

Rapid evolutionary radiations are exceptional systems for interrogating the sources of phylogenetic discordance because such radiations may be influenced by a high prevalence of both ILS and introgression (Suh et al. 2015; Singhal et al. 2021). During rapid speciation events, where there are two or more consecutive short internodes, it is possible that the most common gene tree topology does not match the species tree (Rosenberg 2013). Discordant gene trees of this kind are known as anomalous gene trees and the region of the species tree in which these gene trees correspond has been termed the anomaly zone (Degnan and Rosenberg 2006). Here, it is expected that unlinked loci from across the genome will have gene tree topologies that do not match the species tree topology, even though they are the most common gene tree topology (e.g., Rosenberg and Tao 2008; Degnan and Rosenberg 2009; Degnan et al. 2012; Degnan 2013). Simulation studies have identified when different phylogenetic approaches will recover correct or incorrect topologies under the anomaly zone. For example, concatenation of loci will result in incorrect phylogenetic topologies with strong support in regions of the anomaly zone (Mendes and Hahn 2018). Furthermore, the existence of the anomaly zone has now been shown empirically within many taxa (Linkem et al. 2016; Chafin et al. 2021; Morales-Briones et al. 2021; Owen and Miller 2022). Explicitly testing for the anomaly zone, particularly within groups that have rapidly radiated and have multiple conflicting phylogenies published in the literature is an important consideration for phylogenomic studies.

Once thought to be rare within most taxonomic groups, it is increasingly recognized that hybridization and introgression are widespread (Soltis and Soltis 2009; Payseur and Rieseberg 2016), and that patterns of gene tree discordance may also be driven by these processes. The timescale over which introgression can occur is broad, with examples ranging from recently diverged species (Nicaraguan Midas cichlid fishes <24,000 kya divergent; Olave and Meyer 2020) to taxa that diverged 10's of millions of years ago (*Drosophila*, 20–25 my divergent;

Suvorov et al. 2022). Introgression can serve as a source of genetic variation for local adaptation (Hedrick 2013; Suarez-Gonzalez et al. 2018), influence trait evolution (Ferreira et al. 2020; Karimi et al. 2020), and drive adaptive radiations via a combinatorial effect (Marques et al. 2019). Historical introgression can also lead to the inference of incorrect phylogenetic topologies, ultimately influencing interpretations of speciation and adaptation (Nelson et al. 2021; Myers et al. 2022). Multispecies coalescent network (MSCN) based approaches that scale with genomic datasets and explicitly account for both reticulation (i.e., the signature of introgression among the branches of a phylogeny) and ILS have recently been developed and are likely necessary to accurately reconstruct phylogeny in many groups (Solís-Lemus et al. 2017; Hejase et al. 2018; Wen et al. 2018; Zhang et al. 2018).

Study System

Rattlesnakes are an iconic and well-studied group of snakes, and yet the systematics of the group remains unresolved. This group contains 56 named species within 2 genera (*Crotalus* and *Sistrurus*; Wallach et al. 2014; Uetz et al. 2022). The total number of species has been debated and this dispute is best exemplified by the hypothesized number of species within the *C. viridis* species group; systematic studies have concluded that there are anywhere between 1 species with 9 subspecies and 7 species, with the possibility of multiple unnamed species-level lineages (Pook et al. 2000; Ashton and de Queiroz 2001; Douglas and Schuett 2002; Davis et al. 2016; Holding et al. 2021).

Despite the disagreement in total species numbers, the main rattlesnake species groups have largely remained consistent since early morphological evaluations. For example, early morphological studies identified several major taxonomic groups, including the *C. triseriatus*, *C. durissus*, and *C. viridis* groups (Gloyd 1940; Klauber 1956; Brattstrom 1964). The composition of these groups has been in flux with recent molecular phylogenetic studies (see Supplementary Table S1 for comparisons; Murphy et al. 2002; Reyes-Velasco et al. 2013). In addition, within *Crotalus*, there are 2 main morphological and ecological distinctions, namely, the smaller-bodied, montane taxa (e.g., *C. willardi*, *C. polystictus*, the *intermedius*, and *triseriatus* groups) and the heavy-bodied, primarily low-elevation species (e.g., the *atrox*, *durissus*, and *viridis* groups). But whether these ecologically similar species form reciprocally monophyletic lineages is unclear.

Studies using different datasets or methodological approaches have resulted in contradictory phylogenetic relationships among rattlesnake species (Murphy et al. 2002; Castoe and Parkinson 2006; Pyron et al. 2013; Reyes-Velasco et al. 2013; Zaher et al. 2019; Holding et al. 2021a; Carrasco et al. 2023). Some species groups have been supported by many of these studies; however, the placement of many lineages has been unstable between studies and therefore the phylogeny remains contentious (see Fig. 1). The extent
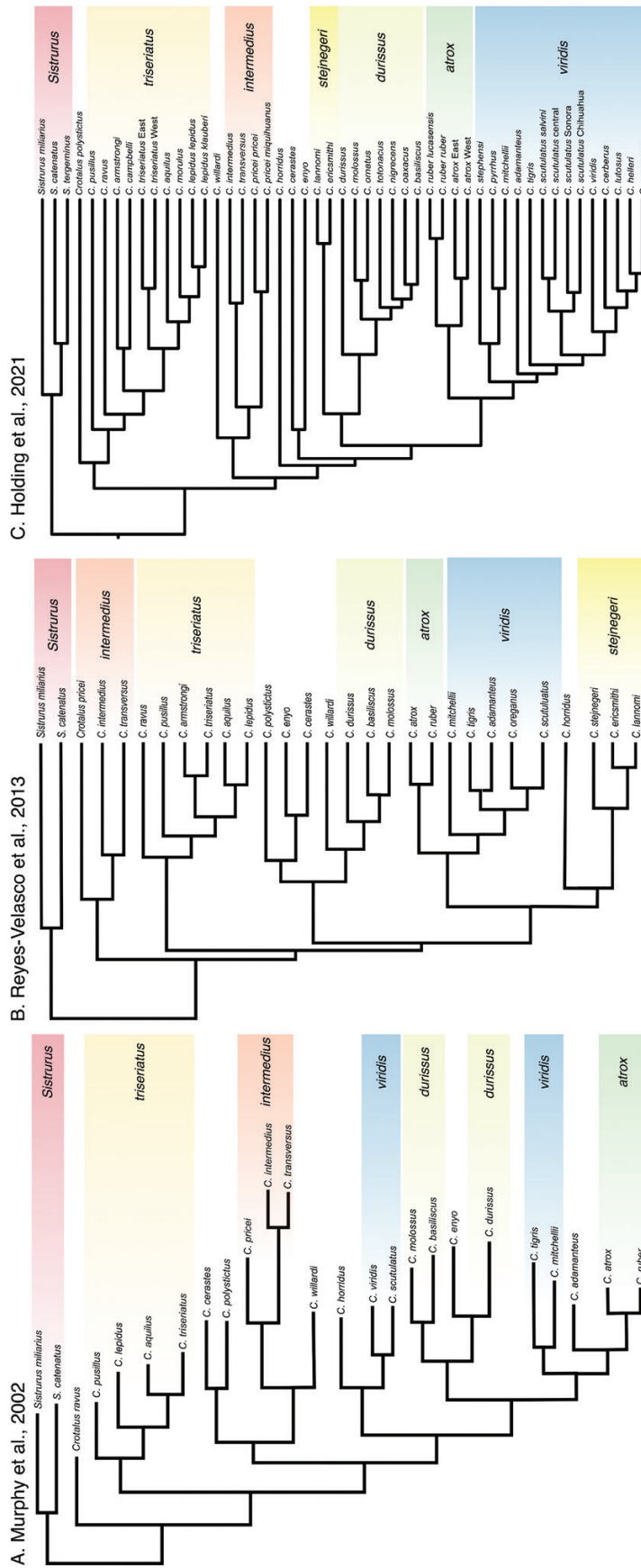
FIGURE 1. Comparison of previously published phylogenetic hypotheses with the species groups highlighted. a) The preferred phylogeny of Murphy et al. (2002) based on mtDNA analyzed with maximum parsimony. b) Species tree presented by Reyes-Velasco et al. (2013) based on 6 loci analyzed using *BEAST. c) Species tree from Holding et al. (2021a) reconstructed from RNA-seq using ASTRAL.

and biological basis of gene tree discordance has yet to be investigated in rattlesnakes. Previous phylogenetic estimates suggest that this group has rapidly diversified since the mid- to late Miocene (Reyes-Velasco et al. 2013; Alencar et al. 2016; Blair and Sánchez-Ramírez 2016), which may have resulted in a high prevalence of ILS. Additionally, introgression has been well-documented in some rattlesnake lineages, particularly the *C. viridis* species group (Meik et al. 2015; Schield, Perry, et al. 2019; Myers 2021). Because of these previous analyses, we suspect that both the presence of past introgression, high levels of ILS, and potentially the anomaly zone have influenced earlier attempts to infer phylogenetic relationships. Here, by incorporating MSCN approaches, we gain a more biologically realistic understanding of the evolutionary history and phylogenetic relationships within this group.

We demonstrate here that the process of rapid species diversification coupled with introgression has led to high levels of gene tree heterogeneity within rattlesnakes. These two processes have jointly affected the evolution of rattlesnakes and have likely influenced previous attempts to infer the phylogeny of this group. We suggest that the complement of these processes may be more pervasive than currently recognized, particularly within groups that have rapidly radiated, regardless of whether that diversification was in the distant past or on a more recent timescale. Therefore, these processes should be jointly considered in phylogenomic studies to better understand phylogenetic discordance and to infer accurate evolutionary relationships.

Materials and Methods

Sampling and Data Generation

We attempted to obtain samples from all rattlesnake species following Wallach et al. (2014) and Uetz et al. (2022). These two taxonomic authorities differ in the total number of species and some of these differences are because of the number of species newly named or elevated since the publishing of Wallach et al. (2014). We sampled 49 named species and several additional phylogeographic lineages for a total of 57 terminal taxa in our phylogenetic analyses. The phylogeographic lineages were sampled because previous analyses have identified them as distinct. For example, up to four lineages have been identified within the Mojave rattlesnake (*C. scutulatus*; Myers et al. 2017; Schield et al. 2018); therefore, we include all of these populations as distinct terminals. In addition, several of the terminal taxa that we use were identified as being monophyletic and deeply divergent in preliminary analyses of these data (e.g., the *C. triseriatus* eastern and western lineages). We have also included all named lineages within the Mexican and Central American *C. durissus* group that have recently been delimited or proposed to

be elevated (Carbajal-Márquez et al. 2020; Muñoz-Mora et al. 2022). The species listed by Uetz et al. (2022) that were not included here are *C. angelensis*, *C. catalinensis*, *C. concolor*, *C. estebanensis*, *C. lorenzoensis*, *C. polisi*, *C. tancitarensis*, *C. thalassoporus*, *C. unicolor*, and *C. vegrandis*. Many of these unsampled species are island endemics, belonging to known species complexes, and, therefore, should not influence our phylogenetic analyses.

We expanded on the phylogenomic data presented in Holding et al. (2021a) by combining their dataset with newly generated transcriptomes from venom gland or blood tissue from 51 additional *Crotalus* specimens, one *Sistrurus tergeminus*, and additional outgroups including three *Cerrophidion tzotzilorum* and six *Agkistrodon* specimens (Supplementary Table S2). In total, we analyzed 298 transcriptomes, 262 samples within *Crotalus* and *Sistrurus* (mean = 4.7 samples per species, range = 1–18), and 36 outgroup samples (mean = 3 specimens per species, range = 1–8). To root the phylogeny and for use in divergence dating, we included the following outgroup taxa, *Micrurus fulvius*, *Thamnophis conanti*, *Azemiops feae*, *Trimeresurus purpureomaculatus*, *Bothrops asper*, *Cerrophidion tzotzilorum*, *Agkistrodon laticinctus*, *Agk. contortrix*, *Agk. piscivorus*, *Agk. conanti*, *Agk. russeolus*, and *Agk. bilineatus* (Zaher et al. 2019).

Specimens were collected throughout the United States, Mexico, and Brazil through recent fieldwork efforts, or donated from USFWS. Blood or venom gland tissue was placed in RNAlater solution. Where permitted, associated voucher specimens were fixed in formalin and deposited in natural history collections (Supplementary Table S2). All samples were ultimately stored for long-term preservation at −80°C. RNA was isolated using a TRIzol extraction protocol following Rokyta et al. (2012). Briefly, tissue was placed in TRIzol solution (Invitrogen), the mixture homogenized, and transferred to phase lock heavy gel tubes (5Prime). After cells were lysed, total RNA was isolated via chloroform and purified via isopropyl alcohol and ethanol precipitation. RNA extraction quantity and quality were checked prior to library prep using a Bioanalyzer 2100 with an RNA 6000 Pico Kit (Agilent Technologies) or Agilent TapeStation 2200 with a RNA ScreenTape.

mRNA was isolated from ~1000 ng total RNA using the NEBNext Poly(A) mRNA magnetic isolation kit (New England Biolabs) and cDNA library preparation was immediately performed using NEB-Next Ultra RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer's protocols. Library quality was checked using an Agilent 2100 Bioanalyzer and concentrations checked via KAPA qPCR. Equal concentrations of samples were pooled in groups of 12 samples and were sequenced with 150 or 250 base pair paired-end reads on an Illumina HiSeq, NextSeq, or NovaSeq platform.

Sequence Data Assembly

We used ALiBaSeq (Knyshov et al. 2021) to identify and extract loci from the RNA-seq data. We extracted

three different types of loci, including (1) the "tetra-pod odb10" set of benchmarking universal single-copy orthologs (BUSCOs; Simão et al. 2015), (2) the squa-mate conserved loci set (SqCL; Singhal et al. 2017) that consists of ultra-conserved elements (Faircloth et al. 2012), anchored hybrid enrichment loci (Lemmon et al. 2012), as well as traditional Sanger loci, and (3) novel orthologous transcript sequences. Additionally, we extracted mitochondrial DNA sequence reads from the trimmed Illumina sequence data for all specimens using MitoSIS (https://github.com/RhettRautsaw/MitoSIS), a wrapper for mitochondrial genome assembly. See Supplementary Text S1 for details on data assembly.

Gene Tree and Species Tree Estimation

We estimated maximum-likelihood gene trees for the nuclear sequence data in IQ-TREE (v.2.2.0) (Minh et al. 2020). For each locus, we ran the standard model selection procedure implemented in IQ-TREE selecting the model with the lowest BIC score using a single model for each alignment (Kalyaanamoorthy et al. 2017), followed by gene tree inference with 1000 ultrafast bootstraps (Hoang et al. 2018). In addition, we estimated a concatenated species tree using all nuclear loci in IQ-TREE, partitioning the concatenated sequence by locus and running model selection by partition, selecting the best-fit module via BIC and assessed support with 1000 ultrafast bootstraps. This was repeated with the concatenated mtDNA sequence alignment.

We estimated an unrooted species tree using ASTRAL-MP (v5.15.5) (Yin et al. 2019). As input, we used our estimated nuclear DNA gene trees from above where all nodes with less than 10% bootstrap support were collapsed (Zhang et al. 2018). Collapsing of nodes was done using commands in the *phytools* and *ips* packages in R (Heibl 2008; Revell 2012). Phased alleles were then assigned to species using the multiple allele per species feature (Rabiee et al. 2019; see Supplementary Table S2 for specimen to species assignments). To test if any nodes of the phylogeny are best represented as polytomies instead of bifurcating nodes we used the quartet-based polytomy test implemented in ASTRAL (Sayyari and Mirarab 2018).

Species tree methods like ASTRAL provide branch support in terms of local posterior probabilities (PP). When analyzing large-scale genomic datasets PP often suggests near-complete confidence in support values, but this metric does not quantify the topological variation present in the underlying data (Ané et al. 2007; Minh et al. 2020; Thomson and Brown 2022). We, therefore, also estimated gene tree concordance factors (gCF) on our ASTRAL species tree topology using IQ-TREE (v.2.2.0) (Minh et al. 2020). This approach involves calculating the fraction of loci consistent with a particular branch and is capable of capturing the underlying agreement and disagreement in the data (Minh et al. 2020). Currently, IQ-TREE can calculate gCFs with missing terminals in the gene trees but not with multiple alleles per

species. Therefore, we dropped all but one allele from a single specimen per species with the least amount of missing data from the above-estimated gene trees. These gene trees were then used as input to IQ-TREE (v.2.2.0) to calculate gCF support on our species tree. This method will also calculate a discordant factor for the proportion of gene trees that match a second resolution of the four clades around the branch in the species tree (gDF1) as well as a third resolution of the four clades (gDF2).

To examine topological differences between the mtDNA gene tree, the full concatenated species tree, and the ASTRAL species tree we calculated the Robinson–Foulds (RF) distance values (Robinson and Foulds 1981). First, both the mtDNA and concatenated tree were reduced to a single allele representative of the species. These reduced trees were then compared to the species tree using normalized RF distances in the R package *phangorn* (Schliep 2011). We also calculated normalized RF distances between individual gene trees and the species tree. This was calculated only for gene trees in which there was at least one allele per species found in the ASTRAL species tree.

Studies have demonstrated that only a few loci can drive the topology of species trees (Shen et al. 2017). We, therefore, tested the sensitivity of our ASTRAL species tree topology based on locus alignment length and the number of phylogenetically informative sites. We chose these two parameters because of their ability to accurately estimate gene trees, where shorter loci and loci with fewer phylogenetically informative sites may introduce noise due to greater gene tree error (Burbrink et al. 2020). The total number of base pairs and phylogenetically informative sites were calculated for each alignment using *ape* and *ips* in R (Paradis et al. 2004; Heibl 2008). We then re-estimated a coalescent-based species tree in ASTRAL using the gene trees in the bottom 25% quantile and top 75% quantile of these two parameters. We compared the four species trees estimated from these subsets of data to the coalescent based species tree based on the full dataset using normalized RF distances.

Effects of Recombination on Species Tree Estimation

Recombination within loci can result in multiple site patterns within a locus and an assumption of coalescent based phylogenetic methods is that recombination occurs between loci but not within loci (Edwards 2009). Recombination hotspots are known to be concentrated within gene-rich regions of the genome within reptiles (Singhal et al. 2015; Kawakami et al. 2017; Schield et al. 2020) and because the loci analyzed here are extracted from RNAseq data it is possible that intralocus recombination is present which may contribute to gene tree to species tree discordance. To test if intralocus recombination has biased our coalescent species tree inference we first used the pairwise homoplasy index (Φ) to test for recombination as implemented in PhiPack (Bruen et al. 2006) with a sliding window size of 100 bp and a P-value threshold of 0.05. We then re-estimated a phylogeny in ASTRAL using only the subset of loci found

to have a significant pattern of recombination as well as those without a signature of intralocus recombination. The two resulting topologies were then compared using RF distances to the ASTRAL species tree based on all gene trees to test if either of these subsets of data has influenced our coalescent species tree inference.

Divergence Dating

We estimated divergence dates using the penalized-likelihood approach in TreePL (Smith and O'Meara 2012). First, we estimated branch lengths on the ASTRAL species tree topology using 1000 concatenated bootstrapped trees with a single allele representing each terminal present in the species tree using the IQ-TREE (v.2.2.0) rapid bootstrap function (Hoang et al. 2018). This was done only with the nuclear genomic data (i.e., the mtDNA was not used to estimate divergence times). With TreePL, we determined the best smoothing parameter that affects the penalty for rate variation over the tree using a cross-validation approach. We used the "random subsample and replicate cross-validation" method which randomly samples, with replacement, multiple terminals, recalculates rates and dates with these terminals removed and calculates the average error over the sampled nodes. We iterated this cross-validation procedure 10 times, with a lambda value from 0.001–1000, and set the "thorough" option to ensure that the run iterated until convergence. These parameters were then implemented to estimate 95% credibility intervals for divergence times over the 1000 bootstrapped replicates from IQ-TREE. The two fossil calibrations used in this analysis followed Zaher et al. (2019): (1) constraining the Colubroidea (sensu Zaher et al. 2009) stem clade minimum age to 35.2 mya and a maximum age of 54 mya between the outgroup specimens *Thamnophis conanti* and *Micrurus fulvius* (Smith 2013) and (2) the Crotalinae stem clade constrained at a minimum of 10.3 mya and a maximum age of 54 mya (Ivanov 1999). With this dated phylogeny, we then plotted a lineage-through time plot of only the genera *Crotalus* and *Sistrurus* using *phytools* in R (Revell 2012).

Detecting the Anomaly Zone

Consecutive speciation events can lead to short internode distances, increasing the occurrence of anomalous gene trees. The boundary of the anomaly zone, $a(x)$ as defined by Degnan and Rosenberg (2006), occurs where x, a species tree branch length in coalescent units, has a descendant internal branch (y), if the length of y is smaller than $a(x)$ then the internode pair is defined as being in the anomaly zone. We used the method developed by Linkem et al. (2016) to test if any branches of the rattlesnake phylogeny fall within this anomaly zone. This method requires a species tree with branch lengths in coalescent units and examines every parent–child internode, calculating whether each node falls within the expected anomaly zone (https://github.com/tkchafin/anomaly_zone; Chafin et al. 2021). We first reran an ASTRAL tree with the multi-locus

bootstrapping method (--gene-only option) to obtain 100 bootstrapped trees. The anomaly zone method was then run across this set of 100 trees to get bootstrap support for the presence of the anomaly zone across our phylogeny.

Introgression

To detect introgression across the rattlesnake phylogeny we used two approaches, a site-based summary statistic and an MSCN method. First, we used Patterson's *D* statistic (also known as the ABBA–BABA test; Green et al. 2010) in the package *Dsuite* (Malinsky et al. 2021), which estimates introgression on a fixed topology based on the expected frequency distribution of site patterns under ILS versus introgression. The *D* statistic assesses the number of shared bi-allelic sites (labeled as A or B alleles) across a 4-taxon statement (an outgroup and three ingroup species). Taxon 1 and taxon 2 are most closely related and taxon 3 has a derived allele (allele B), given introgression or ILS there are two possible configurations in which the derived allele is shared between taxon 1 or 2 and taxon 3 (((A, B), B), A) or (((B, A),B), A). If ILS alone is responsible for the distribution of the B allele between species then the total number of both of these configurations is expected to be equal. However, deviations from this expected pattern can be interpreted as evidence of introgression between taxon 3 and one of the other two ingroup taxa (Durand et al. 2011). The *Dsuite* method is capable of handling a large number of species and integrating over all possible 4-taxon statements across a phylogeny, therefore, allowing for the inference of multiple introgression events. Furthermore, the f-branch test (f_b), which can assign introgression events to specific internal branches on the species tree, was implemented in *Dsuite*. Statistical support of the f-branch tests was assessed using z-scores ≥3.

To call SNPs from the transcriptomic data, we first used STAR (v2.7.5) (Dobin et al. 2013) to map all *Crotalus*, *Sistrurus*, and *Agkistrodon piscivorus* trimmed fastq reads against the *C. viridis* genome (UTA_CroVir_3.0 assembly; Schield, Card, et al. 2019). Then we used samtools (Li et al. 2009) to mark duplicates and bcftools to call SNPs from bam files. From the combined SNP dataset we used vcftools (Danecek et al. 2011) to drop all sites with >50% missing data. With this dataset and our inferred species tree topology we used Dtrios in *Dsuite* to estimate the *D* statistic across all 4-taxon statements with *A. piscivorous* set as the outgroup.

We also used the MSNC approach SNaQ to simultaneously infer the topology of the species relationships and historical reticulations events. This maximum pseudolikelihood approach extends the coalescent model to include reticulation and is implemented in the Julia package *PhyloNetworks* (Solís-Lemus and Ané 2016; Solís-Lemus et al. 2017). Specifically, quartet concordance factors (CFs) of 4-taxon trees are used to estimate a species network with estimated reticulation events and γ-values (inheritance probabilities of ancestral contributions to hybridization events). We initially

attempted to use the full dataset (57 terminals and 298 specimens with phased alleles), however, even an analysis of h_{max} (number of hybrid edges) = 1 was too computationally intensive; using the Clemson University Palmetto HPC the analysis exceeded a wall time of 336 h with 80 CPUs and 1400 GB of memory. We, therefore, ran four SNaQ analyses on different subsets of the data, first we investigated reticulation in each species group identified to have significant introgression from *Dsuite*, this includes (1) the *C. triseriatus* group, (2) the *C. durissus* group, and (3) the *C. viridis* group. Additionally, we ran SNaQ with 24 species that were representative of the rattlesnake radiation but were selected to exclude recently diverged taxa (e.g., phylogeographic lineages within *C. scutulatus* and *C. atrox*). For each of these four analyses, we retained all species' alleles from within these groups from the previously estimated IQ-TREE gene trees and estimated CFs in SNaQ. Phylogenetic networks were estimated with a starting tree topology based on the ASTRAL species tree, the number of h_{max} events ranging from 0 to 5, with 10 independent analyses for each hmax value using the ASTRAL species tree as the starting topology. The best-fit number of reticulation events was inferred using a slope heuristic metric, where adding additional migration events did not improve likelihood scores between h_{max} values. Because we were unable to include all taxa in these SNaQ analyses we ran additional analyses to test the sensitivity of this method to excluding an inferred minor hybrid edge (e.g., lineages that contribute <50% of the genes to a hybrid descendant). We ran two analyses in which a minor edge from the analysis with 24 taxa was excluded; in one *C. cerastes* was excluded and in the other *C. ravus* was excluded. These analyses were both run with 10 independent analyses with h_{max} = 2 and the ASTRAL species tree as the starting topology.

Results

Sequence Data

A total of 8990 loci were assembled (including 2824 UCEs, 353 AHE, 5042 BUSCO loci, 735 ortho-loci, and 36 traditional Sanger loci). After filtering for missing data, we retained a total of 3707 loci. On average 15.1% of the sampled ingroup specimens were missing from the retained loci (4.0–59.6% missing) and each specimen was missing 22.0% of the retained loci (2.3–80.8% missing). Mean locus length was 1665 base pairs (± 1375 bp). mtDNA sequences were recovered from 264 of the 298 sampled specimens. All newly generated fastq files are available from the NCBI BioProject ID PRJNA1092052 (Supplementary Table S2 lists all SRA accession numbers).

Species Tree and Divergence Times

The ASTRAL species tree produced a well-supported phylogeny, recovering many of the previously hypothesized species groups (Fig. 2; Supplementary Table S1).

The lowest supported node is that joining the *C. triseriatus* group + (*C. willardi*, *C. intermedius* group) with a local posterior probability of 0.62. All other nodes have a PP ≥ 0.93. The polytomy test in ASTRAL failed to reject the null hypothesis of a polytomy for the branch uniting *C. triseriatus* group + (*C. willardi*, *C. intermedius* group) (*P* value = 0.44). This suggests that the earliest branch within the genus *Crotalus* is best represented as a polytomy and the relationships between the *C. triseriatus* group + (*C. willardi*, *C. intermedius* group) + all other *Crotalus* species are not resolved (Fig. 3). The gCFs demonstrated that on average each node in the species tree was only supported by 18.4% of the gene trees, and this value ranged from 0.7% to 61.5%. Many of the nodes with high gCFs are among the earliest divergence events in the phylogeny, for example, the split between *Crotalus* and *Sistrurus*, as well as the divergence between the small bodied, montane rattlesnakes and the heavier-bodied species (Fig. 3). Alternative quartet topologies across the species tree topology were supported by very few trees. For example, the second alternative topology had an average gCF of 4.2% and the third alternative topology had an average of 3.6% (Fig. 3). The average percentage of gCFs that do not match alternative quartet topologies presented in the species tree at all is high, with an average of 73.8% of gene trees showing a different topology (range: 22.8–97.2%). These discrepancies in topology are particularly high at regions of the tree with short internode distances, for example, the node subtending the placement of *C. enyo* and *C. horridus* (Figs. 2 and 3). Such low gCFs suggest either that the individual gene trees are difficult to resolve or that there is a conflicting signal among gene trees because of processes like introgression or ILS.

Our resampling of loci based on alignment length and number of phylogenetically informative sites demonstrates the loci in the top 75% quantile of these two parameters estimated coalescent species trees more similar to the one estimated from all loci. For example, the normalized RF distance between the full data coalescent species tree and the top 75% quantile of alignment length was 0.045, and compared to the top 75% quantile of phylogenetically informative sites was 0.015. The species trees constructed with the lower 25% quantile of alignment length and phylogenetically informative sites were, however, still similar in topology with normalized RF distances of 0.06 and 0.076, respectively. The variation in topology among these four subsets of data also differs. For example, the shortest loci species tree varies in the relationships within the *viridis* group, the longest loci species tree differed in the relationships among *C. culminatus*, *C. ehecatl*, and *C. mictlantecuhtli*, the least informative loci placed *C. cerastes* and *C. enyo* as sister as well as having a different placement of the *C. mitchelli* complex, and lastly, the most informative loci differed in the placement of the (*C. willardi*, *C. intermedius* group) (Supplementary Fig. S1). Given that there is no single pattern of discordance between these species trees based on subsets of the data it is unlikely that a small proportion of loci is driving
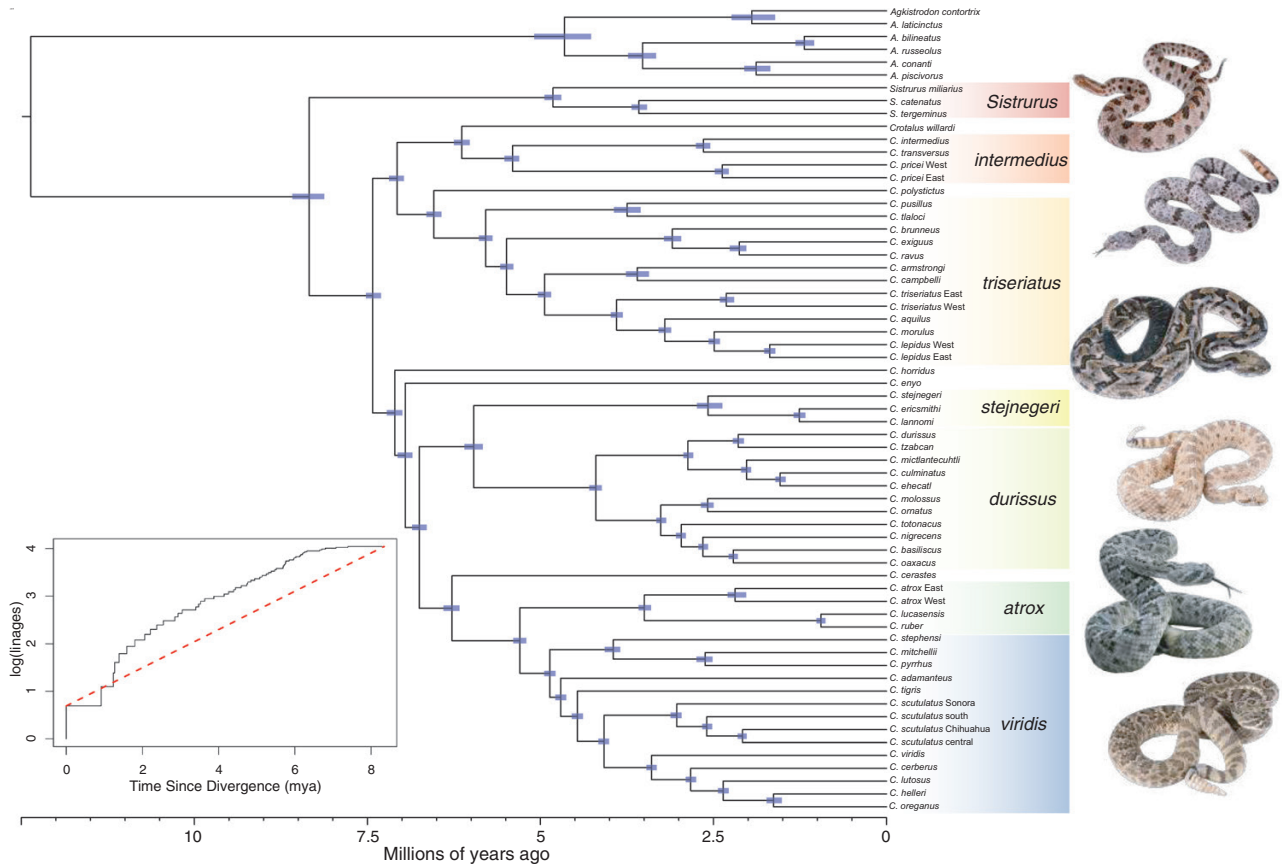
FIGURE 2.   Dated species tree topology with representative rattlesnake species. Blue bars on the phylogeny represent errors around divergence time estimates. A lineage through time plot is shown in the bottom left-hand corner, where the black line is the log number of lineages accumulating over time (in mya) and the dashed line is representative of a constant rate of diversification. Species, from top to bottom, are *Sistrurus miliarius*, *Crotalus lepidus*, *C. horridus*, *C. cerastes*, *C. atrox*, and *C. scutulatus* (photo credits to T. Schramer and E. A. Myers).

the overall topology of the recovered ASTRAL species tree; instead, this suggests that there is extensive heterogeneity in gene tree topology.

Our inferred divergence times indicate that rattlesnakes originated in the middle Miocene at ~12.4 mya (CI = 12.2–12.6 mya). The extant diversity in this group has radiated recently, with the divergence between *Sistrurus* and *Crotalus* estimated at ~8.3 mya (8.1–8.6 mya), with rapid species diversification throughout the Pliocene (Fig. 2).

The concatenated genomic tree and mtDNA gene tree recover many of the same major species groups within rattlesnakes (Supplementary Figs. S2 and S3). However, the phylogenetic position of these groups and several species differ among these trees, including the position of *C. horridus*, *C. enyo*, *C. adamanteus*, and *C. willardi*. For example, both the concatenated genomic and mtDNA trees place *C. enyo* and *C. cerastes* as sister, the ASTRAL species tree does not support this relationship. Additionally, the placement of *C. horridus* is consistent between the ASTRAL species tree and the mtDNA tree but differs from the concatenated genomic tree. Furthermore, the phylogenetic positions of these species are also well supported in the mtDNA gene tree (BS ≥ 92)

and the concatenated tree (BS = 100). The normalized RF distances between these trees are as follows, species tree—concatenated tree = 0.14, species tree—mtDNA tree = 0.32, and concatenated tree—mtDNA tree = 0.30 (Supplementary Fig. S3). The mean normalized RF distances between gene trees and the species tree was 0.84 (Fig. 3b). These high RF distances demonstrate a high level of discordance between individual gene tree topologies when compared to the estimated species tree.

Effects of Recombination on Species Tree Estimation

The pairwise homoplasy index test suggested that 2283 loci (61.5% of all loci) had a signature of intralocus recombination. After re-estimating an ASTRAL species tree with the gene trees estimated from loci that had a signature of recombination we find that this recombination-based coalescent species tree has a normalized RF distance from the ASTRAL species tree based on all data of 0.015. Similarly, after re-estimating a species tree in ASTRAL with only the loci that do not have a signature of recombination we find that this tree also differs from the all-data ASTRAL tree by a normalized RF distance of 0.015. Given that the number of differences
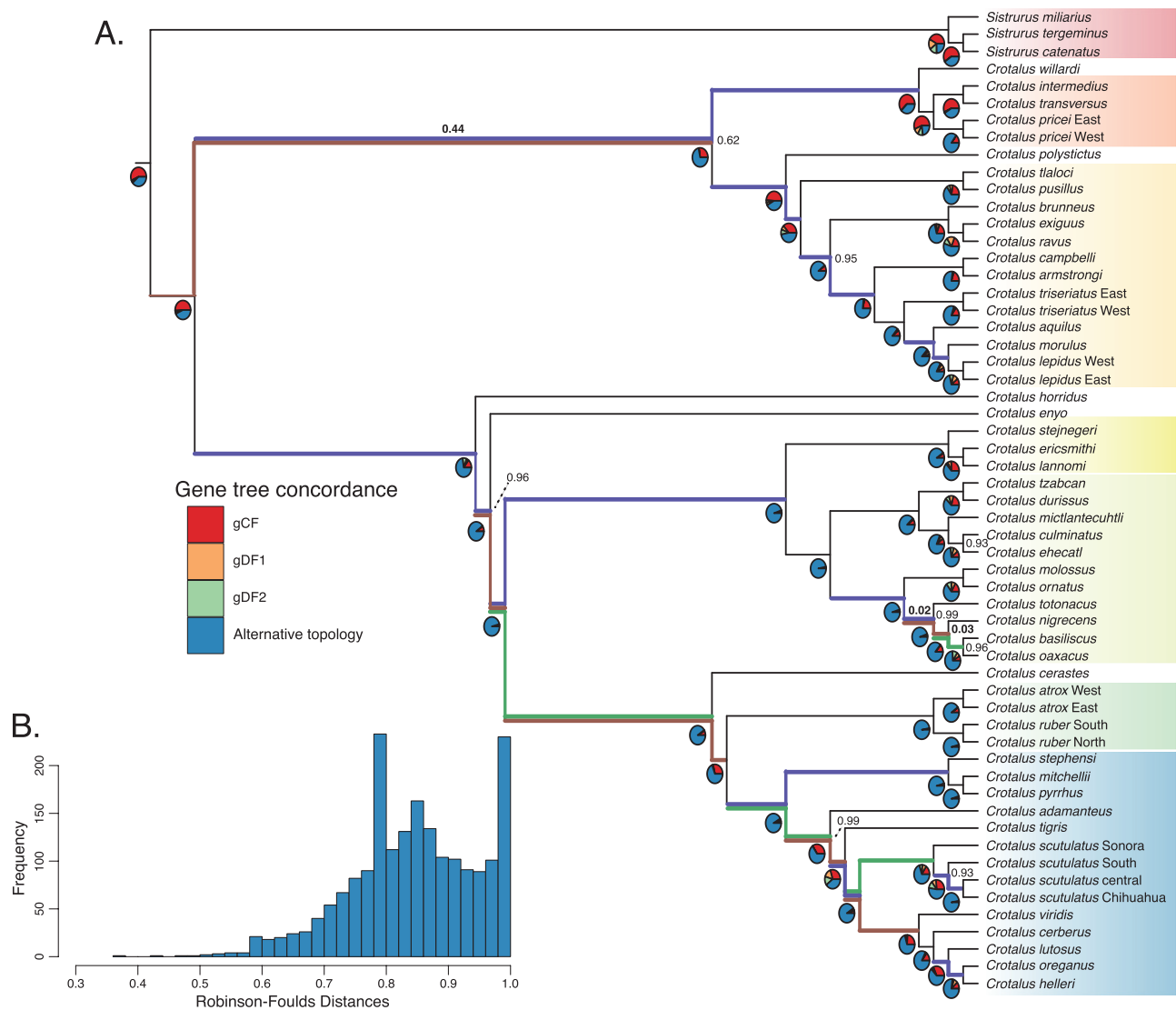
FIGURE 3. a) Species tree topology represented as cladogram where anomaly zone branches are highlighted in bold (in purple, green, or maroon). Numbers at nodes represent local posterior probability values inferred from ASTRAL when less than 1; numbers in bold above branches are *P* values from the ASTRAL polytomy test, and all *P* values greater than 0 are shown. Pie charts at nodes represent the proportions of gene tree concordance factors that support the species tree (gCF in legend), the 2 most common alternative quartet resolutions (gDF1 and gDF2), or support an alternative resolution not found in the species tree (Alternative topology). b) Histogram of the Robins-Foulds distances between gene trees and the inferred species tree topology, demonstrating that individual gene tree topologies rarely match the species tree.

between these two subsets of data (recombination loci vs. no recombination) results in the same number of differences with the coalescent species tree based on all of the loci suggests that intralocus recombination is not biasing our phylogenetic estimates and that these differences are likely a result of random sampling of the total number of loci, similar to the above tests of sampling gene trees by locus length and phylogenetically informative sites. Here, the difference between the recombination tree and the full data set tree is the placement of the (*C. willardi*, *C. intermedius* group), while the difference between the no recombination tree and the full data set tree is the relationships among *C. culminatus*, *C. ehecatl*, and *C. mictlantecuhtli* (Supplementary Fig. S4).

Anomaly Zone

Although the species tree was well supported with estimated local PPs, many parent–child branches were found to be in the anomaly zone (Fig. 3). Using a bootstrap threshold of being present in >0.9 of the bootstrap trees, a total of 21 internal-node pairs in the phylogeny were found to have branch lengths that would produce anomalous gene trees in comparison to the species tree. The deepest branches in the tree (e.g., the split between *Crotalus* and *Sistrurus*, or between the small montane *Crotalus* and the large-bodied species) were not found to be within the anomaly zone. However, many of the branches joining the major species groups and branches

within these species groups were found to be affected by the anomaly zone.

Introgression

We used two methods to infer introgression across the rattlesnake phylogeny. First, the D-statistic calculated across all trios of species in the dataset demonstrated that the largest estimated D-statistic values of introgression that were statistically significant occurred within three of the major species groups; the *C. triseriatus*, *C. durissus*, and *C. viridis* groups (Fig. 4). These results largely agree with several previously estimated or hypothesized instances of gene flow within rattlesnakes (Blair
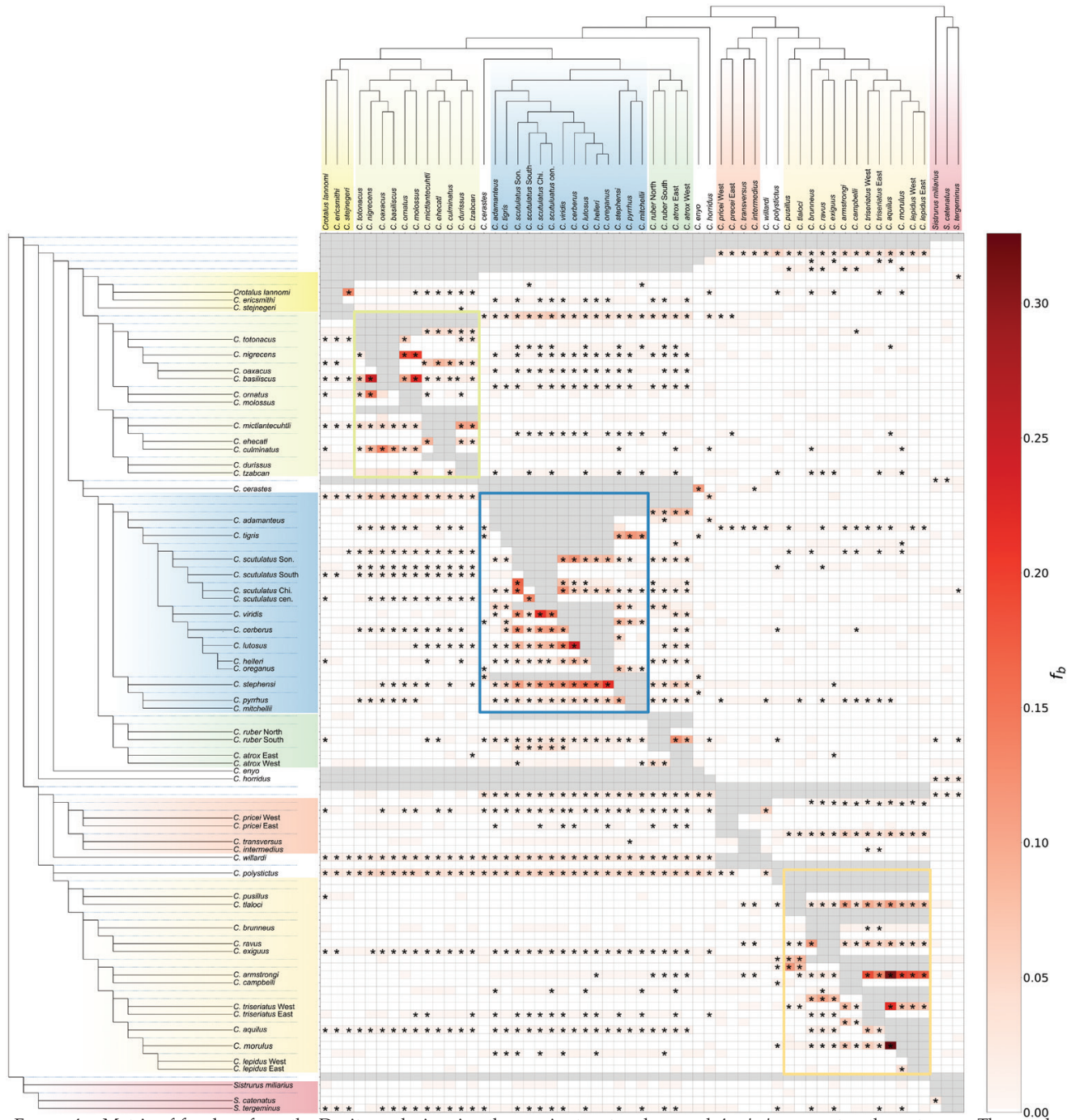
FIGURE 4. Matrix of f_b values from the Dsuite analysis using the species tree topology and *A. piscivorous* set as the outgroup. These values represent the proportion of alleles shared between a donor lineage (column) and the recipient lineage (row) branches. Unlabeled branches with dotted lines along the tree on the *y*-axis represent introgression events that occurred with ancestral lineages. All significant values (*z*-scores ≥ 3) are marked with a "*." These results highlight that introgression has largely occurred within the known species groups, these regions of the matrix are highlighted with boxes.

et al. 2019; Myers 2021). Furthermore, there is a greater weight of evidence for introgression among recently diverged species or between phylogeographic lineages (e.g., among the *C. scutulatus* lineages; Schield et al. 2018). Interestingly, this analysis also suggests that several species that have been hard to place in phylogenetic analyses also have a signature of low levels of introgression. For example, this pattern is seen between *C. cerastes* and *C. enyo*, as well as between both *C. polystictus* and *C. willardi* with the early branch of the 'heavy-bodied' clade of *Crotalus* (Fig. 4). We note that a single instance of gene flow can result in correlated signals of non-zero f_b values across multiple lineages related to a donor lineage (Pyron et al. 2022). This will result in an artifactual horizontal line of significant inference across a row in Figure 4; therefore, the actual number of donor lineages may be smaller than inferred here.

The phylogenetic network approach implemented in SNaQ run with a reduced number of species representative of all major ingroup taxa recovered a similar topology to the ASTRAL species tree with an optimal $h_{max} = 3$ (Fig. 5; Supplementary Fig. S5). Importantly, 2 of these 3 reticulation events involved species groups with alternative placements between the strictly bifurcating tree and the MSNC phylogeny, suggesting that unaccounted for introgression may have influenced the topology of the strictly bifurcating phylogeny. These differences involve the placement of *C. cerates*, where SNaQ infers introgression between this lineage and the ancestral lineage of the *C. viridis* and *C. atrox* groups. In the strictly diverging phylogeny *C. cerastes* is sister to these two species groups (Fig. 2). Additionally, reticulation is inferred between the *C. triseriatus* group and the *C. intermedius* group where the phylogenetic position of the *C. intermedius* group changes with this reticulation event (Fig. 5). The last introgression event is found between *C. ravus* and *C. triseriatus*, which is supported as the only reticulation in the *C. triseraitus* group SNaQ analysis (Fig. 5), although with a different directionality of hybrid edges. Introgression in
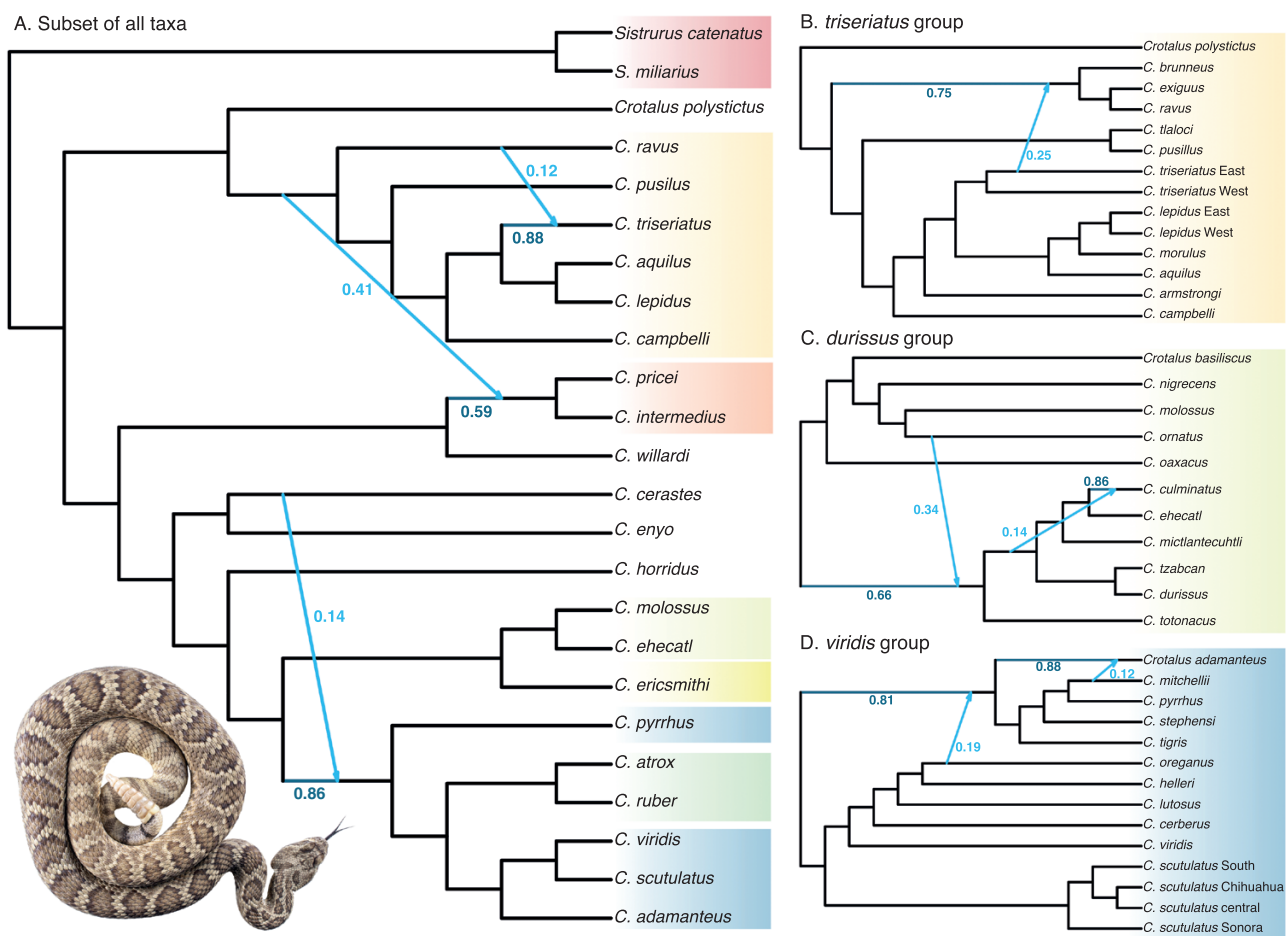

FIGURE 5.   Phylogenetic relationships based on SNaQ analyses. Blue arrows represent introgression events where numbers represent gamma values, or the proportion of alleles shared via introgression. a) SNaQ analyses of a subset of all species yet that are representative of all species groups. An h_{max} of 3 introgression events is supported by these data. b) Introgression within the *C. triseriatus* group, with a single hybridization event between *C. triseriatus* East and the ancestral lineage of (*C. brunneus*, (*C. exiguus*, *C. ravus*)). c) Introgression within the *C. durissus* species group, demonstrating 2 introgression events. d) SNaQ analyses results for the *C. viridis* species group with 2 deep-time hybridization events. Inset photo is of *C. scutulatus* (credit to T. Schramer).

the *C. durissus* group suggests historical introgression between *C. ornatus* and the *C. durissus* complex as well as potential introgression from a ghost lineage into *C. culminatus* (Fig. 5). Finally, in the *C. viridis* species group an $h_{max} = 2$ is best supported with historical introgression from *C. oreganus* into the ancestral lineage of the *C. mitchellii* complex, *C. adamanteus*, and *C. tigris* as well as from *C. mitchellii* into *C. adamanteus* (Fig. 5). All networks with varying values of h_{max} for the 4 SNaQ analyses are presented in the Supplementary Materials (Supplementary Fig. S6).

When a minor hybrid edge was removed and SNaQ analyses rerun, generally the same topology is reconstructed, albeit with some differences in reticulating branches. The difference in topologies includes the placement of the *C. intermedius* group, *C. cerastes*, *C. enyo*, and *C. horridus* (Supplementary Fig. S7). When *C. cerastes* was removed from the analysis, a signal of introgression between *C. ravus* and *C. triseriatus* is still found. However, the second reticulation event is between the *C. triseriatus* and *C. stejnegeri* groups. Similarly, when *C. ravus* is removed, we again find introgression between the *C. triseriatus* group and the *C. intermedius* group. However, instead of finding reticulation involving *C. cerastes*, reticulation is inferred between the *C. durissus* and the ancestral branch of the *C. atrox*/*C. viridis* groups (Supplementary Fig. S7).

Discussion

Previous phylogenetic studies of rattlesnakes have estimated conflicting evolutionary relationships between species using a diverse array of molecular datasets and analytical methods. Here, we addressed the instability of the rattlesnake phylogeny using genomic data generated from transcriptomes sampled from nearly all known species. Using MSC methods, we inferred a species tree that supports many previously recognized species groups, but that is incongruent with previously published phylogenies in their interrelationships. We demonstrate that rattlesnakes have rapidly diversified, and we find that numerous regions of this phylogeny have demographic signatures consistent with them falling within the anomaly zone where there is the expectation of an incongruent gene tree topology that is more probable than the species tree topology. The presence of extensive ILS is suggested by low gene concordance factors and large RF distances between gene trees and the species tree topology, demonstrating high gene tree discordance. In addition, we find that the earliest divergence within *Crotalus* is best represented as a polytomy. Moreover, we find that introgression is common within the major species groups and that historical reticulation between lineages has likely influenced past interpretations of the phylogenetic relationships within this group. These processes may have been the source of incongruences between previous studies of rattlesnake systematics and should be considered in studies of other rapidly radiating groups in which rapid,

successive speciation has occurred and where introgression may be likely.

Reticulate Evolutionary History

During rapid species diversification it is possible for both ILS and on-going introgression to result in high levels of gene tree heterogeneity across the genome. This may be particularly true when speciation proceeds with incompletely separated lineages that maintain large effective population sizes, as has been shown to be common in some rattlesnake species (e.g., Schield et al. 2019; Holding et al. 2021b). If effective population sizes have similarly been large throughout the radiation of rattlesnakes, this, in part, would contribute to high levels of ILS. We also demonstrate that many recently diverged rattlesnake species show a signature of introgression using the MSCN model and D-statistic test. Previous studies have demonstrated that speciation with gene flow is apparently common in rattlesnakes (Harrington et al. 2017; Margres et al. 2021). It is possible that such introgression scales throughout the phylogeny and has occurred throughout the entirety of rattlesnake diversification. These two processes together have led to cascading patterns of high levels of gene tree heterogeneity and have resulted in past difficulties in inferring phylogenies, as has been observed in other taxa (Morando et al. 2004; McGuire et al. 2007; Meyer et al. 2017; Malinsky et al. 2018).

The combined effects of high levels of ILS and interspecific introgression necessitate multispecies network coalescent methods. While MSC-based methods account for ILS and have been shown to be able to infer correct topologies even within the anomaly zone (Liu and Edwards 2009), many taxa may have a reticulate evolutionary history that is not captured by these methods. Here, the strictly diverging and reticulate phylogenies do not have the same evolutionary relationships (Figs. 2 and 5). While we were not able to include all species in the MSCN analysis due to computational demands, our results demonstrate that inferred phylogenetic relationships differ when not accounting for past introgression. First, in the strictly diverging phylogeny, a close relationship between the *C. intermedius* and *C. triseriatus* groups is inferred, suggesting the existence of a monophyletic clade that is similar in body size and habitat associations (e.g., inhabiting mid- to high-elevation montane regions). In our reticulating phylogeny, however, these two groups are not closely related, but are connected via potentially high levels of historical introgression (Fig. 5). These relationships are also inferred to be best represented as a polytomy, a result that could be driven by the unaccounted for signal of past introgression events between these lineages in an analysis that assumes strict divergence. Second, both *C. cerastes* and *C. enyo* have been variably placed in past studies (Murphy et al. 2002; Castoe and Parkinson 2006; Reyes-Velasco et al. 2013), here their phylogenetic affinities also differ between the strictly diverging and reticulating phylogenies. While the two have been

variably inferred to be sister species, our strictly diverging MSC-based phylogeny suggests that *C. cerastes* is sister to the *C. atrox* + *C. viridis* species groups. However, our reticulating phylogeny demonstrates introgressive hybridization between *C. cerastes* and the ancestral lineage leading to the *C. atrox* + *C. viridis* species groups, and a sister relationship between *C. cerastes* and *C. enyo*. Therefore, historical introgression involving these lineages has likely resulted in the difficult placement in past estimates of phylogeny when unaccounted for. Similar patterns in changes to topology driven by past introgression have been observed in other taxa (e.g., *Heliconious* butterflies, *Drosophila* spp.; Edelman et al. 2019; Suvorov et al. 2022). A pattern in which previously recalcitrant phylogenies are best explained by a history of reticulate evolution may be expected given these insights. However, several studies of intractable phylogenetic relationships have been shown to be best explained by extensive ILS alone (Lopes et al. 2021; Meleshko et al. 2021). Future studies should be aware of these issues and test whether a model of the MSCN is better suited to estimate phylogenetic relationships (Blair and Ané 2020; Cai and Ané 2020; Duckett et al. 2020).

Our SNaQ analyses were sensitive to the inclusion of taxa as illustrated by removing an inferred minor hybrid edge and rerunning the analysis. In these cases, the topology and hybrid edges differed from the original analysis. One potential cause of this instability could be repeated bouts of introgression, where, for example, hybrid lineages arise from other hybrids or the radiation contains a lineage that is directly involved in two or more different hybridization events. This is a potentially common outcome for rapidly radiating species (Gante et al. 2016; Kozak et al. 2021; Pyron et al. 2024). However, the model implemented in SNaQ, and most current implementations of the MSCN, assumes a "level-1" network, which is defined as a network where no two cycles share an edge (Rosselló and Valiente 2009). How violating this model assumption will influence the inferred network, including both topology and direction of hybrid edges, is not known. Future simulation-based approaches will be necessary to understand how missing taxa and model violations influence our ability to accurately infer phylogenetic networks.

Rapid Radiation, Speciation, and Admixture

In addition to deeper-time introgression inferred across the phylogeny, our pattern-based test supports extensive introgression within several of the main species groups. These analyses support introgression between several species in the *C. viridis* species group, a result that is expected based on previous reports of gene flow among these species (Schield et al. 2018; Schield, et al. 2019; Myers 2021). We find a particularly strong signal of interspecific gene flow between *C. viridis*—*C. scutulatus* (Chihuahuan Desert), *C. lutosus*—*C. cerberus*, and *C. stephensi*—*C. oreganus*. Of the mentioned pairs with a significant signal of introgression, all of

them have distributions that overlap geographically (*C. viridis*—*C. scutulatus* and *C. stephensi*—*C. oreganus*) or are parapatric (*C. lutosus*—*C. cerberus*). These analyses also find a strong signal of introgression between species within the blacktailed rattlesnake species complex (*C. molossus*). This, in part, corroborates previous hypotheses where researchers have suggested that species within this group freely hybridize. For example, Campbell and Lamar (2004) stated *C. basiliscus* and *C. molossus* hybridize to such an extent that trying to determine distribution limits between these two species is hardly worthwhile. Finally, there are several instances of introgression within the *C. triseriatus* group, particularly involving *C. aquilus*. Previous phylogenomic analyses of this group also found a single deep-time introgression event (Blair et al. 2019); however, the position of this reticulating branch differs from our findings using the same MSCN approach. These contrasting results between our analyses and those published previously could simply be the result of different sampling locations of the specimens sequenced for data analysis, where some populations of a species have a signal of introgression and others do not (Myers 2021).

All three of these species groups, the *C. triseriatus*, *C. durissus*, and *C. viridis* groups, contain taxa with vague species limits, and several recent studies have elevated taxa to species or have named previously unknown cryptic diversity (Bryson et al. 2014; Davis et al. 2016; Carbajal-Márquez et al. 2020; Muñoz-Mora et al. 2022), however, not all authors agree with these delimitations (Reyes-Velasco et al. 2022). These taxonomic disagreements and cases in which specimens are difficult to assign to species (e.g., between *C. basiliscus* and *C. molossus*; Campbell and Lamar 2004) are likely the result of past or ongoing gene flow. While this process may make it empirically difficult to clearly define species limits, it is possible that this introgression has contributed to species diversification by introducing adaptive genetic variation (Marques et al. 2019). In this case, speciation proceeds via a combinatorial mechanism where ancestral genetic variation is reshuffled into unique combinations that can then be acted on by natural selection (Marques et al. 2019). This process of adaptive introgression and lineage divergence has been confirmed in several groups of organisms and is likely common in rapid adaptive radiations (Meier et al. 2017; Lamichhaney et al. 2018; Malinsky et al. 2018). To gain a fuller picture of how introgression has driven adaptation and diversification within adaptive radiations, future studies will benefit from coupling whole genome sequence data with recently developed statistical methods that test for recently introgressed alleles under selection (Gower et al. 2021; Svedberg et al. 2021).

While site-based methods are widely used and an important tool for inferring introgression, there are several potential caveats that should be considered. First, the directionality of introgression is difficult to determine without additional analyses (Pease and Hahn 2015; Svardal et al. 2020). Second, the method implemented here (*Dsuite*; Malinsky et al. 2021) can infer

tip-to-tip or branch-to-tip introgression events but it is not able to infer deep-time branch-to-branch introgression events (Pyron et al. 2022). The identifiability of such introgression events with summary site-based methods is difficult as similar patterns can be generated by several processes including variation in substitution rates (Pease and Hahn 2015), ancestral population structure (Eriksson and Manica 2012), or ghost introgression, which may be common (Ottenburghs 2020; Tricou et al. 2022). However, MSCN-based approaches, such as SNaQ, are capable of identifying the directionality of hybridization as well as deep-time introgression. The coupling of these complementary approaches allows for a fuller understanding of past and ongoing introgression and should be jointly used in tests of introgression among species.

Phylogenetic Discordance and Trait Evolution

Comparative biology is focused on understanding the evolution of traits across a phylogeny (Felsenstein 1985), and most methods developed to-date have focused on the use of strictly bifurcating trees. An often-stated goal of phylogenomics is to produce a "well-resolved" phylogeny to use for comparative analyses. However, as shown here, high gene tree heterogeneity can be driven by both ILS during rapid species diversification and introgressive hybridization, both of which are an intrinsic part of phylogeny and species diversification (Maddison 1997). When mapping character evolution on a strictly bifrucating species tree, an underlying assumption is that the constituent gene trees match this species tree topology. However, this assumption can lead to incorrect inferences regarding parameter estimates, for example, the number of transitions between character states or the timing of these events (Hahn and Nakhleh 2016). Such incorrect inferences of character-state evolution driven by gene tree heterogeneity can be driven by both ILS and introgression (Avise and Robinson 2008).

Given that we demonstrate high gene tree heterogeneity, the underlying genes important for phenotypic differences should also have high levels of heterogeneity complicating our understanding of their evolution across the phylogeny and leading to patterns resembling hemiplasy. A key example of this within our focal group is understanding the evolution of venoms derived from duplications in several gene families. The presence or absence of duplications within these gene families can result in very different venom phenotypes (e.g., neurotoxic vs. hemotoxic venom) and a lack of phylogenetic resolution within rattlesnakes has led to uncertainty in the dynamics of the origins of these phenotypic differences (Mackessy 2010; Dowell et al. 2016). However, given high levels of ILS, it is likely these divergent phenotypes have been maintained via ancestral polymorphism, where lineage sorting determines whether a particular phenotype is maintained as polymorphic, fixed, or lost within a species. It has also

been suggested that hybridization between species has resulted in the introgression of venom genes, further influencing the distribution of venom phenotype across this clade (Rokyta et al. 2015; Dowell et al. 2018). Future interrogation of signals of ILS and introgression may provide further insight into the complexities of venom evolution.

Regardless of the process, gene tree heterogeneity and phylogenetic uncertainty are common (Lopes et al. 2021; Morales-Briones et al. 2021; Feng et al. 2022) and need to be accounted for to understand trait evolution, macro-ecological and -evolutionary patterns, and biogeography. Several recently developed comparative methods now allow for explicit tests of trait evolution via hemiplasy (Hibbins et al. 2020), continuous and discrete character evolution across a reticulate phylogeny (Karimi et al. 2020; Teo et al. 2022), and methods that incorporate gene tree histories into comparative methods that are agnostic to the causes of heterogeneity among trees (Hibbins et al. 2022). These methods and future developments will be necessary for the field to better address questions in comparative biology and the evolution of complex phenotypes.

Rattlesnake Systematics

Determining evolutionary relationships among taxa associated with rapid radiations is difficult and the rattlesnakes are no exception. Within this group, rapid and reticulate diversification explains the observed conflict among gene trees and the previous challenges to resolving evolutionary relationships. Overall, our results suggest that rattlesnakes are the product of rapid lineage diversification that has been accompanied by introgression, where several regions of the phylogeny remain difficult to interpret (e.g., the phylogenetic placement of the *C. intermedius* group, or the sister relationship between *C. cerastes* and *C. enyo*). Our phylogenetic analyses do clarify the position of some previously difficult-to-place taxa, for example, in all analyses *C. polystictus* is sister to the *C. triseriatus* group and *C. willardi* is sister to the *C. intermedius* species group. We recommend that future classifications of rattlesnakes have a more inclusive representation of the known species groups to reflect these close relationships (e.g., *C. willardi* should be included within the *C. intermedius* group; see Supplementary Table S1 for our proposed species groups compared to previously published taxonomies).

Several regions of the phylogeny differ between the strictly diverging and reticulating trees, and we suggest that the more biologically realistic model represented by the MSCN effectively explains these differences by incorporating historical introgression between lineages. Several of these inconsistences were also confounding issues for early taxonomists. For example, Gloyd (1940) listed several taxa as "species of uncertain relationships" based on analyses of morphological data. The inability to sort relationships based solely on morphology within a group like rattlesnakes with high levels of phylogenomic conflict is unsurprising given that the

population-level processes that drive this conflict may also influence rates of phenotypic evolution. It has been recently demonstrated that regions of phylogenies with high gene tree heterogeneity also have the highest rates of phenotypic innovation (Parins-Fukuchi et al. 2021). It is likely that the phenotypic differences among these difficult-to-place rattlesnake species are the result of similar diversification processes (Marques et al. 2019).

Recent phylogeographic and species delimitation studies have demonstrated that there may be substantial unrecognized species-level diversity within rattlesnakes (Kubatko et al. 2011; Anderson and Greenbaum 2012; Bryson et al. 2014; Meik et al. 2015; Schield et al. 2015; Harrington et al. 2017; Myers et al. 2019; Carbajal-Márquez et al. 2020; Margres et al. 2021). Several of these recent studies have relied on single locus or small Sanger-based genetic datasets to cluster individuals and recognize species-level diversity. However, we demonstrate here high gene tree heterogeneity, including between the mtDNA phylogeny and the species tree, and suggest that future studies approach species delimitation with large scale genomic data. Furthermore, much of the species-level diversity within rattlesnakes is likely blurred by contemporary gene flow resulting in incompletely separated lineages. To fully understand species level diversity, especially within the "gray zone" of speciation (de Queiroz 2007; Roux et al. 2016), future studies incorporating dense geographic and genomic sampling coupled with robust analytical approaches (Jackson et al. 2016; Jiao and Yang 2021; Ji et al. 2022) will be necessary to clarify taxonomic issues in a manner that is consistent with the observed complex history of diversification with gene flow and reticulation. Given the extensive introgression at multiple timescales that we demonstrate here, including both recent admixture among terminal species and ancient hybridization across internal branches, it is essential to consider gene flow as an important evolutionary process occurring during speciation within this group.

Resolving Intractable Phylogenies

Numerous rapid radiations have resulted in regions across the Tree-of-Life where phylogenetic discordance is ubiquitous. This discordance is the result of extensive ILS, introgression among taxa, and, in some cases, the coupling of these two processes. The resolution of some regions of the Tree-of-Life relies on the presence of just a few loci and may change given different regions of the genome (e.g., coding vs non-coding; Shen et al. 2017). While many of these nodes are currently unresolved, with additional data and new methods many of these relationships should become recoverable. With chromosome level genome assemblies it is now possible to use synteny breakpoints to estimate phylogenetic trees (e.g., Drillon et al. 2020). Similar methods have recently been used to resolve the phylogenetic relationships among the early branches within Metazoa (Schultz et al. 2023) and teleost fishes (Parey et al. 2023). With the rapid proliferation of high-quality genomic datasets,

it is possible that many previously recalcitrant nodes can now be resolved. Additionally, as MSCN-based approaches continue to develop, a model that allows for higher-level reticulations (e.g., greater than level-1 networks; Pyron et al. 2024) that scale with large numbers of taxa will be of particular interest to empirical biologists and may be necessary to resolve the phylogenies of even the most heavily studied taxa (e.g., among the early branches of the Neoaves and East African cichlids; Hackett et al. 2008; Koblmüller et al. 2010; Suh et al. 2015; Braun and Kimball 2021; Scherz et al. 2022).

Data Availability Statement

All newly generated high throughput sequence data have been archived on the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA1092052 (also see Supplementary Table S2). Additionally, the dated species tree, assembled loci in fasta format, and maximum likelihood gene trees are accessioned on Dryad (https://doi.org/10.5061/dryad.crjdfn396).

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