

## Venom phenotype conservation suggests integrated specialization in a lizard-eating snake

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### ABSTRACT

Biological specialization reduces the size of niche space while increasing efficiency in the use of available resources. Specialization often leads to phenotypic changes via natural selection aligning with niche space constraints. Commonly observed changes are in size, shape, behavior, and traits associated with feeding. One often selected trait for dietary specialization is venom, which, in snakes, often shows variation dependent on diet across and within species. The Neotropical Blunt-headed Treesnake (*Imantodes cenchoa*) is a highly specialized, rear-fanged, arboreal, lizard hunter that displays a long thin body, enlarged eyes, and a large Duvernoy's gland. However, toxin characterization of *I. cenchoa* has never been completed. Here, we use RNA-seq and mass spectrometry to assemble, annotate, and analyze the venom gland transcriptomes of four *I. cenchoa* from across their range. We find a lack of significant venom variation at the sequence and expression levels, suggesting venom conservation across the species. We propose this conservation provides evidence of a specialized venom repertoire, adapted to maximize efficiency of capturing and processing lizards. Importantly, this study provides the most complete venom gland transcriptomes of *I. cenchoa* and evidence of venom specialization in a rear-fanged snake, giving insight into selective pressures of venom across all snake species.

### 1. Introduction

Biological specialization restricts the use of available resources while increasing the efficiency of using specific ones. In turn, specialists have a narrow niche which facilitates their ability to compete against generalists when environmental conditions are constant (MacArthur and Pianka, 1966; Armbruster, 2017). Specialization can lead to the use of a specific habitat almost exclusively, such as feeding on a small suite of prey, or any other extreme reduction in niche space where possible

resources are underutilized (Armbruster, 2017). Diet specialization is a common form of biological specialization (Svanbäck and Persson, 2004), and often coincides with phenotypic modifications that increase prey acquisition and/or handling efficiency (Santos and Cannatella, 2011). Broadly, phenotypic integration due to diet specialization can include changes in morphology, coloration, behavior, and weaponry (Pigliucci, 2003).

In many of the lineages in which venom has evolved, it is thought to be specialized for diet acquisition (Schendel et al., 2019). Venom acts to

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disrupt the physiology of prey and results in faster incapacitation, decreased handling, and a lower risk of retaliation from prey items (Casewell et al., 2013). Because venom is composed of many individual toxins, selection can tailor venom in many different ways resulting in specialization to specific prey (da Silva Jr and Aird, 2001; Davies and Arbuckle, 2019; Margres et al., 2019; Lyons et al., 2020; Holding et al., 2021). An example of venom specialization is in the venom of the cone snail, *Conus leopardus*, which has a reduced number of toxins when compared to closely related species because it exclusively eats acorn worms (Remigio and Duda Jr, 2008). Another way for venom to be specialized is if a specific toxin has clade-specific effects and only disrupts the physiology of that group (e.g., lizards but not mammals) (Pawlak et al., 2006, 2009; Heyborne and Mackessy, 2013; Modahl et al., 2018; Harris et al., 2020). Lastly, venom specialization can also occur when the entire venom cocktail works best on a specific group of taxa as seen in spiders (Pekár et al., 2018) and snakes (da Silva Jr and Aird, 2001; Barlow et al., 2009) with preferred prey.

Rear-fanged snakes are becoming a common study system for understanding venom function and specialization (Mackessy, 2002; Saviola et al., 2014; Junqueira-de Azevedo et al., 2016; Modahl et al., 2016; Mackessy and Saviola, 2016; Modahl and Mackessy, 2019; Schramer et al., 2022; Cerda et al., 2022). Rear-fanged snake venom is homologous with venom from front-fanged species (Viperidae, Elapidae, Atractaspidinae), and some rear-fanged species are medically relevant, even lethal, to humans (Weinstein et al., 2013, 2022). Most rear-fanged species produce venom in a modified gland posterior to their eye called a Duvernoy’s gland which excretes venom passively and introduces it into

prey via grooves in the back teeth while chewing (Minton and Weinstein, 1987; Hill and Mackessy, 2000; Mackessy, 2002; Kardong, 2002). Rear-fanged species generally have either three-finger toxin (3FTx) dominated or metalloproteinase dominated venoms (Modahl and Mackessy, 2019). Cysteine-rich Secretory Proteins (CRISPs) are also a common component of rear-fanged venoms (Junqueira-de Azevedo et al., 2016; Modahl and Mackessy, 2019). Since most rear-fanged species do not appear to use their venom for defense (Ward-Smith et al., 2020; Weinstein et al., 2011), they are ideal to understand the ecological role of venom in prey capture and specialization.

*Imantodes* (Blunt-headed Treesnakes) is a genus of rear-fanged snakes composed of nine species which have similar morphologies including long thin bodies, enlarged eyes, and a large Duvernoy’s gland relative to their head size (Torres-Carvajal et al., 2012; Missassi and Prudente, 2015; Aveiro-Lins et al., 2006). The Neotropical Blunt-headed Treesnake, *I. cenchoa*, is the most widespread and well studied species (Fig. 1). A diet study from the Brazilian Amazon that looked at 103 individuals with prey found that 85% of the diet consisted of two lizard genera, (*Gonatodes* and *Norops*), and the remaining 15% was unidentifiable, but included lizard fragments (de Sousa et al., 2014). Dietary records from Ecuador and Costa Rica have also been documented, showing similar dietary preferences across the species range (Duellman, 1978; Savage, 2005). Additionally, *I. cenchoa* are morphologically and anatomically specialized. The enlarged eyes presumably increases visual acuity in locating sleeping lizards and the long thin body and internal organs being located more posterior in the body shifts the center of gravity which allows *I. cenchoa* to crawl to the end of the branches

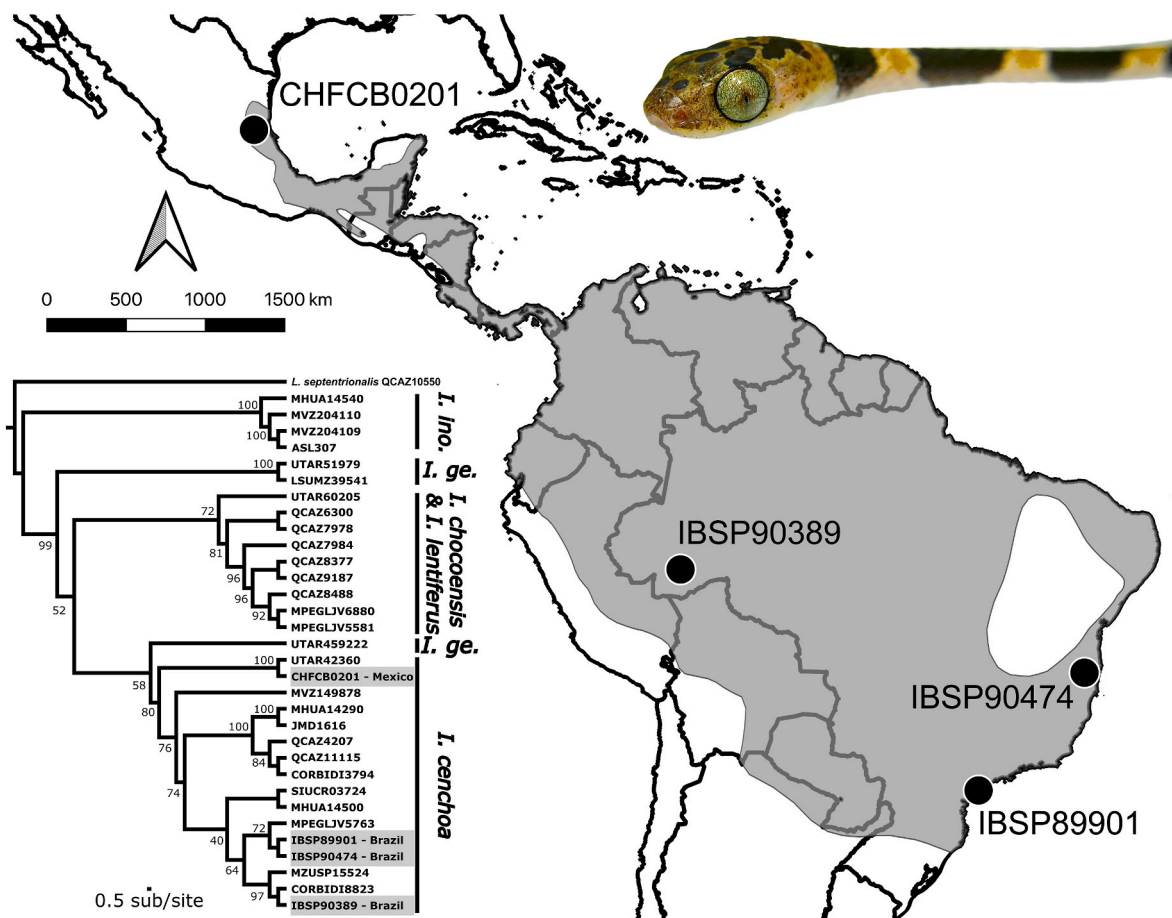


Fig. 1. Distribution map of *Imantodes cenchoa* in grey with the four samples and their locations used in this study marked with black dots. Lower Left Insert: Maximum Likelihood phylogeny of the genus *Imantodes* with the four individuals indicated based on country. *Leptodeira septentrionalis* was used as the outgroup and five of the nine species within *Imantodes* are included: *I. inornatus* (*I. ino.*), *I. gemmistratus* (*I. ge.*), *I. chocoensis*, *I. lentiferus*, and *I. cenchoa*. Photo of *I. cenchoa* (CHFCB0201) by Michael Hogan.

without causing vibrations that might wake the lizards (Aveiro-Lins et al., 2006). The morphology and diet specialization co-occur with behavioral specialization (i.e., nocturnal and arboreal) to hunt lizards that sleep at the ends of branches at night.

*Imantodes cenchoa* demonstrates remarkable morphological, anatomical, and behavioral specialization to hunt lizards, but no studies have been conducted on their venom. To determine if venom specialization is occurring in a species with these adaptations, we sequenced and investigated the venom gland transcriptome of four individuals of *I. cenchoa*, proteomically confirmed the presence of venom toxins, and compared toxin gene expression among individuals. We further analyzed toxin gene evolution by comparing the amount of sequence divergence that has accumulated in the toxin and nontoxin loci. Due to the specialization on lizards in the diet, we hypothesize that the proportion of toxins will be similar in all individuals. Additionally, due to the geographic distance between our samples and the biogeographic barriers that exist between them, we expect sequence divergence has accumulated in both toxin and nontoxin loci at a similar rate due to isolation by distance. If our hypothesis is supported, then we expect that selection is working to maintain a specific phenotype but mutations are able to accumulate due to genetic isolation.

## 2. Materials and methods

### 2.1. *Imantodes cenchoa* sample collection

We collected one adult *Imantodes cenchoa* from Mexico and three adult *I. cenchoa* from Brazil (Table 1 and Fig. 1). Upon capture, all specimens were processed for venom, venom glands, and deposited into a natural history museum in accordance with permit specifications. We collected venom by anesthetizing the snake with isoflurane until righting response was lost. We then subcutaneously injected pilocarpine (7 µg/g in water) into the dorsal side of the snake 3 cm from the back of the head to stimulate venom secretion (Rosenberg, 1992; Hill and Mackessy, 1997). After 5 min, we placed the snake on a stand where the enlarged back tooth of the top jaw on each side of the mouth was positioned onto a capillary tube (Hill and Mackessy, 1997). The snake remained on the stand for 30 min while venom was collected in each of the capillary tubes. Venom was expelled into a cryo tube, lyophilized, and stored at -80 °C until needed. We were unable to get venom from IBSP89901. We sacrificed each snake four days after venom stimulation (Rotenberg et al., 1971), removed the venom glands, placed each gland in RNAlater (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and preserved the specimen in 10% buffered formalin for five days and then transferred to 70% ethanol for deposition. The specimen in Mexico was collected under a scientific collecting permit to M.B. (SEMARNAT: SGPA/DGVS/01090/17) and handled with approval from Clemson University's IACUC (2017-067). One specimen in Brazil was donated to the Animal Reception Service of Instituto Butantan (register number 20180418291) and the other two collected in the field (ICMBio permit Nos 65653 and 65634) and the glands and venom were collected with the approval of the Instituto Butantan's IACUC (CEUA 4479020217).

**Table 1**

Specimen information for the four *Imantodes cenchoa* used in this study.

Museum ID	Field ID	Country	State	SVL	Sex	Mass	Locality	Merged Reads	Toxins/Nontoxins
Number	Number			(mm)		(g)			
CHFCB0201	CLP2288	Mexico	San Luis Potosí	555	Male	10.4	Ciudad Valles	12,780,450	32/1827
IBSP89901	SB0319	Brazil	São Paulo	675	Male	14.9	Iguape	25,522,474	32/2452
IBSP90389	SB0587	Brazil	Acre	644	Male	10.44	Sena Madureira	25,547,560	32/2317
IBSP90474	SB0699	Brazil	Minas Gerais	810	Female	38.12	Santa Maria do Salto	28,126,405	28/2137

SVL—Snout to Vent Length; CHFCB—Colección Herpetológica Facultad de Ciencias Biológicas; IBSP—Coleção Herpetológica do Instituto Butantan; CLP—Christopher L. Parkinson Field Number; SB—Scales of Biodiversity.

### 2.2. Venom gland transcriptome sequencing and assembly

We extracted total RNA from each individual's left and right glands separately using TRIzol and chloroform following Rokytka et al. (2011). We combined RNA in equal concentration from each gland and prepared cDNA libraries for each sample by isolating mRNA with an NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB E7490S) and then reverse transcribed the mRNA to cDNA using the NEB Next Ultra RNA Library Prep Kit for Illumina (NEB E7530) (Strickland et al., 2018a; Ward et al., 2018). For cDNA library synthesis, we used a fragmentation time of 13 min and 30 s and 14 PCR cycles. CHFCB0201 was sequenced on an Illumina HiSeq 2500 at the Florida State University College of Medicine Translational Science Laboratory (Tallahassee, FL, USA). IBSP89901, IBSP90389, and IBSP90474 were sequenced on an Illumina HiSeq 1500 at Instituto Butantan (São Paulo, São Paulo, Brazil). CHFCB0201 and IBSP89901 were sequenced using 150bp paired-end reads and IBSP90389 and IBSP90474 were sequenced using 250bp paired end reads.

After sequencing, we used Trim Galore! 0.5.0 (<https://github.com/FelixKrueger/TrimGalore>) to trim reads and only kept base calls with a phred score at or above 5. Trimmed reads were merged using PEAR v. 0.9.6 (Zhang et al., 2014). The trimmed, merged reads were then assembled using three different de novo methods: NGEN v. 14 (Lasergene DNASTar software package; Madison, WI) with default parameters, Trinity v. 2.6.6 (Grabherr et al., 2011) with a kmer size of 31 and a minimum contig length of 200, and Extender as described in Rokytka et al. (2012) with 120 bp overlaps on 1000 merged read random seeds. Using multiple assembly methods gives the best chance to assemble each of the toxins families and is in line with best practices for venom gland transcriptomics (Holding et al., 2018a).

### 2.3. Species verification and phylogenetic analysis

Because *I. cenchoa* has a large distribution and nothing is known about the phylogeography of the species or the evolutionary history of the genus, we used phylogenetics to verify species identity and ensure monophyly of the four individuals in this study. We used available *Imantodes* sequences from GenBank which limited our analysis to two mitochondrial genes, *cytb* and *ND4*, and five of the nine species (Supplemental Data). We used MITGARD to extract the mitochondrial genome from the transcriptome sequences (<https://github.com/pedr-onachtigall/MITGARD>) and extracted both genes in Geneious. We aligned each gene in Geneious using MAFFT v. 7.407 (Katoh and Standley, 2013) and then concatenated the two alignments. We generated a Maximum Likelihood phylogenetic tree using IQTREE v. 1.6.12 (Nguyen et al., 2015) and partitioned by gene and codon position (-sp), with 1000 ultrafast bootstrap replicates (-bb 1000) (Hoang et al., 2018), and used ModelFinder (Kalyanamoorthy et al., 2017) within IQTREE.

### 2.4. Mass spectrometry

For the three individuals with venom, we used mass spectrometry (MS) to ensure no toxins were missed during transcriptome annotation and to verify annotated toxins in the transcriptome (Rokytka et al.,

2015a). We followed Rokyta and Ward (2017) and Ward et al. (2018) for MS data generation and analysis with the following modifications. Proteomics was performed at the Florida State University Department of Biological Sciences Core Facility. We used the Nanodrop 2000c (Thermo Scientific) to determine the protein quantification of each of the three venom samples. To prepare the sample for trypsin digestion, we used 11 µg of venom and added 150 µL of 100 mM Ammonium Bicarbonate. After 20 min, we added 30 µL of 10 mM DTT (Dithiothreitol) and incubated at 60 °C for 1 h. Then, we added 30 µL of 50 mM IAA (Iodoacetamide) and after 30 min added 150 µL of 50 mM Ammonium Bicarbonate. We digested the sample by adding 1 unit of Trypsin (Promega V511 at 0.5 µg/2.5 µL) diluted in 50 mM Ammonium Bicarbonate and incubated at 37 °C for 18 h. To stop digestion, we added 1% TFA (Trifluoroacetic acid) at 5% volume of the solution.

Following digestion, we lyophilized the venom and resuspended in 0.1% formic acid to a concentration of 250 ng/µL. We ran each venom sample in triplicate at room temperature and used three digested *Escherichia coli* proteins (Abcam) of known concentration as standards: 25 fmol of Beta-lactamase ampC (P00811), 250 fmol of Protein deglycase 1 (P31658), and 2500 fmol of Chaperone protein FimC (P31697). For MS, 2 µL of digested venom was run on a Thermo Q Exactive HF high-resolution electrospray tandem mass spectrometer (Thermo Scientific) with an UltiMate3000 RSLCnano System (Thermo Scientific). To separate the protein fragments, we used an Acclaim PepMap RSLC 75 µm 15 cm nanoviper column (Thermo Scientific) and a flow rate of 300 nL/min. We ran a linear gradient over 1 h from 3% to 45% B (solvent A—99.9% H<sub>2</sub>O (EMD Omni Solvent) and 0.1% formic acid; solvent B—99.9% acetonitrile and 0.1% formic acid). We nanosprayed the eluent into the spectrometer and operated the detector in data-dependent mode with the Thermo Excalibur 3.1.66 data system software (Thermo Scientific). We targeted ions with a value of 10<sup>5</sup> during high energy collisional dissociation and scanned from 350 to 1700 m/z at a resolution of 60,000 in profile mode.

We compared the MS results to all coding sequences in our combined venom gland transcriptome (EMBOSS function: getorf -find 1 -minsize 90; Rice et al. (2000)) using Proteome Discover 2.2 software (Thermo Scientific) and SequestHT as the search engine with the following parameters: enzyme name = Trypsin, precursor mass tolerance = 10 ppm, minimum peptide length = 6, maximum peptide length = 144, maximum delta Cn = 0.05, dynamic modifications, carbamidomethyl +57.021 Da(C), oxidation +15.995 Da(M), maximum missed cleavage = 2, and fragment mass tolerance = 0.2 Da. We confirmed the identified proteins in Scaffold v. 4.10.0 (Proteome Software Inc., Portland, OR, USA) with a false discovery rate of 1.0%. Any coding sequence with a peptide match in one of the three runs was added to the combined assembly for that individual from above and used in transcript annotation. If a protein passed all quality checks and was detected in one of the three replicates, it was considered proteomically confirmed.

## 2.5. Reversed-phase High Performance Liquid Chromatography

We were able to conduct proteomic profiling for the three *I. cenchoa* with venom using Reversed-phase High Performance Liquid Chromatography (RP-HPLC). We used an Aeris 3.6 µm C18 column (Phenomenex, Torrance, CA) connected to the Shimadzu Prominence HPLC system with a SIL-30AC autosampler. We used two standard solvents (A = 0.1% trifluoroacetic acid (TFA) in water and B = 0.06% TFA in acetonitrile) at a flow rate of 0.2 mL/min for 125 min as follows: 10% B for 5 min, gradual increase to 55% B over 110 min, gradual increase to 75% B over 5 min, 5 min at 75%B, and then a wash at 10%B for 15 min in preparation for the next sample. We used the retention time and peak intensity to visualize the RP-HPLC profiles for the three samples and injected 15 µg of venom in 50 µL of LC/MS water for each individual.

## 2.6. Transcriptome annotation and visualization

To annotate transcripts, we first used a toxin and nontoxin database compiled from previous studies (Rokyta et al., 2011, 2012, 2013, 2015b, 2017). Transcripts from the combined file were annotated if they matched at ≥90% or better using cd-hit-est v. 4.7 (Li and Godzik, 2006; Fu et al., 2012). All transcripts that were not annotated were then annotated with respect to the Uniprot animal venom protein and toxins database (downloaded 22 January 2019) via blastx v. 2.2.31+ searches. We kept the top ten matches with an e-value of 0.0001 or better and annotated in Geneious Prime v 2019.0.4 (Biomatters Ltd., Auckland, New Zealand. <https://www.geneious.com>). We extracted the coding sequence from annotated sequences for each individual and removed duplicates with the BBtools package Dedupe (Joint Genome Institute, Department of Energy, Walnut Creek, California, USA) implemented in Geneious. We removed chimeric transcripts by aligning merged reads to the transcripts with BWA-MEM v. 0.7.17 (Li, 2013) using a custom script (<https://github.com/masonaj157/ChimeraKiller>). Any transcripts with uneven or zero coverage were removed.

To reduce allelic variation in each individuals' transcriptome, we clustered each individual 98% using cd-hit-est (Fu et al., 2012). We ensured that each transcript was expressed in the venom gland by aligning the merged reads for each individual to their consensus transcriptome with BWA-MEM (Li, 2013). We removed reads with four or more gaps or nucleotide differences (Rokyta et al., 2017). We considered transcripts absent if there was less than 5x coverage over ≥10% of the transcript (Hofmann et al., 2018). To generate transcriptome profiles, we mapped the merged reads for each individual to their consensus transcriptome with the Bowtie2 aligner (Langmead et al., 2009; Langmead and Salzberg, 2012) in RSEM (Schridder et al., 2011). We imported the transcripts per million reads (TPM) data into RStudio v 1.2.5033 with R v. 3.6.2 (R Core Team, 2019). We removed zero TPM values using the cmultRepl function in zCompositions (Palarea-Albaladejo and Martín-Fernández, 2015) which also maintained the compositional structure of the dataset (Aitchison, 1986; Rokyta et al., 2015a).

## 2.7. Ortholog identification

We used OrthoFinder v. 2.2.6 (Emms and Kelly, 2015, 2019) to identify orthologous transcripts among the four *I. cenchoa* transcriptomes to test for differential expression and for sequence comparisons. OrthoFinder uses the protein sequence and determines orthogroups by identifying genes that likely share a common ancestor among the individuals being compared (Emms and Kelly, 2015, 2019). Ideally, each orthogroup would contain one transcript from each of the four individuals. However, for some toxin families, due to sequence conservation and recent expansion, some orthogroups contained more than four sequences. For these, we used the nucleotide sequence and clustered based on similarity so that orthogroups contained one-to-one matches for all individuals. Nontoxins that were not found in all four individuals were excluded. All venom components were kept as there were no toxins unique to a single individual. We used MAFFT v. 7.407 (Katoh and Standley, 2013) to align the sequences for each orthogroup and exported the consensus sequence using a majority rule for ambiguities for the orthologous transcript consensus transcriptome.

## 2.8. Differential expression analysis

To test for differential expression of any of the orthologous transcripts, we used country as treatment in DESeq2 (Love et al., 2014). We first mapped merged reads in RSEM v. 1.3.1 (Schridder et al., 2011) with default parameters and the Bowtie2 aligner (Langmead et al., 2009; Langmead and Salzberg, 2012) to the consensus orthologous transcripts. We imported the count data into RStudio v. 1.2.5033 with R v. 3.6.2 (R Core Team, 2019) using the tximport function (Soneson et al., 2015). For analyses, we used a false discovery rate (FDR) of 0.05 and a pairwise

comparison of our two treatments, Mexico and Brazil. We used the log-fold change to identify the direction of expression differences and a DESeq2 adjusted p-value threshold of 0.05 as significant. If the mean expression was too low to calculate an adjusted p-value in DESeq2, the unadjusted p-value was used. Expression analysis was conducted on proteomically confirmed toxins and all other venom-related proteins, such as Proprotein Convertase (PCSK1), VEGF, and Venom Factor. These related proteins have been described in other snake venoms; however, there are many described proteins in these families and their distinction as true toxins is debated (Ferreira et al., 2021; Vogel and Fritzing, 2017).

## 2.9. Toxin sequence divergence

To examine evolution of toxin sequences in *I. cenchoa*, we called single-nucleotide polymorphisms (SNPs) using a combination of BWA-MEM, Picard Tools, and GATK. Specifically, merged reads were mapped to the consensus orthologous transcriptome using BWA-MEM (Li, 2013). Reads with more than two mismatches were removed. Picard Tools (broadinstitute.github.io/picard) was used to sort and index aligned reads prior to using GATK (software.broadinstitute.org/gatk/) for local realignment. Next, GATK was used to remove reads less than 120 bp and with a mapping quality less than 40. GATK HaplotypeCaller and GenotypeGVCFs were used to call SNPs which were filtered with the following criteria: QD  $\geq$  2.0, FS  $\leq$  60.0, MQ  $\geq$  40.0, MQRankSum  $\leq$  -12.5, ReadPosRankSum  $\leq$  -8.0. Finally, SNPs were phased using WhatsHap (Patterson et al., 2015) and alleles were generated using bcftools (<http://samtools.github.io/bcftools>) and the consensus ortholog transcriptome. We masked regions which had no coverage using bedtools (Quinlan and Hall, 2010; Quinlan, 2014). For analyses, phased SNPs were merged into a single file using bcftools.

To summarize variant data and look for differences between toxins and nontoxin transcripts, we first used SnpEff to determine which SNPs encoded nonsynonymous vs. synonymous mutations (Cingolani et al., 2012). With these data, we calculated SNPs per kilobase (kb) for each transcript as well as the total number of nonsynonymous and synonymous mutations. To assess if toxin transcripts had a greater number of SNPs than nontoxins, we used a *t*-test on 1) the total number of SNPs/kb, 2) the number of synonymous SNPs/kb, and 3) the number of nonsynonymous SNPs/kb. In addition, we performed a  $\chi^2$  test to determine if there is a significant association between the type of mutation (i.e., synonymous vs. nonsynonymous) and the type of transcript (i.e., toxin or nontoxin). Next, we calculated Tajima's D using vcftools (Danecek et al., 2011). If toxins have undergone selective sweeps or purifying selection, we expect to find a negative Tajima's D value that is significantly different from nontoxin transcripts, which we expect to evolve neutrally or under purifying selection. Finally, we calculated nucleotide diversity ( $\pi$ ) using vcftools (Danecek et al., 2011). We tested for significant differences using *t*-tests in R with the package ggpubr (R Core Team, 2019; Kassambara, 2020).

To test for evidence of positive selection in toxin loci, we calculated dN/dS ( $\omega$ ) for each transcript (Rautsaw et al., 2019). First, we estimated a species tree using the nontoxin allele sequences generated by bcftools for input in PAML (Yang, 2007). Specifically, unrooted nontoxin gene trees were inferred using IQTREE with 1000 bootstrap replicates (Nguyen et al., 2015). Gene trees were concatenated and an unrooted species tree estimated using ASTRAL-III (Zhang et al., 2018). This tree was used as input for PAML CODEML to estimate  $\omega$  and compare models of selection. For each gene, we tested CODEML models M0, M1, and M2. We used M0 estimates of  $\omega$  to determine if toxin  $\omega$  was significantly different from nontoxins using a *t*-test and – more importantly – if toxins had an  $\omega > 1.0$ . In addition to estimating  $\omega$ , we performed likelihood ratio tests (LRT) comparing model M1 or M7 (neutral) to models M2 and M8 (selection), respectively. We assessed if the calculated LRT statistic was significant different between toxins and nontoxins using a *t*-test and similarly assessed if M2 or M8 was a significantly better fit for toxins (*p* <

0.05).

## 2.10. Data availability

Raw data for venom gland transcriptomes are on the National Center for Biotechnology Information (NCBI) under BioProject PRJNA88989: Sequence Read Archive (SRA) accession SRR12266697 (CHFCB0201); BioProject PRJNA625548: Sequence Read Archive (SRA) SRR12266696 (IBSP89901), SRR12266695 (IBSP90389), and SRR12266694 (IBSP90474) and Biosample accessions SAMN15581662 (CHFCB0201), SAMN15581663 (IBSP89901), SAMN15581664 (IBSP90389) and SAMN15581665 (IBSP90474). The consensus transcriptome for each individual was submitted to the NCBI Transcriptome Shotgun Assembly (TSA) database. This TSA project has been deposited at DDBJ/EMBL/GenBank under accession GKDN00000000. The version described in this paper is the first version, GKDN01000000. Additional data files, including mass spectrometry data, and code used are available at [https://github.com/jstrickland63/Imantodes\\_cenchoa\\_transcriptomics](https://github.com/jstrickland63/Imantodes_cenchoa_transcriptomics).

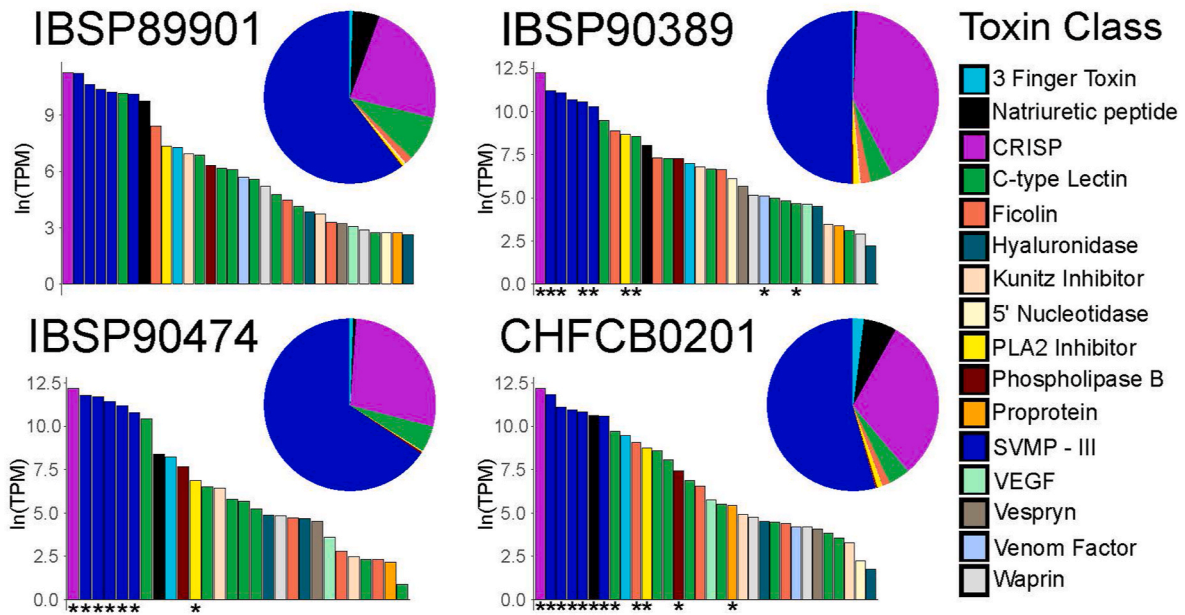
## 3. Results

### 3.1. Venom components of *Imantodes cenchoa*

First, using mitochondrial genes retrieved from the transcriptomes, we confirmed that all four samples fell within a monophyletic clade of *I. cenchoa*, including representatives of the northern and southern distribution limits of the species (Fig. 1 and Table 1). We then analyzed the transcriptomes and recovered venom related transcripts from 16 venom related protein families and identified between 28 and 32 venom components in each transcriptome (Fig. 2). In CHFCB0201, we recovered 32 venom related protein transcripts and 1827 nontoxin transcripts. IBSP89901 had 32 venom related protein transcripts and 2452 nontoxin transcripts. IBSP90389 had 32 venom related protein transcripts and 2317 nontoxin transcripts. IBSP90474 had 28 venom related protein transcripts and 2137 nontoxin transcripts. We recovered 32 unique venom related protein transcripts and 1128 nontoxin transcripts in our consensus transcriptome. Snake venom metalloproteinases (SVMPs) were the most abundant toxin family in each of the four samples, accounting for between 49.6% and 65.5% of the transcriptomes. One cysteine rich secretory protein (CRISP) was the most common single transcript in each individual, representing between 23.2% and 41.5% of the final transcript. The remaining toxin families each accounted for less than 10% of the transcriptomes (Fig. 2). We were able to confirm 7–12 venom components using MS in the three samples with data (Fig. 2). Five SVMPs were identified and confirmed, in each of the four individuals. The one cysteine rich secretory protein (CRISP) was confirmed in the venom for the three individuals with MS data. Natriuretic peptides, Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) Inhibitors, Ficolin, Proprotein Convertase, Phospholipase B, Venom Factor and C-type Lectins were the other families confirmed in the venom of at least one individual (Fig. 2). The RP-HPLC profiles (Fig. S1) show peaks likely corresponding to the CRISP (~79 min) and SVMPs (~85–105 min) (Fry et al., 2003; Osipov et al., 2005; Wagstaff et al., 2009; Chen et al., 2019; Mackessy et al., 2020; Koua et al., 2022); however, fractionation was not performed in this study. OrthoFinder identified 32 venom component orthologs among the four individuals. Three of the four individuals contained all 32 orthologs and the fourth, IBSP90474, was missing five orthologs (Waprin- OG0001497, VenomFactor-1- OG0001496, Nucleotidase-OG0001495, Hyaluronidase- OG0001494, and a C-type lectin-OG0001493). None of the individuals had any unique venom components that were specific to their transcriptomes.

### 3.2. Venom component transcript expression of *Imantodes cenchoa*

Venom component transcript expression was similar among the four individuals and only five components were differentially expressed



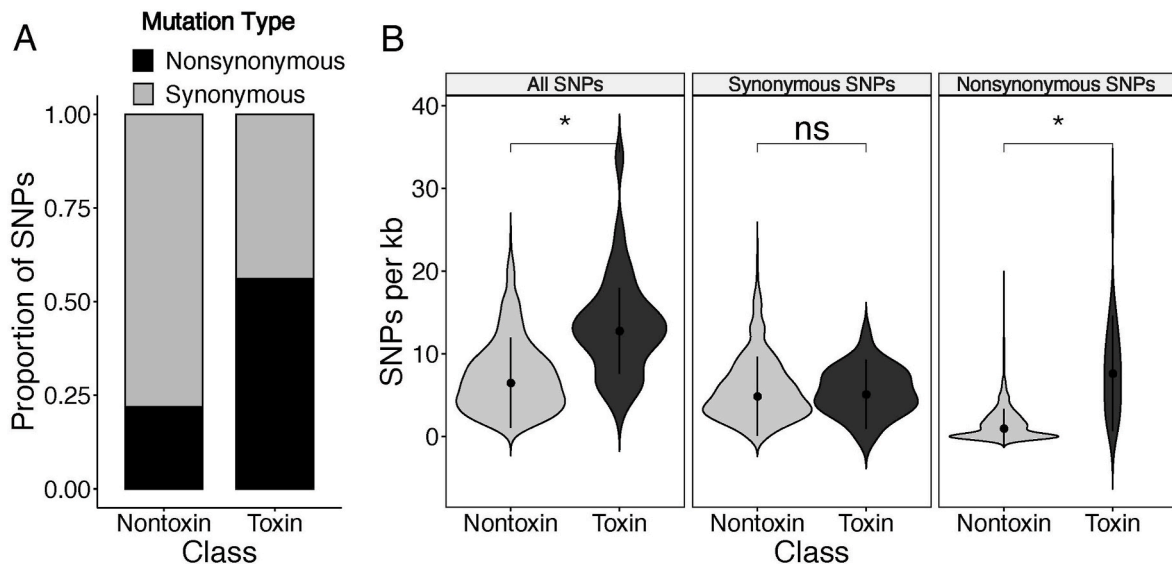
**Fig. 2.** Representations of the venom gland transcriptomes of four *Imantodes cenchoa*. Pie charts represent the proportion of each toxin class and the bar graphs are individual toxin transcripts colored by toxin class. Asterisks indicate toxins confirmed in the venom for the three individuals with venom collected. TPM - Transcripts Per Million reads; CRISP - Cysteine Rich Secretory Protein; PLA2 - Phospholipase A2; SVMP-III - Snake Venom Metalloproteinase Type III; VEGF - Vascular Epithelial Growth Factor.

when we compared the three Brazil individuals to the Mexico individual using DeSEQ2 (Supplemental Data). All five were lowly expressed in each individual. The first, Proprotein Convertase (PCSK1), was expressed at 4.6 times higher in the individual from Mexico (CHFCB0201;  $p < 0.001$ ) and does not have a known toxic function. It was most similar in sequence to proprotein convertase subtilisin/kexin type 1 (PCSK1) from the *Pantherophis guttatus* (GenBank Locus ID: XM\_034440713) and *Thamnophis sirtalis* (XM\_014057760) genomes. The second, VEGF-1, was expressed 3.42 times higher in the Mexico individual ( $p = 0.007$ ), but was lowly expressed with an average TPM of 118.78. It was most similar in sequence to vascular endothelial growth factor A isoform X2 (VEGF-A) from the *Thamnophis elegans* (GenBank Locus ID: P\_032072241) genome and is also not known to have a toxic function. The third, KUN-1, was expressed 1.83 times higher on average

in the three Brazil individuals ( $p = 0.028$ ), but the Mexico individual value was higher than the lowest Brazil individual. One of the 8 CTL orthologs, CTL-OG0001297, was expressed 3.09 times higher in the Mexico individual ( $p = 0.037$ ) and was the third highest expressed CTL in three of the four individuals. Finally, Waprin was expressed 3.41 times higher in the Mexico individual but was lowly expressed overall (average TPM = 98.47). The p-value was 0.0005 which is similar to the unadjusted p-value for CTL-OG0001297.

### 3.3. *Imantodes cenchoa* toxin sequence evolution

Toxin transcripts are significantly associated with nonsynonymous mutations (Fig. 3A). Specifically, toxins have significantly more SNPs per kb (median: 12.80) than nontoxins (median: 6.51), with



**Fig. 3.** The number and type of SNPs found in toxin and nontoxin transcripts. (A) Toxins maintain a higher ratio of nonsynonymous to synonymous SNPs. (B) Toxins have a higher number of SNPs per kilobase (kb) with nonsynonymous SNPs driving this difference (\*: significant; ns: not significant).

nonsynonymous mutations driving this difference (Fig. 3B). This suggests that toxins accumulate mutations — particularly nonsynonymous mutations — at a higher rate than nontoxin transcripts. This pattern can also be seen in the mean pairwise alignment distances across orthologous transcripts where toxins exhibit greater divergence (median: 0.06) than nontoxins (median: 0.04) (Fig. 4C). Despite observing more nonsynonymous mutations in toxins, there is no difference between toxin and nontoxin transcripts in either Tajima's D or nucleotide diversity ( $\pi$ ; Fig. 4A & B). Nucleotide diversity for toxins and nontoxins had medians of 0.29 and 0.33, respectively. Both toxins and nontoxins have positive Tajima's D with medians at 1.80 and 1.70, respectively. However, toxins and nontoxins are not significantly different from one another (Fig. 4A).

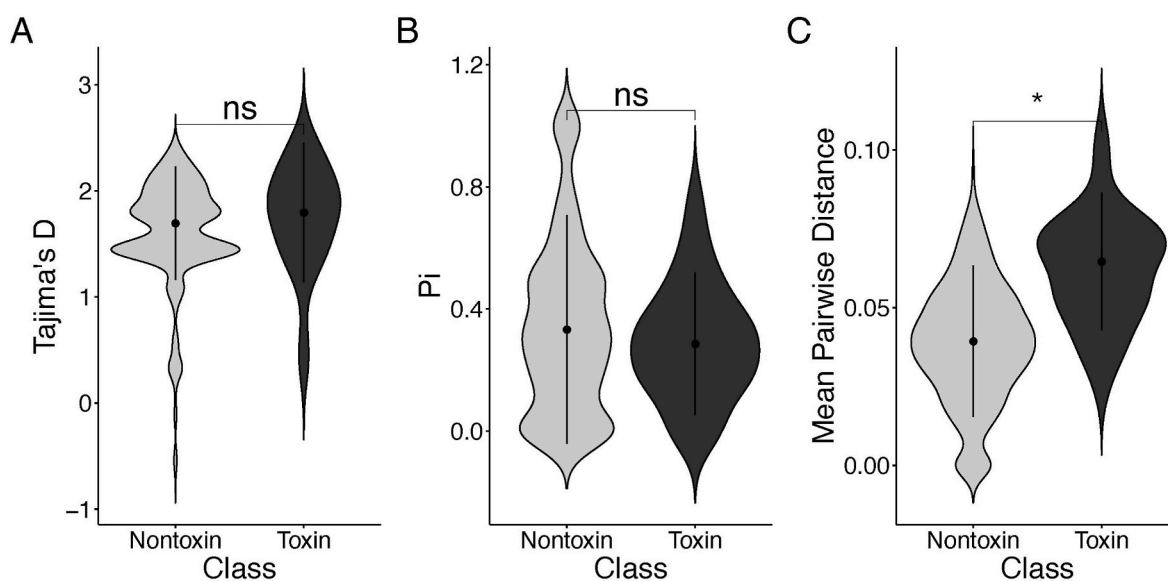
To explicitly test whether positive selection plays a role in shaping toxin sequence divergence, we ran codeml with models M0, M1, M2, M7, and M8. There was a significant difference in  $dN/dS$  ( $\omega$ ) between nontoxins and toxins (Fig. 5A); however, both nontoxins and toxins were found to have a mean  $\omega$  less than one (toxin median: 0.41; nontoxin median: 0.21) suggesting that the majority of sites do not experience positive selection. These results were confirmed by likelihood ratio tests between codeml models M1-M2 and M7-M8 (Fig. 5B & C). Specifically, both comparisons found that toxins are subject to significantly greater selection pressures than nontoxins, but both toxins and nontoxins have a mean likelihood ratio less than the  $\chi^2$  critical value for a significant difference between these two models suggesting that the majority of sites do not experience positive selection in *I. cenchoa*. When we examined specific toxins for signatures of positive selection, there were four orthologs that had an  $\omega > 1$  with model M0: Ficolin-OG0000053a, CTL-OG0001294, CTL-OG0001298, and CTL-OG0001300. When we compared M1 to M2 and M7 to M8, there were only two toxins, SVMPIII-OG0000000c and Ficolin-OG0000053b, that were under positive selection for either comparison.

#### 4. Discussion

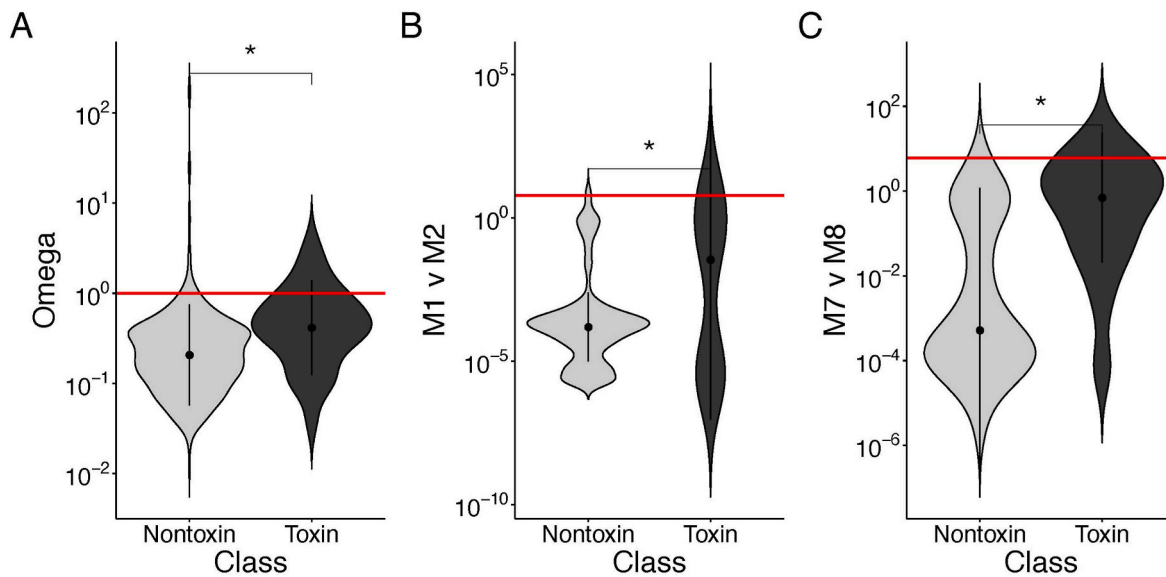
We provide the first characterization of the venom and venom gland transcriptome of a species of Blunt-headed Treesnake, *Imantodes cenchoa*, and find that there is venom conservation across the distribution of the species. While intraspecific variation of venom has also been documented in generalist species (Mora-Obando et al., 2023), when

considering the expansive distribution of *I. cenchoa* (Fig. 1) and the low venom variation at the expression (Fig. 2) and sequence level (Figs. 3 and 4), our data suggest that venom specialization may accompany morphological, anatomical, and behavioral specialization. Among the two primary venom phenotypes for rear-fanged snake species, *I. cenchoa* fits within the SVMP dominated venom category (Modahl and Mackessy, 2019) as only a single 3FTx transcript was identified and it was lowly expressed (Fig. 2). While prey specificity of SVMPs in rear-fanged snakes has not yet been tested, SVMPs have been observed to have adaptations to local prey in rattlesnakes (Holding et al., 2016, 2018b; Margres et al., 2017; Robinson et al., 2021) and have been shown to exhibit different types of structural damage (Herrera et al., 2015). While not exclusive to lizard specialists, SVMP dominated venoms can be found in other rear-fanged snakes that possess a diet largely composed of lizards, such as *Philodryas* (López and Giraudo, 2008; Modahl and Mackessy, 2019). This suggests the different phenotypic traits are likely integrated to increase efficiency of finding, incapacitating, and eating lizard prey.

The individuals used in this study fall within a monophyletic *I. cenchoa* group even though the two most distant samples are separated by a 7500 km straight-line distance and span several recognized geographic breaks (Fig. 1). Based on the size of the distribution, it is surprising that there is so little venom variation at the transcriptomic level (Fig. 2). For many venomous species that have been studied across their distribution, there is intraspecific venom variation present even if the source of the variation is unknown (da Silva Jr and Aird, 2001; Hofmann et al., 2018; Strickland et al., 2018b). However, the lack of variation in *I. cenchoa* is not unprecedented. Coral snakes (*Micrurus fulvius*) in the Southeast USA also lack venom variation which could be due to their diet consisting primarily of other snakes and lizards (Margres et al., 2015). Although we identified up to 32 toxins in the transcriptome, no more than 12 were proteomically confirmed in any single individual. Four of the five SVMP transcripts were proteomically confirmed in all three individuals and were the most abundant toxin family. The most abundant single transcript was a CRISP and was confirmed in the three individuals with proteomic data (Fig. 2). The function of CRISPs in this species and most snake species is unknown and requires further study (Tadokoro et al., 2020). However, there is some evidence that, in snakes, they inhibit ion channels, prevent the growth of new blood vessels, increase vascular permeability, and promote



**Fig. 4.** Divergence and selection on toxin transcripts compared to nontoxin transcripts. (A) Positive Tajima's D values with no significant difference between toxins and nontoxins indicates a lack of purifying or positive selection. (B) Similarly, there is no difference in nucleotide diversity ( $\pi$ ) suggesting no difference in the amount of polymorphism between toxins and nontoxins. (C) However, multiple-sequence alignments demonstrate that toxins are on average more dissimilar to one another than nontoxins (\*: significant; ns: not significant).



**Fig. 5.** PAML estimates of positive selection on toxin and nontoxin transcripts. Despite being significantly different, estimates of  $\omega$  and likelihood ratios do not support positive selection for either toxins or nontoxins. Red lines indicate thresholds supporting positive selection. (A) Model M0 estimates of  $\omega$  were less than one for all but four toxins. (B & C) Likelihood ratios were less than the significance cutoff for all but two toxins when comparing (B) models M1 and M2 or (C) models M7 and M8 (\*: significant; ns: not significant). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inflammatory responses (Tadokoro et al., 2020). The simplicity of our HPLC profiles also support the simplicity of our venom gland transcriptomes; however, peaks differ between samples (Fig. S1). This could be due to nonsynonymous changes shifting elution times or low quality venom samples because of difficulties that come with extracting venom from rear-fanged snakes (Modahl et al., 2016).

Further evidence of venom conservation is shown by our findings that there was almost the exact same suite of venom orthologs identified across our samples (Supplemental Data). The only individual that was different was IBSP90474 which lacked five orthologs that were present in the other three individuals. We attempted to locate these orthologs in the transcriptome but were unable to recover them. Their absence could be due to the lack of genes present in the genome or the lack of expression. Additionally, IBSP90474 was the only female in our study and the largest individual sequenced (Table 1). Both sex and size have been shown as sources of potential differences in venom composition (Menezes et al., 2006; Borja et al., 2018; Mackessy et al., 2018). However, due to the low sample size, we are unable to confirm if there are size or sex based differences within *I. cenchoa*. Of the 32 orthologs identified, only 5 were differentially expressed between Mexico and Brazil: KUN-1, CTL-OG001297, Waprin, VEGF-1, and Proprotein Convertase. KUN-1, CTL-OG001297, Waprin, and VEGF-1 were among the lowest expressed venom-related proteins, and none were confirmed in the proteome. Proprotein Convertase was proteomically confirmed in one individual; however, further work is needed to determine if Proprotein Convertase or VEGF-1 contribute to the actual venom phenotype as neither have known toxic functions (Fig. 2).

With regard to sequence divergence, we found little evidence for selection acting on toxin transcripts in *I. cenchoa* (Fig. 3 and 4). Toxins do appear to accumulate more mutations, but positive selection does not appear to cause the differences in the number of nonsynonymous and synonymous mutations (Figs. 3 and 5). Specifically, we did not find a difference in Tajima's D or nucleotide diversity between toxins and nontoxins among our samples despite the large geographic distance between them (Fig. 4). These results suggest that rare alleles are scarce across our samples which may be due to balancing selection favoring maintenance of genetic variation. Additionally,  $\omega$  was significantly higher for toxins compared to nontoxins,  $\omega$ , however, for both groups was less than one, suggesting a lack of positive selection acting on toxin

transcripts (Fig. 5). Of the 32 toxins, only six showed signatures of positive selection in either of the model comparisons. Only one of those, SVMPIII-OG000000c, was expressed highly and it was also the only one confirmed in the proteome (Fig. 2). The other five were lowly-expressed Ficolins and CTLs which are relatively diverse toxin families in *I. cenchoa*. Together, these results may indicate a potential role of purifying selection to maintain the venom phenotype of *I. cenchoa*. Alternatively, given the significantly higher number of mutations, higher  $\omega$ , and slightly higher values of Tajima's D ( $\neq 0$ ), it is more likely that balancing selection is acting to maintain genetic variation in this species. However, this genetic variation is not related to selective sweeps occurring in either population. It should be noted that our estimates of Tajima's D,  $\pi$ , and  $\omega$  may be strongly influenced by our small sample size (Subramanian, 2016). Nonetheless, these results suggest that toxins and nontoxins evolve very similarly and that *I. cenchoa* has a stable venom phenotype with little variation in regards to both toxin expression and sequence divergence.

*Imantodes cenchoa* is one of nine species of Blunt-headed Treesnakes, and previous work has identified diet (de Sousa et al., 2014) and anatomical (Aveiro-Lins et al., 2006) specialization. Studies of other snake genera have demonstrated a link between dietary breadth and venom complexity, with species feeding on a wide range of prey items producing more complex venom phenotypes (Davies and Arbuckle, 2019; Lyons et al., 2020; Holding et al., 2021). Additionally, local adaptations of snake venoms in response to prey has been demonstrated between populations in other systems (Holding et al., 2016; Margres et al., 2017). Therefore, by showing genetic evidence of the maintenance of venom complexity and composition across distant populations, our results provide evidence there is likely further phenotypic integration of venom to capture lizard prey. Based on work from other snake species, it is also possible there are lizard specific SVMP toxins within the venom of *I. cenchoa* (Pawlak et al., 2006, 2009; Heyborne and Mackessy, 2013; Margres et al., 2017; Holding et al., 2016, 2018b; Modahl et al., 2018). Unfortunately, due to a lack of venom, we were unable to functionally verify if specific toxins within the venom act on lizards. Our results may extend to the other species within *Imantodes*, such as *I. gemmistratus*, which likely specialize on lizards and have similar anatomical and behavioral specialization as well (Henderson and Nickerson, 1976;



Myers, 1982). Several species within *Imantodes* occur sympatrically in several locations (de Sousa et al., 2014). Further work could test whether specialization occurs on different lizard groups and if there is niche partitioning among sympatric lineages. In doing so, *Imantodes* could be developed into a powerful system to study phenotypic integration and biological specialization by combining natural history data, morphology, behavior, and venomics.

#### Credit author statement

**Tucker C. Heptinstall:** Writing – review & editing, Formal analysis. **Jason L. Strickland:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Ramses A. Rosales-Garcia:** Writing – review & editing, Formal analysis. **Rhett M. Rautsaw:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Cassandra L. Simpson:** Writing – original draft, Formal analysis. **Gunnar S. Nystrom:** Data curation, Formal analysis. **Schylar A. Ellsworth:** Data curation, Formal analysis. **Michael P. Hogan:** Data curation, Formal analysis. **Miguel Borja:** Data curation, Funding acquisition. **Pollyana Fernandes Campos:** Data curation, Funding acquisition. **Felipe G. Grazziotin:** Conceptualization, Data curation, Funding acquisition. **Darin R. Rokyta:** Conceptualization, Data curation, Funding acquisition. **Inácio L. M. Junqueira-de-Azevedo:** Conceptualization, Funding acquisition. **Christopher L. Parkinson:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

#### Ethical statement

**Reporting standards:** The authors declare that our manuscript describes original research and every effort was made to ensure the accuracy of the results and the account.

**Data Access and Retention:** Raw data for venom gland transcriptomes are on the National Center for Biotechnology Information (NCBI) under BioProject PRJNA88989: Sequence Read Archive (SRA) accession SRR12266697 (CHFCB0201), SRR12266696 (IBSP89901), SRR12266695 (IBSP90389), and SRR12266694 (IBSP90474) and Bio-sample accessions SAMN15581662 (CHFCB0201), SAMN15581663 (IBSP89901), SAMN15581664 (IBSP90389), and SAMN15581665 (IBSP90474) The consensus transcriptome of orthologs was submitted to the NCBI Transcriptome Shotgun Assembly (TSA) database. This TSA project has been deposited at DDBJ/EMBL/GenBank under accession GKDN00000000. The version described in this paper is the first version, GKDN01000000. Additional data files, including mass spectrometry data, and code used are available at [https://github.com/jstrickland63/Imantodes\\_cenchoa\\_transcriptomics](https://github.com/jstrickland63/Imantodes_cenchoa_transcriptomics).

**Originality and Plagiarism:** The authors declare that our manuscript is an original work with proper citations as needed.

**Multiple, Redundant or Concurrent Publication:** The authors declare that the data and work described in our manuscript has not and will not be submitted for consideration to another journal.

**Authorship of Paper:** All authors of the manuscript made significant contributions, and no one making significant contributions was excluded from authorship. All authors have seen and read the submitted version of the manuscript and have approved the submission.

**Hazards and Human or Animal Subjects:** The specimen in Mexico was collected under a scientific collecting permit to M.B. (SEMARNAT: SGPA/DGVS/01090/17) and handled with approval from Clemson University's IACUC (2017–067). One specimen in Brazil was donated to the Animal Reception Service of Instituto Butantan (register number 20180418291) and the other two collected in the field (ICMBio permit Nos 65653 and 65634) and the glands and venom were collected with the approval of the Instituto Butantan's IACUC (CEUA 4479020217).

**Fundamental Errors in Published Works:** If a fundamental error or inaccuracy is discovered in the results, the authors will immediately notify the editor or publisher.

#### Declaration of competing interest

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

#### Data availability

Data used herein are deposited in public databases listed in the manuscript

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxicol.2023.107135>.

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