



# Sequence Divergence in Venom Genes Within and Between Montane Pitviper (Viperidae: Crotalinae: *Cerrophidion*) Species is Driven by Mutation–Drift Equilibrium

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## Abstract

Snake venom can vary both among and within species. While some groups of New World pitvipers—such as rattlesnakes—have been well studied, very little is known about the venom of montane pitvipers (*Cerrophidion*) found across the Mesoamerican highlands. Compared to most well-studied rattlesnakes, which are widely distributed, the isolated montane populations of *Cerrophidion* may facilitate unique evolutionary trajectories and venom differentiation. Here, we describe the venom gland transcriptomes for populations of *C. petlalcalensis*, *C. tzotzilorum*, and *C. godmani* from Mexico, and a single individual of *C. sasai* from Costa Rica. We explore gene expression variation in *Cerrophidion* and sequence evolution of toxins within *C. godmani* specifically. *Cerrophidion* venom gland transcriptomes are composed primarily of snake venom metalloproteinases, phospholipase A<sub>2</sub>s (PLA<sub>2</sub>s), and snake venom serine proteases. *Cerrophidion petlalcalensis* shows little intraspecific variation; however, *C. godmani* and *C. tzotzilorum* differ significantly between geographically isolated populations. Interestingly, intraspecific variation was mostly attributed to expression variation as we did not detect signals of selection within *C. godmani* toxins. Additionally, we found PLA<sub>2</sub>-like myotoxins in all species except *C. petlalcalensis*, and crotoxin-like PLA<sub>2</sub>s in the southern population of *C. godmani*. Our results demonstrate significant intraspecific venom variation within *C. godmani* and *C. tzotzilorum*. The toxins of *C. godmani* show little evidence of directional selection where variation in toxin sequence is consistent with evolution under a model of mutation–drift equilibrium. *Cerrophidion godmani* individuals from the southern population may exhibit neurotoxic venom activity given the presence of crotoxin-like PLA<sub>2</sub>s; however, further research is required to confirm this hypothesis.

**Keywords** Transcriptomics · Gene family evolution · Mutation–drift equilibrium · Selection

## Resumen

El veneno de las serpientes puede variar entre y dentro de las especies. Mientras algunos grupos de viperidos del Nuevo Mundo—como las cascabeles—han sido bien estudiadas, muy poco se sabe acerca del veneno de las nauyacas de frío (*Cerrophidion*) que se encuentran en las zonas altas de Mesoamérica. Comparadas con las extensamente estudiadas cascabeles, que están ampliamente distribuidas, las poblaciones de *Cerrophidion*, aisladas en montañas, pueden poseer trayectorias evolutivas y diferenciación en su veneno únicos. En el presente trabajo, describimos el transcriptoma de las glándulas de veneno de poblaciones de *C. petlalcalensis*, *C. tzotzilorum*, y *C. godmani* de México, y un individuo de *C. sasai* de Costa Rica. Exploramos la variación en la expresión de toxinas en *Cerrophidion* y la evolución en las secuencias genéticas en *C. godmani* específicamente. El transcriptoma de la glándula de veneno de *Cerrophidion* está compuesto principalmente de Metaloproteinasas de Veneno de Serpiente, Fosfolipasas A<sub>2</sub> (PLA<sub>2</sub>s), y Serin Proteasas de Veneno de Serpiente. *Cerrophidion*

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*petlalcalensis* presenta poca variación intraespecífica; sin embargo, los transcriptomas de la glándula de veneno de *C. godmani* y *C. tzotzilorum* difieren significativamente entre poblaciones geográficamente aisladas. Curiosamente, la variación intraespecífica estuvo atribuida principalmente a la expresión de las toxinas ya que no encontramos señales de selección en las toxinas de *C. godmani*. Adicionalmente, encontramos miotoxinas similares a PLA<sub>2</sub> en todas las especies excepto *C. petlalcalensis*, y PLA<sub>2</sub>s similares a crotoxina en la población sureña de *C. godmani*. Nuestros resultados demuestran la presencia de variación intraespecífica presente en el veneno de *C. godmani* y *C. tzotzilorum*. Las toxinas de *Cerrophidion godmani* muestran poca evidencia de selección direccional, y la variación en la secuencias de las toxinas es consistente con evolución bajo un modelo de equilibrio de mutación-deriva. Algunos individuos de *C. godmani* de la población del sur potencialmente tienen un veneno neurotóxico dada la presencia de PLA<sub>2</sub>s similares a la crotoxina, sin embargo, se necesita más evidencia para corroborar esta hipótesis.

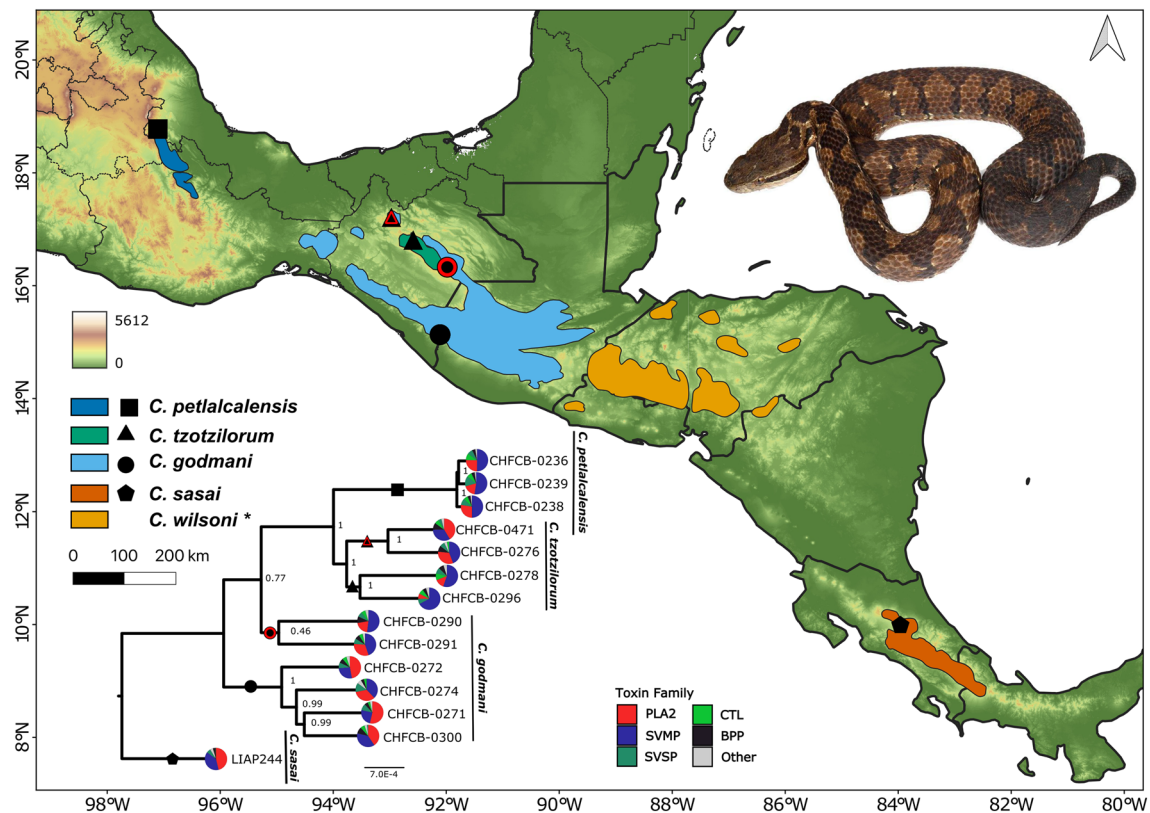
## Introduction

Venoms have emerged as an excellent system for investigating trait evolution given their phenotype–genotype tractability, clear contribution to fitness, and rapid rate of evolution resulting from mutations in protein-coding regions and/or changes in gene expression (Casewell et al. 2011, 2012, 2013; Rokyta et al. 2015b; Aird et al. 2015; Margres et al. 2017). Venom toxins can be linked to specific genes, which facilitates the investigation of how evolutionary processes generate genomic and corresponding phenotypic variation (Brahma et al. 2015; Junqueira-de Azevedo and Ho 2002; Margres et al. 2014b; Rokyta et al. 2015b, 2011). Snake venoms in particular consist of 10–100 secreted toxins, many of which are isoforms from paralogous gene duplications within 10–20 gene families (Casewell et al. 2014; Calvete 2017). Several studies have found evidence for strong, positive directional selection in snake venom toxins (Rokyta et al. 2013; Aird et al. 2015, 2017; Mason et al. 2020; Strickland et al. 2018b); however, others have shown that drift and balancing selection might also have an important role given a lack of differential selection between toxins and non-toxin genes (Ochoa et al. 2020; Rautsaw et al. 2019; Schield et al. 2022) and less selective constraint in toxins than in nontoxins (Aird et al. 2013). These contrasting results may reflect the influence of several key factors, such as the phylogenetic diversity and complex resistance mechanisms of prey (Holding et al. 2021b; Gibbs et al. 2020), the adaptive landscape (Rautsaw et al. 2019), or the demographic history of the species (Ochoa et al. 2020; Margres et al. 2021; Holding et al. 2021a; Rautsaw et al. 2019; Aird et al. 2017). Targeted examinations of specific toxin families have also yielded insights into how genomic variation affects adaptive phenotypes. One example of such gene families are the phospholipase A<sub>2</sub>s (PLA<sub>2</sub>s)—a primary component of many pitviper venoms (Tasoulis and Isbister 2017).

The PLA<sub>2</sub>s in New World pitviper venom evolved via gene duplication and neofunctionalization with gene loss shaping much of the current variation in PLA<sub>2</sub> content (Dowell et al. 2016). For example, certain species possess a heterodimeric PLA<sub>2</sub>  $\beta$ -neurotoxin (herein referred to as

“crotoxin-like” PLA<sub>2</sub>) which is formed by an interaction of an acidic and a basic subunit (Rübsamen et al. 1971). Despite an ancient origin of this neurotoxin, the corresponding genes have been lost in several lineages despite being associated with higher lethality in prey (Mackessy 2008; Dowell et al. 2016, 2018; Borja et al. 2018; Rokyta et al. 2015b). Most of the research on PLA<sub>2</sub> variation has been done in rattlesnakes, specifically in the large-bodied, lowland clade, as several species in this group exhibit intraspecific variation in the presence/absence of crotoxin-like PLA<sub>2</sub>s (Strickland et al. 2018b; Dowell et al. 2018; Margres et al. 2021; Zancolli et al. 2019; Neri-Castro et al. 2019). Variation in specific toxins and in venom composition more broadly is expected to be higher among lineages with substantial population genetic structure, with high environmental variation across their distribution, and between populations with low amounts of gene flow (Ochoa et al. 2020; Margres et al. 2017, 2019). These factors are often present in high elevation species, however, these groups are generally not well studied.

The genus *Cerrophidion* comprises five species of small montane pitvipers distributed throughout Mesoamerica: *C. petlalcalensis* and *C. tzotzilorum* in Mexico; *C. godmani* from Mexico and Guatemala; *C. wilsoni* primarily in Honduras, Guatemala, Nicaragua, and El Salvador; and *C. sasai* in Costa Rica and Panama (Campbell and Lamar 2004; Jadin et al. 2012; Fernández et al. 2017) (Fig. 1). Until recently, *C. godmani*, *C. sasai*, and *C. wilsoni* were thought to be a single, wide-ranging species with substantial morphological variation, however, phylogeographic analyses revealed dynamic biogeographic processes which have resulted in species diversification (Jadin et al. 2012). In particular, montane lineages of pitvipers from Middle America have complex evolutionary histories driven by the geologic events and climatic changes that occurred in the Pliocene and Miocene (Castoe et al. 2009; Daza et al. 2010). *Cerrophidion* species have likely evolved in response to several vicariant events across well-known biogeographic barriers (Castoe et al. 2009; Daza et al. 2010): *C. sasai* and *C. wilsoni* are separated by the Nicaraguan Depression; *C. tzotzilorum* and *C. petlalcalensis* are separated by the Tehuantepec Isthmus;



**Fig. 1** *Cerrophidion* distribution in Mesoamerica. Map modified from VenomMaps (Rautsaw et al. 2022), with the localities of samples of each species used herein. Species are represented by different shapes, different outline colors correspond to Northern populations of that species. \**Cerrophidion wilsoni* was not included in this work. Species tree scaled with IQtree from the inferred Astral tree; support

values correspond to the Astral species tree. Node shapes correspond with the populations in the map. Pie charts in the tips show the percentage of expression of the five most abundant toxin families; the remaining toxin families are included in the category “Other.” Photo Credit: Jason M. Jones (*C. tzotzilorum*)

and *C. godmani* is separated from *C. sasai* and *C. wilsoni* by the Motagua–Polochoic Faults (Castoe et al. 2009; Daza et al. 2010). *C. godmani* and *C. tzotzilorum* are the only species that are not separated by a major biogeographic barrier (Fig 1). As a more clear understanding of their evolutionary history emerges, we are now able to further investigate other biological aspects of *Cerrophidion*.

A thorough understanding of the venom composition of *Cerrophidion* is lacking; published proteomic data on venom composition are limited to *C. sasai* and individuals of *C. godmani* with no associated locality data that may correspond to any one of three distinct species (Gutiérrez et al. 1985; Gené et al. 1989; Arni et al. 1999; Lizano et al. 2000; Chen et al. 2004; Durban et al. 2011; Lomonte et al. 2012, 2014). *Cerrophidion sasai* venom is composed

mostly of snake venom metalloproteinases (SVMPs) and PLA<sub>2</sub>s, inducing intravascular coagulation, hemorrhages, and myonecrosis due to the presence of D49 and K49 type myotoxic PLA<sub>2</sub>s (Lomonte et al. 2012). Our knowledge of *C. petlalcalensis* venom is restricted to an envenomation report (López-Luna et al. 1999): the venom was described as relatively potent, where localized pain and swelling of the hand and forearm started 15 minutes post bite and lasted for three days despite the administration of polyvalent antivenom (López-Luna et al. 1999). *Cerrophidion godmani*, *C. tzotzilorum*, and *C. wilsoni* venom composition is unknown; however, coagulotoxic effects from their venom have been described (Jones et al. 2022).

Given their complex phylogeographic history, montane distribution, and the general lack of information regarding

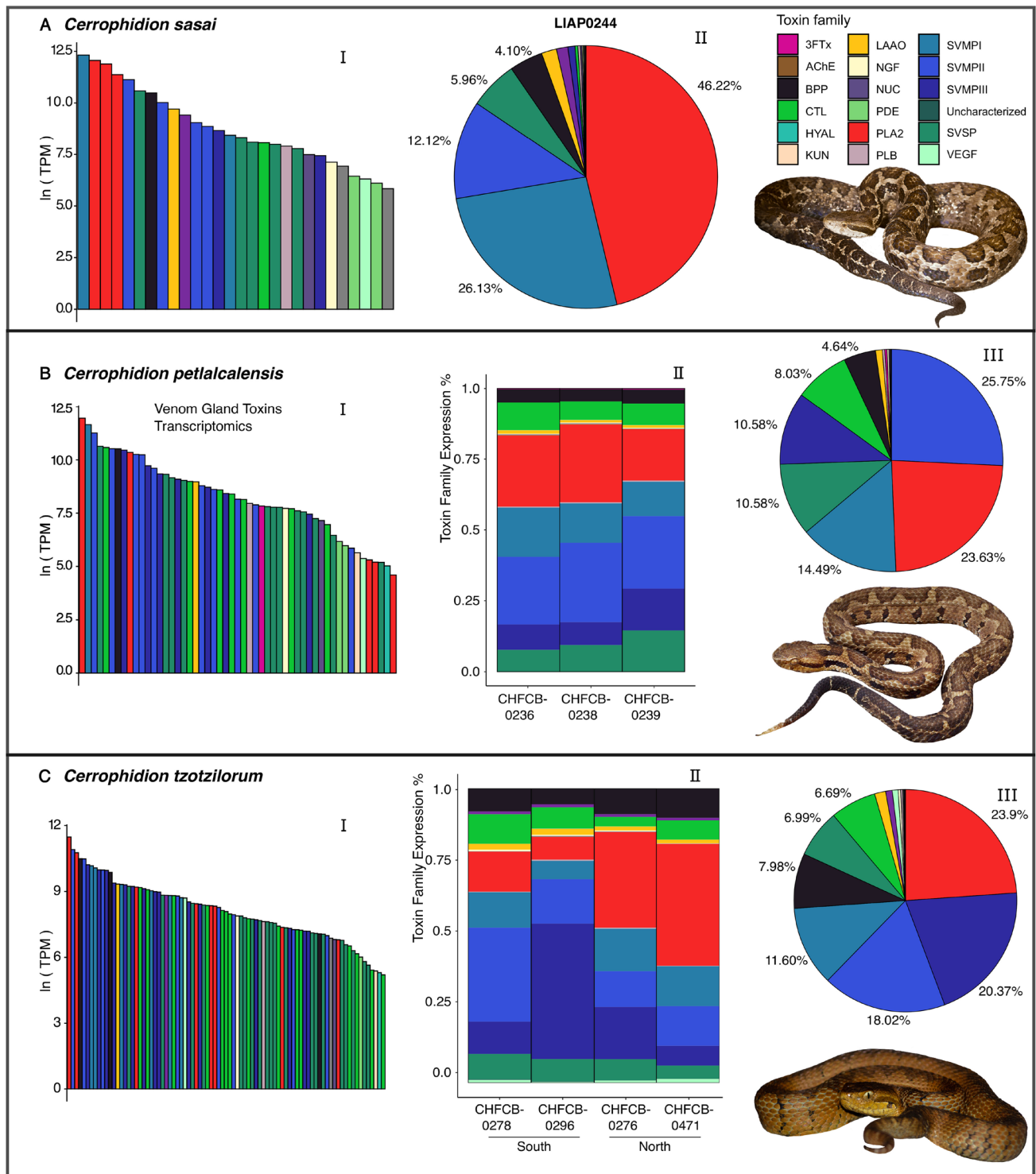
**Table 1** Species information for *Cerrophidion* individuals used in this work

| Collector ID             | Museum ID  | Sex | Locality                       | Age Class | SVL (cm) | Nontoxin Count | Toxin Count |
|--------------------------|------------|-----|--------------------------------|-----------|----------|----------------|-------------|
| <i>C. godmani</i>        |            |     |                                |           |          |                |             |
| CLP2359                  | CHFCB-0271 | F   | Union Juarez, MX               | A         | 46.5     | 1957           | 82          |
| CLP2360                  | CHFCB-0272 | F   | Union Juarez, MX               | A         | 34.3     | 2697           | 82          |
| CLP2362                  | CHFCB-0274 | F   | Union Juarez, MX               | A         | 42.0     | 3385           | 85          |
| CLP2377                  | CHFCB-0290 | F   | Las Margaritas, MX             | A         | 36.3     | 3462           | 78          |
| CLP2378                  | CHFCB-0291 | M   | Las Margaritas, MX             | A         | 45.0     | 3188           | 73          |
| CLP2388                  | CHFCB-0300 | F   | Union Juarez, MX               | J         | 17.1     | 3362           | 68          |
| Average                  | –          | –   | –                              | –         | –        | 2933           | 78          |
| Consensus                | –          | –   | –                              | –         | –        | 5424           | 117         |
| <i>C. petlalcalensis</i> |            |     |                                |           |          |                |             |
| CLP2324                  | CHFCB-0236 | M   | San Andres Tenejapan, MX       | A         | 36.1     | 3140           | 63          |
| CLP2326                  | CHFCB-0238 | F   | San Andres Tenejapan, MX       | A         | 41.9     | 5204           | 65          |
| CLP2327                  | CHFCB-0239 | M   | San Andres Tenejapan, MX       | A         | 36.7     | 3093           | 65          |
| Average                  | –          | –   | –                              | –         | –        | 3812           | 64          |
| Consensus                | –          | –   | –                              | –         | –        | 5252           | 53          |
| <i>C. sasai</i>          |            |     |                                |           |          |                |             |
| -                        | LIAP244    | M   | Las Nubes, San Jose, CR        | A         | 42.4     | 2387           | 52          |
| Consensus                | –          | –   | –                              | –         | –        | 1879           | 28          |
| <i>C. tzotzilorum</i>    |            |     |                                |           |          |                |             |
| CLP2364                  | CHFCB-0276 | F   | Rayon Mescalapa, MX            | A         | 35.5     | 3272           | 81          |
| CLP2366                  | CHFCB-0278 | F   | San Cristobal de las Casas, MX | A         | 37.0     | 3120           | 64          |
| CLP2383                  | CHFCB-0296 | M   | San Cristobal de las Casas, MX | J         | 21.1     | 3327           | 78          |
| CLP2903                  | CHFCB-0471 | F   | Rayon Mescalapa, MX            | A         | 40.6     | 2477           | 81          |
| Average                  | –          | –   | –                              | –         | –        | 3239           | 76          |
| Consensus                | –          | –   | –                              | –         | –        | 4746           | 85          |

Collector ID: CLP = Christopher L. Parkinson. Museum ID: CHFCB = Colección Herpetológica Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango (UJED), Durango, Mexico, and LIAP = Laboratorio de Investigaciones en Animales Peligrosos, Instituto Clodomiro Picado, San José, Costa Rica. Collection locality, age class: Adult (A) and Juvenile (J), measurements: snout-vent length (SVL) for specimens used herein. Nontoxin count and toxin count refer to the number of annotated contigs. Average is the mean contigs by species; Consensus is the number of contigs in the cleaned species consensus transcriptome

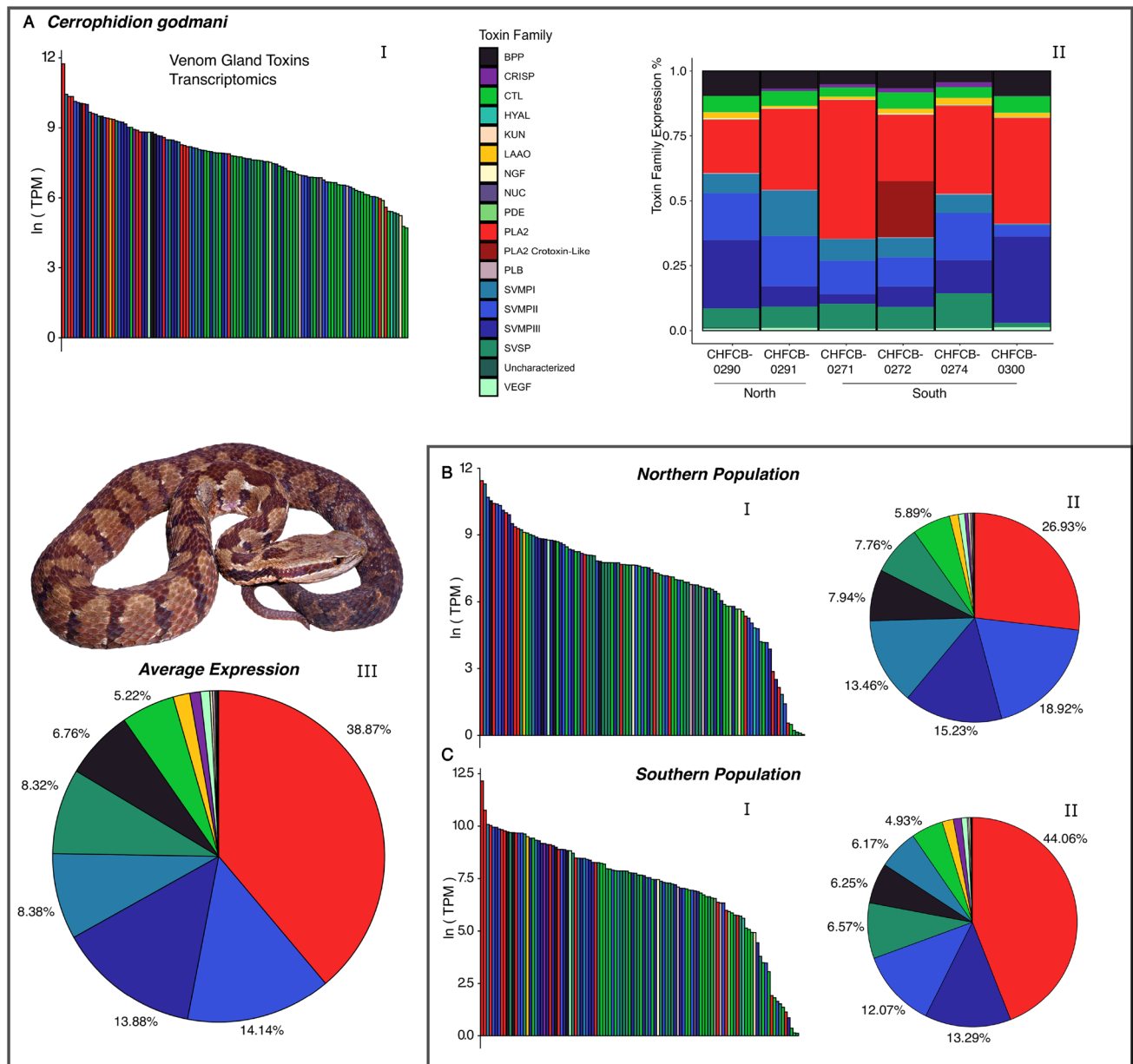
venom composition and variation within *Cerrophidion*, we aimed to describe their venom gland transcriptomes, determine if there is differential toxin expression among populations, and determine if selection is driving toxin evolution in the venom. To do this, we collected representatives of four of the five described *Cerrophidion*: six *C. godmani*, four *C. tzotzilorum*, three *C. petlalcalensis*, and one *C. sasai* (Table 1, Fig. 1). Based on previous proteomic work on *C. sasai*, we expected to see venoms with a high content of PLA<sub>2</sub>s, SVMPs, and snake venom serine proteases (SVSPs), including myotoxic PLA<sub>2</sub>s (Lomonte et al. 2012). Additionally, we inferred a PLA<sub>2</sub> gene-tree using our newly generated transcriptomic data, and previously published sequence data (Whittington et al. 2018;

Neri-Castro et al. 2020b; Mason et al. 2020) to classify *Cerrophidion* PLA<sub>2</sub> groups and infer their evolutionary history. To investigate potential intraspecific and ontogenetic variation, we tested for differential expression of toxins between populations and body sizes. Last, we tested for signals of selection in toxins, comparing toxins against nontoxins genes. If toxins have a greater mean genetic signal of selection than nontoxins, then an adaptive evolution hypothesis would be supported for *Cerrophidion* venom. Alternatively, comparable levels of toxin and nontoxin divergence would suggest *Cerrophidion* venom is largely impacted by drift, possibly influenced by



**Fig. 2** RSEM results for the consensus transcriptomes of **A** *C. sasai*, **B** *C. petlalcalensis*, and **C** *C. tzotzilorum*. In **A** **(I)** barplot of the log ranked expression of toxin genes, **(II)** pie charts of the percent expression of each toxin family average of all individuals. In **B** and **C**, **(I)** barplot of the log ranked expression of toxin genes, **(II)** stacked

barplots with the percent expression of each toxin family by sampled individual, **(III)** pie charts of the percent expression of each toxin family average of all individuals. Photo Credit: **A** R. Wayne VanDevender (*C. sasai*), **B** Carlos E. Montaña-Ruvalcaba (*C. petlalcalensis*), and **C** Ramses A. Rosales-García (*C. tzotzilorum*)



**Fig. 3** RSEM results for the **A** average transcriptome of all individual of *C. godmani*; **B** average of the northern population; and **C** average of the southern population. In **A** **(I)** barplot of the log ranked expression of toxin genes, **(II)** stacked barplots with the percent expression of each toxin family by sampled individual, **(III)** pie chart of the

percent expression of each toxin family for individual populations and for all the individuals. In **B** and **C**, **(I)** barplot of the log ranked expression of toxin genes; **(II)** pie chart of the percent expression of each toxin family for individual populations and for all the individuals. Photo Credit: Carlos E. Montaña-Ruvalcaba (*C. godmani*)

their distribution in isolated mountain ranges and low dispersal capability.

## Results

*Cerrophidion* venom gland transcriptomes were dominated by PLA<sub>2</sub>s and SVMPs, followed by bradykinin-potentiating peptides (BPPs), C-type lectins (CTLs), L-amino acid oxidases (LAAO), and SVSPs. Other toxins families were present in lower percentages (Figs. 2 and 3). *Cerrophidion*

*petlalcalensis* transcriptomes all had largely similar toxin composition, while *C. godmani* and *C. tzotzilorum* both exhibited variation in toxin composition and expression corresponding to the geographic origin of individuals. Toxin composition inferred from the *C. sasai* venom transcriptome was similar to the results of previous proteomic studies (Fig. 2A II; Lomonte et al. 2012). Interestingly, a single individual of *C. godmani* expressed PLA<sub>2</sub>s that were inferred, based on phylogenetic placement, to be closely related to both subunits of the crotoxin-like PLA<sub>2</sub>s of other pitviper genera, suggesting intraspecific polymorphism for neurotoxic venom.

Our species tree—used to test for selection—did not recover *C. godmani* as a monophyletic lineage; instead we recovered the northern population as sister to *C. tzotzilorum* and *C. petlalcalensis* (Fig. 1). This structure might be a result of possible introgression between the sympatric populations of northern *C. godmani* and *C. tzotzilorum* (Fig. 1), which future work may help elucidate.

### Venom Gland Transcriptome Composition

Toxins were more highly expressed than nontoxins in all species analyzed (70.83 – 87.59% of the total expression in transcripts per million (TPM; online resource 2, Table S3, S4, S5). For *C. petlalcalensis* and *C. tzotzilorum*, SVMs were the most highly expressed toxin family followed by PLA<sub>2</sub>s and SVSPs. In comparison, *C. godmani* and the individual *C. sasai* had higher expression of PLA<sub>2</sub>s, followed by SVMs and SVSPs.

Interestingly, *C. godmani* and *C. tzotzilorum* both exhibited intraspecific expression variation corresponding to geographic location. For example, PLA<sub>2</sub> expression in the northern population of *C. tzotzilorum* was higher than in the southern population (36.79% compared to 10.58%, respectively), though SVMs were the most highly expressed toxin family in both populations (39.07% compared to 61.25%, respectively; Fig. 3C II; online resource 1, Fig. S1). In *C. godmani*, the southern population exhibited a higher expression of PLA<sub>2</sub>s compared to the northern population (44.06% compared to 26.93%, respectively) and SVMs were more highly expressed in the northern population (47.61% compared to 31.53%, respectively; Fig. 3 B II and C II).

### PLA<sub>2</sub> phylogeny

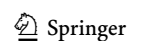
Previous studies on New World pitvipers have characterized five paralogs within PLA<sub>2</sub>s: the K49 myotoxic gK PLA<sub>2</sub>s; two basic PLA<sub>2</sub>s gB1 and gB2 (the basic subunit of crotoxin), and two acidic PLA<sub>2</sub>s gA1 and gA2 (the acidic subunit of crotoxin) (Whittington et al. 2018). We identified

PLA<sub>2</sub> sequences similar to the groups gK, gB1 and gA1 in most of the species, with the exception of gK PLA<sub>2</sub>s in *C. petlalcalensis*, and gB1 in *C. sasai* (Fig. 4A). Expression of both PLA<sub>2</sub>s related to the crotoxin-like subunits (gA2 and gB2) occurred only in a single individual, a small female from the southern population of *C. godmani* (CHFCB-0272; Fig. 4). Expression of these PLA<sub>2</sub> subunits suggests that some individuals in the southern population of *C. godmani* may possess a neurotoxic venom. However, this hypothesis requires isolation of the toxins and confirmation of biological activity from the venom.

### Differential Expression

To assess expression variation within a species, we tested for significant differences in toxin expression between geographically discrete populations using DESeq2 and edgeR R packages (Lov et al. 2014; Robinson et al. 2010). *Cerrophidion petlalcalensis* and *C. sasai* were excluded from these analyses due to a lack of population-level sampling. We found 29 toxins and 77 nontoxins differentially expressed between populations of *C. godmani* (Fig. 5; online resources 2, Table S12), including the toxin families CTL (10), SVM (10), PLA<sub>2</sub> (5), SVSP (4). The differentially expressed PLA<sub>2</sub>s were similar to the gA1 (*C. godmani\_1*, *C. godmani\_4*) and gK myotoxins PLA<sub>2</sub>s (*C. godmani\_26*, *C. godmani\_41*) based on our tree (Fig. 4). Additionally, we tested whether venom expression varied by body size by using snout-vent length (SVL) as a proxy for discrete “adult” and “juvenile” categorization, as we lacked replicates to directly test for differences between these groups. Four toxins and 38 nontoxins were differentially expressed (Fig. 5), including the toxins families CTL (1), PLA<sub>2</sub> (1), SVMPII (1), SVSP (1). All of the differentially expressed toxins had lower expression in the smaller individual, and the differentially expressed PLA<sub>2</sub> grouped with the gK myotoxins (*C. godmani\_42*). Testing differential expression on the added expression of toxin families, we also found that SVMPIs and SVMPIIs are differentially expressed across SVL, with higher expression in the larger individuals (online resource 1, Fig. S2; online resources 2, Table S13). These data suggest that there are ontogenetic changes in the venom. At the level of entire toxin gene families, no families were differentially expressed by population with either method.

For *C. tzotzilorum*, we found 10 toxins and 42 nontoxins differentially expressed between populations, including the toxin families CTL (3), PLA<sub>2</sub> (1), SVMPIII (2), and SVSP (4) (online resource 1, Fig. S3). The differentially expressed PLA<sub>2</sub> (*C. tzotzilorum\_1*) had the highest average expression, and grouped with the gA1 PLA<sub>2</sub>s in our phylogeny. Only one



**Fig. 4** **A** Consensus maximum likelihood tree of the PLA<sub>2</sub>s in *Cerrophidion* including PLA<sub>2</sub>s used in Whittington et al. (2018), Mason et al. (2020), Neri-Castro et al. (2020b), and from Genbank (accession numbers in online resource 1, Table S1). The *Cerrophidion* PLA<sub>2</sub>s (names highlighted in blue) are numbered by the toxin's average expression for each species. Acidic and basic PLA<sub>2</sub>s are identified by red and blue branches, respectively, based on the hypothetical isoelectric point of the amino acid sequences. Nodes with a black dot have > 75 bootstrap support. *Cerrophidion* crotoxin subunit homologs are identified by a star (\*) next to the name. **B** Species tree scaled with IQtree from the inferred Astral tree; lineages with crotoxin-like subunit homologs are purple; support values correspond to the Astral species tree. **C** Amino acid alignment of the gA2 clade and the hypothetical homolog from *Cerrophidion*, dots represent no change from the reference sequence (*C. godmani*\_11). Sites represented with bars match cleavage sites identified in Whittington et al. (2018); a black star (\*) at site 5 is the key substitution known in *Bothriechis*, *Crotalus*, and *Gloydus*; a red star (\*) is at the alternative cleavage site in *C. godmani* based on the protein cutter tool from ExPASy server ([https://web.expasy.org/peptide\\_cutter/](https://web.expasy.org/peptide_cutter/); Gasteiger et al. 2005) (Color figure online)

toxin and 16 nontoxins were differentially expressed across SVL; however, DESeq2 alone indicated that 12 toxins were differentially expressed including the toxin families CTL (3), PLA<sub>2</sub> (2), SVMp (5), SVSP (1), and vascular endothelial growth factor (VEGF; 1) (online resource 2, Table S14). The PLA<sub>2</sub> *C. tzotzilorum*\_1 was differentially expressed for SVL and between populations. Testing for the added expression of toxin families (online resources 1, Fig. S4), we found that PLA<sub>2</sub>s were differentially expressed only by DESeq2, with higher expression in the northern population (Fig. 2C; online resource 2, Table S15). Across SVL, DESeq2 found hyaluronidase (HYAL), SVMPIII, and VEGF as differentially expressed with higher expression of HYAL and SVMPIII in smaller individuals and higher expression of VEGF in larger individuals.

### Signatures of Selection in *C. godmani*

We tested for signatures of selection within *C. godmani* given our sample size and geographic coverage. However, we were unable to compare among species as identification of orthologous toxins is difficult due to the high abundance of paralogs in several toxin families.

### Sequence Diversity

After filtering by coverage and excluding invariant genes, we had information on 68 toxins and 4,508 nontoxins (Table 2; online resource 2, Table S7). While the number of variants per Kbp was similar between toxins and nontoxins ( $\bar{x}_{\text{toxins}} = 6.42 \pm 3.58$ ,  $\bar{x}_{\text{nontoxins}} = 6.77 \pm 4.31$ ), nonsynonymous SNPs were proportionally greater in toxins

than nontoxins (61 and 26%, respectively;  $\chi^2 = 230.22$ ,  $df = 1$ ,  $p < 0.01$ ). The number of variants per Kbp (squared root transformed for normality) was not explained by gene type ( $R^2 < 0.01$ ,  $p = 0.68$ ,  $b = 0.01$ ); however, the number of nonsynonymous variants per Kbp (centered ratio log transformed) was explained by gene type ( $R^2 = 0.01$ ,  $p < 0.01$ ,  $b = 99.1$ ) (online resource 1, Fig. S5). We found no significant relationship between nucleotide diversity and gene type ( $R^2 < 0.01$ ,  $p = 0.30$ ,  $b = 0.04$ ) (online resource 1, Fig. S7).

### Test of Selection

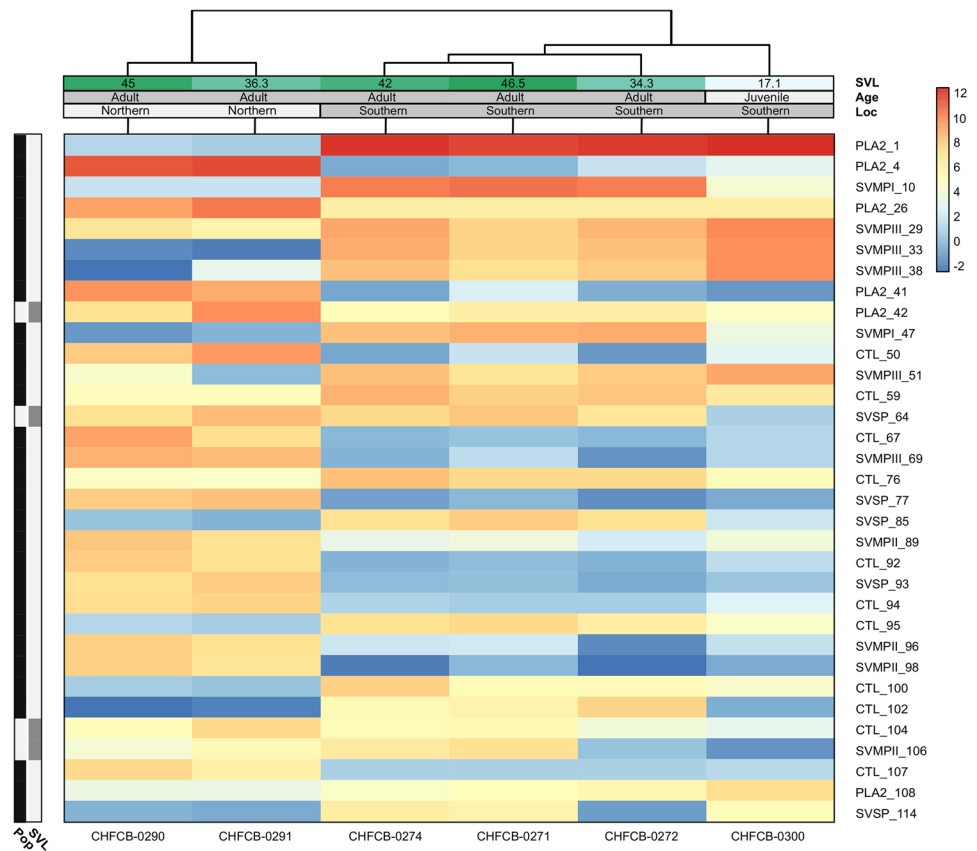
We first used Tajima's D to test for signatures of selection. The mean Tajima's D for nontoxins was significantly less than 0 ( $\bar{x} = -0.19 \pm 0.78$ ,  $t = -16.19$ ,  $df = 4507$ ,  $p < 0.01$ ,  $b = 0.48$ ), providing evidence for either directional selection of nontoxins or population expansion after a recent bottleneck (Fig. 6A). However, the mean Tajima's D for toxins is not significantly different from 0 ( $\bar{x} = -0.04 \pm 0.82$ ,  $t = -0.35$ ,  $df = 67$ ,  $p = 0.73$ ). This potentially implies that toxins are evolving along a mutation–drift equilibrium or that toxins are under highly variable selection pressures, which neutralizes the signal of the mean value. However, the variance of estimates of Tajima's D for toxins and nontoxins is not significantly different ( $F = 0.89$ ,  $df_1 = 4507$ ,  $df_2 = 67$ ,  $p = 0.46$ ). Additionally, toxins and nontoxins are not significantly different in their estimates of Tajima's D ( $R^2 < 0.01$ ,  $p = 0.11$ ,  $b = 0.10$ ), suggesting that there are no differences in selection for toxins and nontoxins.

We also calculated Tajima's D independently for nonsynonymous and synonymous variants (online resource 1, Fig. S7). For both substitution classes, mean Tajima's D values for nontoxins and toxins were < 0. However, only nontoxins were significantly different from 0 for synonymous substitutions. For both substitution types, toxins and nontoxins did not differ significantly (Synonymous  $R^2 < 0.01$ ,  $p = 0.21$ ,  $b = 0.05$ ; Nonsynonymous  $\chi^2 = 1.84$ ,  $df = 1$ ,  $p = 0.17$ ,  $b = 0$ ).

Next, we calculated  $F_{ST}$  between the northern and southern populations of *C. godmani* (Fig. 6B). The  $F_{ST}$  values were not significantly explained by the gene type ( $R^2 < 0.01$ ,  $p = 0.60$ ,  $b = 0.01$ ). Although the difference was nonsignificant, toxin genes had a higher mean  $F_{ST}$  and higher standard deviation than the nontoxin genes ( $\bar{x}_{\text{nontoxins}} = 0.19 \pm 0.21$ ,  $\bar{x}_{\text{toxins}} = 0.21 \pm 0.26$ ).

We used the Branch-Site Unrestricted Statistical Test for Episodic Diversification (BUSTED) model of HyPhy to detect signals of selection (Murrell et al. 2015). This model is based on the rate of synonymous

**Fig. 5** Heatmap showing the log TPM expression of toxins identified as differentially expressed in *C. godmani* ordered by the average expression. In the left columns (Pop & SVL) the darker colors represent significant differential expression agreement by both DESeq2 and edgeR ( $FDR < 0.05$ )



**Table 2** SNPs statistics and selection metrics. Toxins and nontoxins with SNPs from *C. godmani*, the number of SNPs in total and per Kbp (mean  $\pm$  standard deviation), the number/proportion of nonsyn-

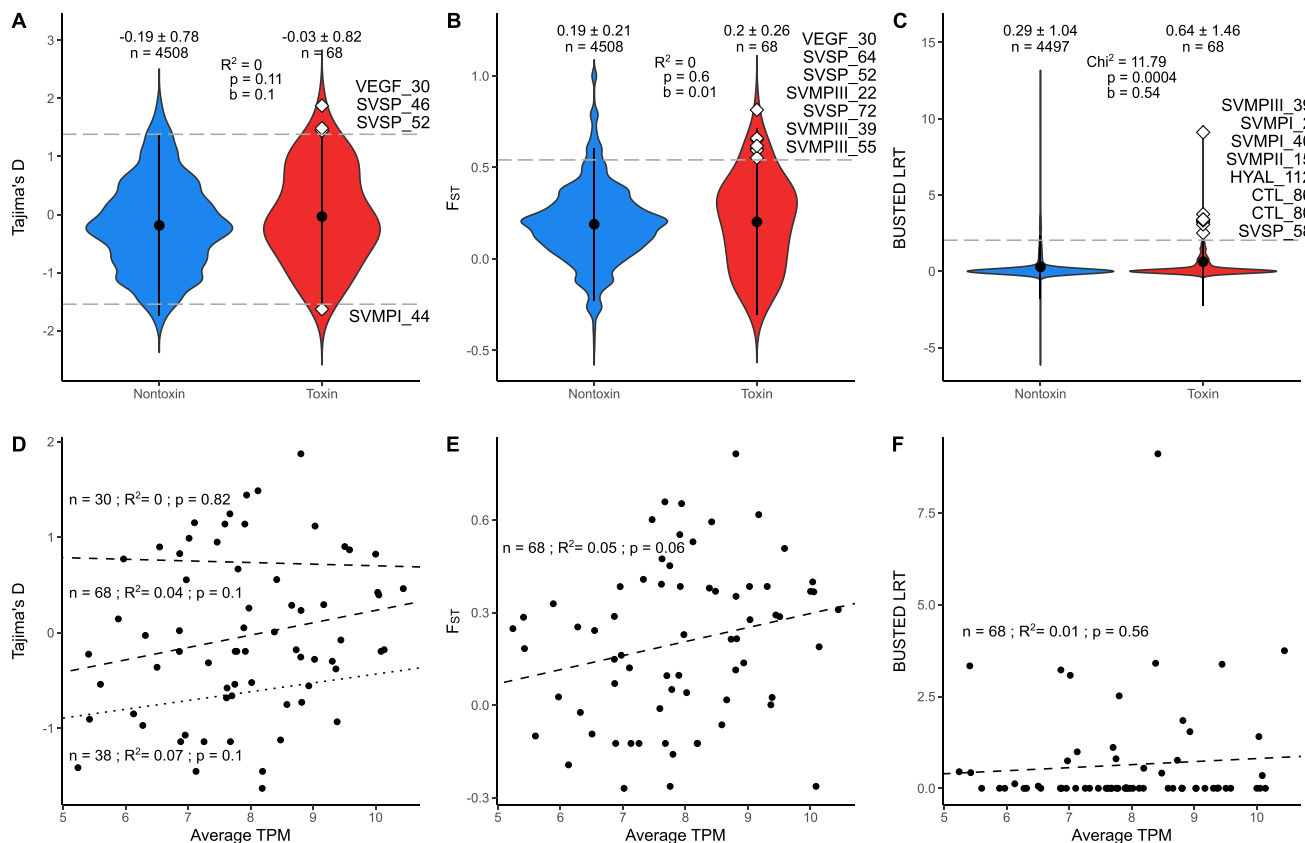
onymous and synonymous variants, and the mean  $\pm$  standard deviation values of nucleotide diversity ( $\pi$ ), Tajima's D,  $F_{ST}$ , and BUSTED model likelihood ratio test ( $LRT$ )

| Class    | Transcripts | SNPs   | SNPs/Kbp          | Total Non-synonymous | Total Synonymous | Nucleotide Diversity ( $\pi$ ) | Tajima's D         | $F_{ST}$          | BUSTED ( $LRT$ )  |
|----------|-------------|--------|-------------------|----------------------|------------------|--------------------------------|--------------------|-------------------|-------------------|
| Nontoxin | 4,508       | 32,657 | 6.766 $\pm$ 4.308 | 8,563 (26%)          | 24,094 (74%)     | 0.315 $\pm$ 0.081              | -0.187 $\pm$ 0.777 | 0.189 $\pm$ 0.209 | 0.287 $\pm$ 1.038 |
| Toxin    | 68          | 390    | 6.423 $\pm$ 3.580 | 236 (61%)            | 154 (39%)        | 0.325 $\pm$ 0.084              | -0.035 $\pm$ 0.824 | 0.203 $\pm$ 0.255 | 0.638 $\pm$ 1.458 |

and nonsynonymous variants ( $\frac{dN}{dS}$ ) and tests for evidence that at least one site on at least one test branch has experienced positive selection. To compare toxins against nontoxins, we used a nonparametric Kruskal–Wallis test as the  $LRT$  results were not normal (Fig. 6C). We found a significant relationship between the class of genes and the  $LRT$  ( $\chi^2 = 11.79$ ,  $df = 1$ ,  $p < 0.01$ ,  $b = 0.54$ ) with the toxins having a higher mean which indicates that they are more likely to experience selection than nontoxins ( $\bar{x}_{nontoxins} = 0.29 \pm 1.04$ ,  $\bar{x}_{toxins} = 0.64 \pm 1.46$ ). However, only one of the toxins (SVMPIII\_39) analyzed was found to be under significant positive selection ( $LRT$   $p < 0.05$ ).

### Relationship Between Expression and Sequence Evolution in Toxins

To test for a relationship between the expression level in toxins and the sequence divergence, we used the natural log of the average expression of the toxins (TPM). We then evaluated if the differentially expressed genes between populations were under stronger selective pressures or had higher sequence divergence. First, we performed linear regression between the average expression and Tajima's D. Our models showed that Tajima's D increased with average expression, however, the relationship was not significant and explained very little of the variation ( $R^2 = 0.04$ ,  $p = 0.10$ ). Next, we tested if toxins with Tajima's D  $> 0$  or  $< 0$  (i.e.,



**Fig. 6** Selection plots. Top: estimates of selection using **A** Tajima's D, **B**  $F_{ST}$ , and **C** Likelihood Ratio Test (LRT) for the BUSTED model for Toxins and Nontoxins, each with the Nontoxin 95th percentile (dotted lines) to identify outlier toxins. The toxin family and the rank based on highest-to-lowest average expression in the transcriptome is displayed for toxins which fall outside the 95th percentile. Bottom:

Linear regressions of the Toxin's mean expression (Average TPM) and estimates of selection including **D** Tajima's D, **E**  $F_{ST}$ , and **F** LRT of the BUSTED model. For Tajima's D, dotted lines are regressions considering all the transcripts (center), just positive values (top) and just negative values (bottom)

those experiencing balancing or positive selection pressures, respectively) had different relationships with expression. In concordance with the full analysis, toxins with Tajima's D  $< 0$  show a positive trend with higher selection pressures being exerted on lowly expressed toxins. This trend was inverted in toxins with Tajima's D  $> 0$ ; however, neither group was significant (Fig. 6D). Similarly, differential expression did not predict the strength of selection in toxins ( $R^2 < 0.01$ ,  $p = 0.91$ ) (online resource, Fig. S8). Expression level also failed to significantly predict  $F_{ST}$ , but did show a trend of increased population differentiation in the toxins with higher expression ( $R^2 = 0.05$ ,  $p = 0.06$ ) (Fig. 6E). Differential expression between populations was also not a significant predictor of toxin  $F_{ST}$  ( $R^2 = 0.04$ ,  $p = 0.10$ ), which suggests that changes in the expression are not correlated with divergence in the sequences of the genes between populations (online resource, Fig. S8). The nonparametric test of the LRT and the differentially expressed toxins was significant ( $\chi^2 = 4.76$ ,  $p = 0.03$ ); however, the LRT was not significantly correlated with the average expression of

toxins ( $R^2 = 0.01$ ,  $p = 0.56$ ) (Fig. 6F). Nucleotide diversity ( $\pi$ ) is not correlated with the differential expression between populations ( $R^2 < 0.01$ ,  $p = 0.72$ ), or to the average expression of the genes (log transformed for normality;  $R^2 = 0.03$ ,  $p = 0.14$ ; online resource 1, Fig. S8). Overall, our results suggest that there is no correlation between expression level and signals of selection in toxins.

### Selection on Individual Toxins

We used the distribution of nontoxin values as a reference to identify individual toxins that are outside the 95th percentile of the nontoxins for Tajima's D,  $F_{ST}$ , and BUSTED model LRT (Fig. 6A–C). With Tajima's D, we identified four toxins, three with values greater than 0 which suggests balancing selection (1 VEGF, 2 SVSPs), and one with a value less than 0 suggesting positive selection (SVMPI). Similarly, we found seven toxins with significantly higher  $F_{ST}$  values (1 VEGF, 3 SVMPIIIs, 3 SVSPs), and eight toxins with a

significantly higher LRT value (3 CTLs, HYAL, 2 SVMPIs, SVMPII, SVMPIII, SVSP). From these toxins, only three were found across multiple methodologies (VEGF\_30, SVSP\_52, SVMPIII\_39).

## Discussion

### Venom Composition and Expression Variation

The venom of *Cerrophidion* is dominated by five toxin families: SVMPIs, PLA<sub>2</sub>s, SVSPs, CTLs, and BPPs (Fig. 2, 3). These toxin families are generally found in high abundance within the venoms of Crotalinae species (Amazonas et al. 2018; Hofmann et al. 2018; Almeida et al. 2021; Strickland et al. 2018a; Tasoulis and Isbister 2017). The venoms of *Cerrophidion* species likely have proteolytic and hemolytic activity given the high percentages of SVMPIs, as well as myotoxic activity due to the presence of PLA<sub>2</sub>s similar to the gK PLA<sub>2</sub>s of *Crotalus* (e.g., Gutiérrez et al. 1985; Gené et al. 1989; Arni et al. 1999; Lizano et al. 2000; Chen et al. 2004; Durban et al. 2011; Lomonte et al. 2012, 2014).

We found significant intraspecific variation in toxin expression in *C. tzotzilorum* (Fig. 2C), which could indicate local adaptation between the northern and southern populations (Fraser et al. 2011; Strickland et al. 2018b). This has been observed in other pitviper species, where rapid toxin expression evolution occurred due to local adaptation between mainland and island populations; however, this was restricted to lowly expressed toxins (Margres et al. 2016). Here, we found both lowly and highly expressed toxins, including the most highly expressed toxin, were differentially expressed between populations of *C. tzotzilorum* (online resource 1, Fig. S3). According to Margres et al. (2016) the observed difference herein might be explained by the difference in divergence time. *Cerrophidion* lineages are more ancient compared to the intraspecific lineages studied by Margres et al. (2016). Margres et al. (2014a), observed a similar pattern to ours when looking at range-wide geographic variation in toxin expression as opposed to island vs mainland in *C. adamantus*.

Lastly, the distribution of *C. tzotzilorum* is relatively small and discontinuous, which may facilitate either local adaptation or drift in these isolated populations resulting in the differential expression of toxins. Such local adaptation in different geographic regions has been observed in other species with broad distributions, such as *Crotalus scutulatus* (Strickland et al. 2018b). In contrast, our toxin sequence evolution results for *C. godmani* suggest that mutation–drift could also be driving evolution in toxin differential expression between populations as it does for sequence evolution.

We similarly found that *C. godmani* showed significant variation between the southern and northern populations

(Fig. 3B and C). In the northern population of *C. godmani*, the PLA<sub>2</sub>s were expressed less than in the southern population, however, the toxin with the highest expression in the northern population was a PLA<sub>2</sub> not expressed in the southern population. This toxin is an acidic PLA<sub>2</sub> (gA1) related to PLA<sub>2</sub>s from *Bothrops* and sister to a clade of PLA<sub>2</sub>s from *Gloydius* (Figs. 4, 5). The southern population of *C. godmani*, in general, exhibited higher expression of PLA<sub>2</sub>s than the northern population; however, this difference was not significant for total PLA<sub>2</sub> expression. Nonetheless, there were several PLA<sub>2</sub> isoforms differentially expressed between the two populations. Notably, one individual (CHFCB-0272) expressed PLA<sub>2</sub>s similar to both subunits of crotoxin-like toxins of other genera. The remaining individuals showed nearly no expression of these PLA<sub>2</sub>s. The venom of the southern population also showed a large percentage of SVMPIs. The venom of CHFCB-0272 was composed of 21.60% crotoxin subunits, 25.48% other PLA<sub>2</sub>s, 26.62% SVMPIs, and 26.30% other toxins families.

*Cerrophidion petlalcalensis* exhibited little variation in the composition of toxins in its venom (Fig. 2B). Our samples came from populations in Veracruz, Mexico. New populations have recently been discovered in Oaxaca, Mexico, several kilometers south of Veracruz (Torre-Loranca et al. 2019). We lack information regarding the venom of these populations and additional sampling might recover intraspecific venom variation. However, *C. petlalcalensis* diverged from *C. tzotzilorum* in the Pliocene, probably by a vicariant event due to the tectonic movement that removed the highlands connections between both sides of the Isthmus of Tehuantepec (Daza et al. 2010; Castoe et al. 2009). It is likely that this lineage underwent a bottleneck post-divergence resulting in a population with low genetic diversity given the short branch lengths between *C. petlalcalensis* individuals in our species tree. This low genetic diversity may have resulted in largely homogeneous venom phenotypes within this population. Samples from the newly discovered population would facilitate our understanding of the evolutionary history of the species as they would allow us to assess the genetic diversity of the species and test for differential expression of the toxins among populations as we did with *C. godmani* and *C. tzotzilorum*.

The sampled individual of *C. sasai* differed in the percentages of toxin families from what has been previously described for this species using proteomics (Fig. 2A). Here, we found a higher expression of PLA<sub>2</sub>s and lower expression of SVSPs than Lomonte et al. (2012). The differences might be methodological artifacts, given that we investigated the venom gland transcriptome and Lomonte et al. (2012) analyzed the venom directly. Transcriptome and proteome comparisons have variable results, with high or low correlation depending on the methodologies used (Rokytka et al. 2015a; Durban et al. 2011). Another potential source of variation

might be that Lomonte et al. (2012) used pooled venom samples from several individuals and therefore profiling within species variation in the expression of toxin families. However, given the isolated distribution of *C. sasai* and the low genetic variation within the species (Sasa 1997), the venom likely has little variation similar to *C. petlalcalensis*.

### PLA<sub>2</sub>s in *Cerrophidion*

The evolution of PLA<sub>2</sub>s has been extensively studied, particularly in rattlesnakes (Glenn et al. 1994; Wooldridge et al. 2001; Mackessy 2008; Dowell et al. 2016, 2018; Whittington et al. 2018). Unique combinations of different PLA<sub>2</sub> paralogs have been found in the venom of rattlesnakes both between and within species, and ancestral character reconstruction suggests that the specific arrangement is the result of gene loss (Dowell et al. 2016, 2018). The most recent common ancestor (MRCA) of rattlesnakes likely possessed an expanded array of PLA<sub>2</sub> paralogs, including the two subunits that form the crotoxin-like neurotoxins (Dowell et al. 2016). *Cerrophidion* species show a high diversity of PLA<sub>2</sub>s with unique paralog compositions based on our phylogeny. *Cerrophidion petlalcalensis* has gB1 and gA1 PLA<sub>2</sub>s and lacks gK PLA<sub>2</sub>s; *C. sasai* has gK and gA1 PLA<sub>2</sub>s and lacks gB1 PLA<sub>2</sub>s. *Cerrophidion tzotzilorum* has copies of gA1, gB1, and gK PLA<sub>2</sub>s in both populations and differs in the expression of the paralogs. *Cerrophidion godmani* seems to have different PLA<sub>2</sub> gene composition between populations as only the northern population has the gA1 PLA<sub>2</sub> *C. godmani*\_4, while only the southern population has the gA1 PLA<sub>2</sub> *C. godmani*\_1. Additionally, the southern population seems to be polymorphic given the presence of the crotoxin-like subunits in CHFCB-0272, this individual expresses all the other paralogs as well. The composition of PLA<sub>2</sub> genes in *Cerrophidion* seems to have evolved as in *Crotalus*, with unique arrangements originating from gene loss. However, there are individuals of *C. godmani* that express all the PLA<sub>2</sub> groups, as in the hypothetical rattlesnake MRCA proposed by Dowell et al. (2016). This suggests that the genotype with all proposed PLA<sub>2</sub> paralogs was present in the MRCA of *Cerrophidion* and rattlesnakes, and likely in the MRCA of all New World pitvipers.

The *C. godmani* acidic PLA<sub>2</sub>, similar to the gA2 group, exhibits a proline in position 127 (Fig. 4C at site 5), hypothesized to be the ancestral state of acidic PLA<sub>2</sub>s prior to the evolution of the gA2 group (Whittington et al. 2018). A proline in that position blocks the cleavage recognition residual (phenylalanine) in position 126 (Keil 1992) and cleavage of this site is necessary for the folding of a functional acidic crotoxin subunit (Whittington et al. 2018). A substitution of proline for other amino acids unlocks the cleavage recognition residual specific for chymotrypsin-like SVSPs (Whittington et al. 2018; Keil 1992). *Bothriechis*, *Crotalus*, and

*Gloydus* have a serine at position 127 in their homologs of gA2 PLA<sub>2</sub>s and the recently discovered melanurutoxin from *Mixcoatlus melanurus* has a leucine, demonstrating that different amino acids at position 127 have the potential to unlock this site (Neri-Castro et al. 2020b; Whittington et al. 2018) (Fig. 4C). Whether the toxins present in *C. godmani* are functional as a chaperone and phospholipase activity agonist for the basic subunit is still unknown (Radvanyi and Bon 1982). If they are neurotoxic, this would mean that a different cleavage recognition residue at site 5 might facilitate cleavage. The homolog of the acidic subunit of *C. godmani* shows sequence differences upstream of position 127, with a tryptophan and two consecutive phenylalanines at positions 124–126. This may cause local changes in secondary structure or protease recognition that may facilitate an alternative cleavage site in this homolog. According to the peptide cutter tool from the ExPASy server ([https://web.expasy.org/peptide\\_cutter/](https://web.expasy.org/peptide_cutter/); Gasteiger et al. 2005) (online resource 1, Fig. S9), tryptophan or phenylalanine residues might act as cleavage recognition sites, although the tryptophan at position 124 has a higher probability of being cleaved (Fig. 4C, red asterisk). Additionally, this tryptophan residue is highly solvent exposed (site-specific solvent accessible surface area (SASA) 104.31 at position 126, online resource 2, Table S2), which would permit attack by a protease. An alternative hypothesis is that the acidic subunit homologs of *C. godmani* have functions similar to other acidic PLA<sub>2</sub>s given that the sequence of *C. godmani*\_4 and the gA1s from different taxa in the same group (*Gloydus* and *Bothrops*) have tryptophan and two phenylalanines at positions 124–126. The expression levels of the subunits support the former hypothesis for the presence of a crotoxin-like toxin; representing 11.89 and 9.70% of the total venom expression for the acidic and basic subunits, respectively, within CHFCB-0272. Further research on the biological activity and on the proteomics of the venom is needed to discover the function of these PLA<sub>2</sub>s. Clinical records could also help to understand effects of the venom, although snake bite reports from Mexico and Central America usually lack a trustworthy identification of the species involved in the envenomation (Neri-Castro et al. 2020a; Gutiérrez 2014).

### Toxin Sequence Variation and Selection

Our results suggest that toxins and nontoxins are evolving under the same evolutionary pressures supporting a main influence of mutation–drift equilibrium. Tajima's D distributions for toxins and nontoxins were similar with slightly more variation in toxins. However, an F-test showed that the variance of toxins and nontoxins do not differ significantly ( $F = 0.85$ ,  $p = 0.13$ ), suggesting it is unlikely that diverse selection pressures are acting on the toxins and therefore masking a true significant difference (Rautsaw et al. 2019).

A pattern of toxin evolution by mutation–drift equilibrium has been found in other species including *Bothriechis nigroviridis*, *B. nubestris*, *Crotalus cerastes*, *Protobothrops mucrosquamatus*, and *Sistrurus catenatus* (Rautsaw et al. 2019; Aird et al. 2017; Ochoa et al. 2020; Mason et al. 2020).

Several factors might influence the evolutionary history of toxins, particularly effective population size and diet (Ochoa et al. 2020; Holding et al. 2021b; Mason et al. 2022). In the case of *C. godmani*, data regarding population sizes are lacking, though Campbell and Lamar (2004) suggested they are abundant within their range. It is likely that they form isolated populations with low gene flow given that they inhabit high elevation, discontinuous mountain ranges. The mean  $F_{ST}$  (Nontoxins = 0.181, Toxins = 0.182) suggest moderate differentiation between populations, indicating low gene flow at least between the populations sampled herein. Their isolated, patchy distribution might result in smaller effective population sizes, thereby increasing the influence of drift in these populations and potentially masking the effects of selection. Including more populations in future studies might change our current interpretation as the population genetic structure of the species is unknown. Margres et al. (2019) found that species with more generalist diets do not diverge in venom unless there is absence of gene flow. *Cerrophidion godmani* is considered a diet generalist (Schramer et al. 2018; Campbell and Solórzano 1992), and the mean values of  $F_{ST}$  suggest partial differentiation between southern and northern populations. Both toxin and nontoxin genes show differentiation between populations but were not significantly different from one another, suggesting that toxins are not experiencing directional selection. Instead, balancing selection may play a larger role in toxin differentiation in this species (Margres et al. 2019). The venoms of species with phylogenetically diverse diets evolve under balancing selection that maintains an increased number of toxins (Holding et al. 2021b). Our results partially support this pattern in *C. godmani*, as its diet is phylogenetically diverse and its venom could be considered complex considering the number of toxins in the consensus transcriptome (a total of 117). However, our results do not suggest a predominant influence of balancing selection on the toxins. Instead, toxins appear to be in mutation–drift equilibrium within *C. godmani*. These results are limited by the sample size and filtering protocols used here. Specifically, toxins that could be experiencing directional selection in some individuals or are new paralogs of toxin genes might have not been considered as they are not present in most of the individuals and thus not analyzed. A population-wide study would help to further clarify the evolutionary history of these toxins, including genes homologous to crotoxin subunits found in

one individual. Only a small number of toxin genes seem to be experiencing directional or balancing selection. According to Holding et al. (2021b), the primary toxin families associated with more complex venom in snakes with phylogenetically diverse diets are PLA<sub>2</sub>s, SVMs, and SVSPs. Further, Schield et al. (2022) found signals of balancing selection driving sequence evolution in PLA<sub>2</sub>s, SVMs, and SVSPs within and between *Crotalus oreganus* and *Crotalus viridis*. We would expect that these families evolved by balancing selection, assuming a complex venom and a phylogenetically diverse diet. In *Cerrophidion godmani*, the toxins subjected to balancing selection belong to the toxin families VEGF and SVSP(2), according to Tajima's D. Therefore, our results do not support the balancing selection hypothesis as more toxins appeared to be in mutation–drift equilibrium. However, our analysis is focused only on intraspecific variation, whereas the study of Holding et al. (2021b) addressed variation on both larger taxonomic and time scales. A more extensive study on Neotropical pitvipers might show agreement with what is known for Nearctic pitvipers, though it is also possible that given the different evolutionary pressures and demographic histories experienced by Nearctic and Neotropical snakes, similar patterns may not be expected to be maintained.

A few toxins—including SVM, SVSP, and VEGF toxin families (Fig 6A–C)—were regularly found outside of the nontoxin's 95<sup>th</sup> percentile distribution in our selection analyses. These toxin families have a wide variety of biological functions; however, they might be most closely associated with hemorrhagic effects in their prey (Fox and Serrano 2005; Yamazaki et al. 2009; Jones et al. 2022). *Cerrophidion godmani* has a hemorrhagic venom (Jones et al. 2022), which suggests that these few toxins have key functions in prey acquisition by causing massive tissue degradation and internal bleeding.

Expression is not correlated with signals of selection on toxin genes. We found a nonsignificant trend of increasing values of Tajima's D,  $F_{ST}$  and LRT with higher average expression in the toxins of the venom. This trend suggests that highly expressed toxins are evolving by balancing selection and toxins with low expression have more signals of positive selection. This pattern contrasts with what was found for *Crotalus cerastes*, where the lower expressed toxins are evolving by balancing selection (Rautsaw et al. 2019). Although expression does not correlate with the signals of sequence selection there is clear expression differentiation between populations in *C. godmani* given the number of genes that show differential expression (Fig. 5), which might indicate that selection in the venom is acting on levels of expression instead of on sequence evolution.

## Conclusions

We found evidence of venom variation between and within species: *Cerrophidion petlalcalensis* has a homogeneous venom, with little variation between individuals; *C. godmani* and *C. tzotzilorum* showed intraspecific variation in the toxins present and in the expression of individual toxins. This variation was observed between geographic populations, as well as by body size, suggesting possible ontogenetic shifts in the venom composition. Overall, sequence variation in toxins and nontoxins in *C. godmani* was not significantly different across multiple tests for selection. Instead, the evolution of toxin genes seems to be driven by mutation–drift equilibrium with no relationship to toxin expression. The presence of myotoxic PLA<sub>2</sub>s gK seems to be widespread in most *Cerrophidion* species, except *C. petlalcalensis*. Additionally, acidic and basic subunits of homologous crotoxin-like PLA<sub>2</sub>s were discovered in one specimen of *C. godmani*, which suggests that this species might have the heterodimeric crotoxin-like neurotoxin. These PLA<sub>2</sub>s were found in a single, southern population individual, which suggests there is intraspecific variation for the presence of this trait as observed in other pitviper genera such as *Crotalus*. Overall, our combined results support the hypothesis that drift is potentially the key evolutionary force shaping sequence evolution in highland pitviper species.

## Materials and Methods

### Sample Collection

We collected six specimens of *C. godmani* from two populations in Mexico: two from the Central Highlands of Chiapas (Las Margaritas), and four from the Sierra Madre de Chiapas, close to the border with Guatemala (Union Juarez); four specimens of *C. tzotzilorum* from two populations in the Central Highlands of Chiapas, Mexico (Rayon Mescapala and San Cristobal de las Casas); three specimens of *C. petlalcalensis* from San Andres Tenejapa (Veracruz, Mexico); and one specimen of *C. sasai* from Las Nubes Coronado, San Jose, Costa Rica (Fig. 1, Table 1). We extracted venom from the individuals by allowing the snakes to bite a sterile cup covered with parafilm. Four days after the venom extraction, we euthanized the snakes with an injection of sodium pentobarbital ( $100 \frac{\text{mg}}{\text{kg}}$ ) and excised the venom glands in order to maximize mRNA transcription (Rotenberg et al. 1971). We preserved the glands in RNAlater at 4°C, then moved the glands to permanent storage at –80°C. All protocols involving live snakes followed ASIH guidelines and were approved by the Clemson University Animal Care and Use Committee (Animal Use Protocol 2017-067).

## cDNA Libraries Prep and Sequencing

RNA extraction and sequencing were done following Hofmann et al. (2018). Briefly, we diced the venom glands and placed them in a TRIzol solution (Invitrogen). The homogenized mixture was transferred to a phase-lock heavy gel tube (5Prime). We waited until the cells were lysed, and then isolated and purified RNA using chloroform followed by isopropyl alcohol and ethanol precipitation. We quantified RNA using a Qubit RNA BroadRange kit, and checked the quality of the RNA using a Bioanalyzer 2100 with an RNA 6000 Pico Kit (Agilent Technologies). The mRNA was isolated with NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB#E7490). We prepared cDNA libraries using a NEB Next Ultra RNA Library Prep Kit for Illumina (NEB#E7530) following the manufacturer's recommendations. We used a fragmentation time of 13 minutes, 30 seconds to achieve a target mean fragment size of 400 bp, and 14 PCR cycles for amplification of double-stranded cDNA libraries. We quantified library yield and quality with a Bioanalyzer 2100, and determined the total amplifiable concentration of cDNA using KAPA qPCR. We pooled equal concentrations of samples and assessed the final concentration and quality of our pooled libraries with a Bioanalyzer and with KAPA qPCR. The libraries were sequenced on an Illumina HiSeq2500 or NovaSeq 6000 platform at the Translational Science Laboratory in the College of Medicine at Florida State University.

### Transcriptome Assembly

We trimmed the adaptors and low-quality sequences of the raw Illumina sequences with TrimGalore! (v0.6.6, <https://github.com/FelixKrueger/TrimGalore>), set to trim reads with a quality score lower than 5 and a length below 75 bp. We merged the forward and reverse reads with PEAR (v0.9.6) (Zhang et al. 2014). For the subsequent analyses we used GNU parallel 20201122 software for multiprocess computing (Tange 2020). We used Trinity (v2.11.0) (Grabherr et al. 2011), SeqMan Ngen (v14) (using the Lasergene DNASTar software package; Madison, WI, USA: <https://www.dnastar.com/t-nextgenseqman-ngen.aspx>), and Extender (Rokyta et al. 2012) to *de novo* assemble contigs following the recommendations of (Holding et al. 2018). We merged the assemblies and ran cd-hit-est (Fu et al. 2012) with a sequence identity threshold of 1 to filter redundancy in our combined assembly. We annotated toxin and nontoxin sequences with ToxCodAn (Nachtigall et al. 2021), a method that uses general Hidden Markov Models (gHMM) to identify the toxin genes. We complemented the output of ToxCodAn with a manual annotation of toxin sequences.

Manual annotation was done following the methodology in (Hofmann et al. 2018). Briefly, we ran blastx (v2.10.1) against UniProt animal venom proteins and toxins database (<https://www.uniprot.org/program/Toxins>), then we used SignalP (Petersen et al. 2011) and cd-hit-est to automatically annotate the genes and extract the coding sequences from the contigs by comparing the contigs to a database of existing pitviper transcripts (Rokyta et al. 2013). The genes that were not automatically annotated were manually annotated using the output of blastx. Specifically, we compared all potential open reading frames (ORF) to the blastx results and annotated the best-matched ORF, with regard to percent identity and sequence length, with the appropriate toxin family. We concatenated the output of ToxCodAn and of the manual annotation and ran cd-hit-est with a threshold of 1 to remove the redundant sequences. We ran ChimeraKiller (<https://github.com/masonaj157/ChimeraKiller>) to eliminate chimeric sequences. Briefly, reads are mapped to the annotated transcriptome and transcripts with zero coverage at any position are removed. Chimeric transcripts are then reported by searching for a difference > 75% (-d 0.75) in the average length of reads on either side of a given site based on the average read size. A final manual revision was done to check for misplaced sequences. We merged the sequences cleaned by ChimeraKiller and ran cd-hit-est with a threshold of 0.99 to keep only one copy of each gene. We made a consensus species transcriptome by concatenating the transcriptomes of all individuals for each species and running cd-hit-est with a threshold of 0.98 to remove any variants between individuals. Additionally, we filtered sequences with internal stop codons, no stop codons, or an incomplete translation frame.

### Expression Quantification and Differential Expression

We used the consensus transcriptomes generated for each species and the merged reads to calculate the expression of the genes using RSEM (Li and Dewey 2011) with Bowtie2 as the aligner with default settings. We then removed the toxins that were below the 5% percentile of average expression to avoid including contamination from highly expressed toxins from other samples sequenced with our samples. We repeated the RSEM (Li and Dewey 2011) expression analysis with the reduced data set (see online resource 2, Table S3, S4, S5, S6). For visualization, we took the mean transcripts per million reads (TPM) for each toxin in a given species. Results were plotted using (R Core Team 2020) with the plotting script included in ToxCodAn. Names of the toxins in the figures were set as the toxin family and the ranking of average expression of the toxin for the species (see online resource 2, Table S8, S9, S10, S11). We used expected counts from RSEM and R

packages DESeq2 (Lov et al. 2014) and edgeR (Robinson et al. 2010) to test for differences in expression between northern and southern populations for *C. godmani* and for *C. tzotzilorum*. *Cerrophidion petlalcalensis* and *C. sasai* were excluded from differential expression analyses given a lack of population-level sampling and body size variation in the samples. We used SVL to test if body size influences gene expression as a proxy for ontogenetic shifts. Although sexual dimorphism of the venom has been found in some species (Zelanis et al. 2012, 2016), that is not always the case (Saviola et al. 2015; Franco-Servín et al. 2021). We did not add sex as a factor in our experimental design as we lacked of biological replicas of at least one of the sexes in every species. For DESeq2, we used a Wald significance test with a local fit dispersion and used a false-discovery rate (FDR) < 0.05 as the threshold for differentially expressed genes. For edgeR, we used a likelihood ratio test (LRT) and, as in DESeq2, we used a FDR < 0.05 as the significance threshold. We repeated DESeq and edgeR test for the accumulated expression of each gene family including all nontoxin genes as both packages use all information to fit the dispersion. We created heatmaps of the expression of the toxins in R with package pheatmap (Kolde 2019) to visualize differences in expression and differential expression among populations or across SVL.

### PLA<sub>2</sub> Phylogeny and Proteomic Alignment

To reconstruct the PLA<sub>2</sub> phylogeny for *Cerrophidion*, we combined the PLA<sub>2</sub> sequence data from (Whittington et al. 2018; Neri-Castro et al. 2020b; Mason et al. 2020), and additional PLA<sub>2</sub> sequences available in GenBank (see online resource 2, Table S1). We extracted the annotated PLA<sub>2</sub>s of the consensus transcriptome for each of our species and aligned them with the other sequences using MAFFT v7.475 (Katoh and Standley 2013). We used CIALign v1.0.10 and trimal v1.4.rev15 to clean and trim the alignment (Tumescheit et al. 2020; Capella-Gutiérrez et al. 2009). As the *Python bivittatus* PLA<sub>2</sub> was discarded by CIALign we concatenated the sequence at the end of the cleaning and trimming process and realigned with MAFFT. We used this alignment as input for IQTree using ModelFinder and 1000 ultrafast bootstrap replicates (Kalyaanamoorthy et al. 2017; Hoang et al. 2018; Nguyen et al. 2015). The PLA<sub>2</sub>s we generated were translated with Biopython (Cock et al. 2009) while the PLA<sub>2</sub>s from GenBank were downloaded as amino acid sequences. The theoretical isoelectric points were calculated with biopython (Cock et al. 2009). The translated sequences were aligned with omega clustal algorithm in Geneious Prime 2020.2.4 (<https://www.geneious.com/>; Sievers and Higgins 2018). The cleavage sites of the sequences similar to the acidic subunit of crotoxin were estimated using the

ExPASy Peptide Cutter tool ([https://web.expasy.org/peptide\\_cutter/](https://web.expasy.org/peptide_cutter/); Gasteiger et al. 2005). We modeled the structure of the homolog of the gA2 following (Whittington et al. 2018). We used the X-ray crystal structure of the Crotoxin from *Crotalus durissus terrificus* (pdb:3R0L; Faure et al. 1991) as template for modeling. The model was built using Modeller v 10.2 (Eswar et al. 2006). We built 10 independent structures with the method slow VFTM optimization for 500 iterations, and slow MD refinement. The refinement was repeated 4 times. The models were evaluated using the DOPE-HR. We choose the model with the lower DOPE-HR score to calculate the SASA using GetArea (<http://curie.utmb.edu/getarea.html>; Fraczkiewicz and Braun 1998).

### SNPs Calling and Selection Analysis

We followed the methodology of Rautsaw et al. (2019) for variant calling with our *C. godmani* data. We excluded the other species for this analysis due to low sample size or lack of population sampling. Briefly, we mapped the merged reads to the consensus transcriptome with BWA-MEM (Li 2013). We used Picard v2.12.1 (<http://broadinstitute.github.io/picard/>) to sort and index the aligned reads. We followed GATK v3.8.1 and v4.1.9 ([software.broadinstitute.org/gatk/](http://software.broadinstitute.org/gatk/)) SNP calling pipeline (Auwera et al. 2013); we performed a local realignment of regions with indels, removed reads shorter than 120 nucleotides, called variants, performed joint genotyping, and filtered SNPs. We phased the variants using WhatsHap v0.15 (Martin et al. 2016). We then filtered mapped contigs with a transcript coverage of 0 for more than 5% of total length in order to avoid partial transcripts and poorly mapped reads. We kept genes for analysis if they were present in at least three individuals based on the coverage filter and if they had at least one SNP. We additionally removed toxins that were under the 5% percentile in the first RSEM analysis.

We used Tajima's D, Weir and Cockerham's  $F_{ST}$ , and Nucleotide Diversity ( $\pi$ ) to test if toxins have more selective pressures than the nontoxin genes following (Rautsaw et al. 2019). We used several selection metrics as some of them can potentially be influenced by demographic history and population structure (e.g., Tajima's D; Nielsen 2005). Additionally, we used the BUSTED model from HyPhy 2.5.31(MP) (Murrell et al. 2015) to test for signals of positive selection in whole genes and used the LRT results to test for significant differences in selection between toxins and nontoxins. First we separated nonsynonymous and synonymous SNPs using SnpEff v.5.0 using our consensus transcriptome as a reference (Cingolani et al. 2012). We calculated the SNPs per kilobase for each gene. We use this value to test for significant differences between toxins and nontoxins using linear regression. We also tested if the type of mutation (i.e., nonsynonymous or synonymous) was

significantly associated with the type of gene (toxin or nontoxin) with a  $\chi^2$  test. We used vcftools v0.1.16 (Danecek et al. 2011) to calculate the nucleotide diversity ( $\pi$ ), Tajima's D per gene and per site, and Weir and Cockerham's  $F_{ST}$  comparing northern and southern populations. We performed all statistical analyses in R software (R Core Team 2020).

Testing for selection with HyPhy 2.5.31(MP) (Murrell et al. 2015) requires a species tree. To generate a phylogeny, we recovered 3,530 single-copy BUSCO loci with BUSCO v5.2.2 (Simão et al. 2015). Next, we aligned each locus with MAFFT v7.475 (Katoh and Standley 2013), and cleaned the alignments with CIALign v1.0.10 (Tumescheit et al. 2020). We made preliminary gene trees with IQtree using ModelFinder and 1000 ultrafast bootstrap replicates (Kalyaanamoorthy et al. 2017; Hoang et al. 2018; Nguyen et al. 2015). We used the consensus trees and the cleaned alignments to run TreeShrink (Mai and Mirarab 2018). TreeShrink detects and removes outlier long branches in a collections of trees. We recovered the output alignments from TreeShrink and cleaned them again using CIALign. Finally, we trimmed the alignments with trimAl v1.4.rev15 (Capella-Gutiérrez et al. 2009). We made final gene trees with IQtree using ModelFinder and 1000 ultrafast bootstrap replicates. We concatenated the final 2,122 gene trees with the highest likelihood, and used ASTRAL v5.7.7 (Zhang et al. 2018) to obtain a species tree with default settings (Fig. 1). As the branch lengths from ASTRAL are expressed in coalescent units and all terminal tips have the same length, we constrained the resulting ASTRAL tree topology and re-scaled branch lengths using IQtree. Briefly, we concatenated the BUSCO loci present in all the individuals (306), then used a custom script to concatenate the genes and delimit the partitions of the concatenated alignment to check for the best model for each gene. We used IQtree to select the better evolution model for each partition and scale the tree generated with Astral (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017; Nguyen et al. 2015). We trimmed the branches of *C. godmani* of the species tree using the ape R package (Paradis and Schliep 2019). We chose one of the two WhatsHap phased alleles at random for each locus and each individual as input for the BUSTED model (HyPhy v2.5.31) using the species tree as the reference phylogeny (Murrell et al. 2015; Pond et al. 2020). The BUSTED models test for evidence that at least one site on at least one test branch has experienced diversifying selection. We analyzed 4,497 nontoxins and 68 toxins with this method; for ten nontoxins the substitution resulted in one internal stop codon for one individual.

For nucleotide diversity, we calculated the mean  $\pi$  (Nei and Li 1979) for each gene and used a linear regression to test for significance in  $\pi$  between toxin and nontoxin genes. To avoid the effect of the sample size driving significant differences regardless of effect size given that the number of nontoxins is higher than the toxins, we performed

bootstrapping by taking a random sample of the nontoxins matching the sample size of toxins and repeated the linear regression 1,000 times. We report the proportion of replicates with  $p < 0.05$  in the linear regression as bootstrap support. Similarly, we use linear regression and bootstrapping to test for significant differences with Tajima's D (Tajima 1989), and  $F_{ST}$  (Wright 1949) between toxins and nontoxins. For Tajima's D, we ran a t-test to evaluate if the mean value from toxins and nontoxins was different from 0; and repeated the linear model using only synonymous SNPs. We performed a nonparametric Kruskal–Wallis test to compare toxins and nontoxins Tajima's D for nonsynonymous SNPs due to their nonnormal distribution. We used a nonparametric Kruskal–Wallis test to compare the values of the likelihood ratio test (LRT) for the BUSTED model between toxins and nontoxins. As for the linear regression tests, we used bootstrapping for the nonparametric tests with 1000 iterations. To identify specific toxins under selection, we generated a null distribution from the nontoxin transcripts and identified toxins outside the 95<sup>th</sup> percentile of the distribution for Tajima's D (two-tail),  $F_{ST}$  (right tail), and LRT of BUSTED model (right tail). To test if low-expression toxins were under stronger positive or balancing selection pressures, we used linear regression with average toxin expression (ln average TPM) as the response variable and Tajima's D,  $F_{ST}$ , and LRT of BUSTED model, we ran a regression for each variable. We tested positive and negative values of Tajima's D separately to account for potential differences in expression related to alternate selection pressures. We used linear regression and bootstrapping as in the previous steps.

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**Author Contributions** RARG, EPH, and CLP conceived the study. CIG, HFC, ITAC, RRC, MADL, MB, GCG, MS, MLH, and RMR collected samples used in this study. RMR, EPH, MLH, JLS, AJM, and RARG generated data, RARG, RMR, and EPH processed data and performed analyses. RARG drafted the manuscript. JLS, AJM, DRR, EAM, MLH, and CLP provided analytical and conceptual input. RMR, EAM, EPH, AJM, JLS, MLH, and CLP reviewed and edited the manuscript. All authors reviewed and approved the final manuscript.

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**Data Availability** The raw data-sets generated and analyzed during the current study are available in the National Center for Biotechnology Information (NCBI) repository under the accession numbers SRR19227107, SRR19227106, SRR19227101, SRR19227100, SRR19227099, SRR19227098, SRR19227097, SRR19227096, SRR19227095, SRR19227094, SRR19227105, SRR19227104, SRR19227103, and SRR19227102. The consensus transcriptomes generated and analyzed during the current study are available at the github repository [https://github.com/RamsesRosales/Cerrophidion\\_Selection](https://github.com/RamsesRosales/Cerrophidion_Selection), a similar version is available at the DDBJ/EMBL/GenBank under the accession numbers: *C. sasai* GJZX000000000, the version described in this paper is the first version, GJZX010000000; *C. petalcalensis* GKAV000000000, the version described in this paper is the first version, GKAV010000000; *C. godmani* GKCA000000000, the version described in this paper is the first version, GKCA010000000. *C. tzotzilorum* GKIB000000000, the version described in this paper is the first version, GKIB010000000. Scripts utilized in this work can be found in the online resources 3 and 4, and in the github repositories: [https://github.com/RamsesRosales/Cerrophidion\\_Selection](https://github.com/RamsesRosales/Cerrophidion_Selection), <https://github.com/RamsesRosales/ModelCrotA>.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Consent for Publication** Not applicable

**Ethics Approval** All protocols involving live snakes followed the ASIH guidelines and were approved by the University of Central Florida and Clemson University Animal Care and Use Committees (16-17W: UCF and 2017-067: CU). The sample (LIAP244) was collected in and exported from Costa Rica under Investigation Permit SINAC-ACC-OSJ-re-428-2021. Samples (CHFCB-0271, CHFCB-0272, CHFCB-0274, CHFCB-0290, CHFCB-0291, CHFCB-0300, CHFCB-0236, CHFCB-0238, CHFCB-0239, CHFCB-0276, CHFCB-027, CHFCB-0296, CHFCB-0471) were collected in and exported from Mexico under SEMARNAT:SGPA/DGVS/01090/17;SGPA/DGVS/002288/18;SGPA/DGVS/08831/20.

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