UNIVERSIDAD TECNOLÓGICA INDOAMÉRICA

DIRECCIÓN DE POSGRADO

MAESTRÍA EN BIODIVERSIDAD Y CAMBIO CLIMÁTICO

TEMA:

TRANSCRIPTOMIC ANALYSES FOR THE CHARACTERIZATION OF VENOM TOXIN DIVERSITY IN TOADHEADED PITVIPERS (*BOTHROCOPHIAS*: VIPERIDAE: SERPENTES).

Trabajo de Investigación previo a la obtención del título de Magister en Biodiversidad y Cambio Climático

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QUITO – ECUADOR

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DEDICATORIA

A mis padres Pablo, Marthy, y a mi hermana Ammy por su amor y apoyo.

AGRADECIMIENTO

Al Ph.D. David Salazar Valenzuela, director de mi disertación, quien me dio la oportunidad de realizar esta investigación, por todas las oportunidades que se abrieron gracias a él, y por sus comentarios en el proyecto. Al Dr. Inácio Junqueira-de-Azevedo, investigador principal del Laboratorio de Toxinología Aplicada del Instituto Butantan, por permitir el acceso a su laboratorio, base de datos y su apoyo durante mi estadía allá. A Juan David Bayona-Serrano, quien me ayudó en todo el proceso de análisis de datos y quien ha sido el mayor apoyo durante el desarrollo del proyecto, tanto en Ecuador como en Brasil. A Pedro G. Nachtigall por sus consejos y apoyo con la revisión de datos. A Diego Quirola por su esfuerzo en la colecta de especímenes y el procesamiento de los mismos.

A mis padres Pablo y Marthy por enseñarme todo, por su amor y apoyo incondicional, por su ejemplo, su sacrificio y esfuerzo para permitirme seguir adelante, siempre gracias. A mi hermana Ammy por su apoyo y comprensión, su ejemplo de sinceridad y fortaleza. Un agradecimiento muy especial a George por ser quien me impulsó dentro del mundo de la Biología, quien me compartió el amor por las serpientes y principalmente por ser un excelente primo, guía y amigo durante todos estos años.

A Andrés, Leo, Daya, por el apoyo durante todo el proceso.

ÍNDICE DE CONTENIDO

ÍNDICE DE TABLAS

ÍNDICE DE FIGURAS

UNIVERSIDAD TECNOLÓGICA INDOAMÉRICA
DIRECCIÓN DE POSGRADO
MAESTRÍA EN BIODIVERSIDAD Y CAMBIO CLIMÁTICO

TEMA: Análisis transcriptómicos para la caracterización de diversidad de toxinas de veneno en Víboras cabeza de sapo (*Bothrocophias:* Viperidae: Serpentes).

AUTOR: Mauricio Mejía Guerrero
TUTOR: David Salazar Valenzuela, Ph.D.

RESUMEN EJECUTIVO

Bothrocophias (víboras cabeza de sapo) es un género sudamericano de serpientes que pertenece a la familia Viperidae. Mientras que la composición y la función del veneno han sido ampliamente estudiadas en *Bothrops* (género filogenéticamente hermano), solo el veneno de unas pocas especies de *Bothrocophias* ha sido analizado mediante estudios proteómicos. Aquí, nuestro objetivo es caracterizar la diversidad de toxinas del veneno en cuatro especies de víboras de cabeza de sapo de Ecuador utilizando análisis transcriptómicos. La transctiptómica es una técnica que permite estudiar los genes que se transcriben en un determinado tejido; en nuestro caso, las glándulas de veneno. De esta forma obtener una visión general de las toxinas que potencialmente se encontrarán en el veneno de cada especie. La anotación y detección de toxinas se llevó a cabo mediante análisis bioinformáticos. Además inferimos árboles filogenéticos por cada familia principal para determinar la relación evolutiva entre los transcritos. Obtuvimos tres resultados principales: 1) identificamos dos tipos diferentes de veneno: uno dominado por Fosfolipasas A2 (PLA2s) y un segundo tipo donde las Metaloproteinasas (SVMPs) corresponden a la familia de toxinas más expresada; 2) se identificó variación ontogenética en una especie (*B. lojanus*) para la composición entre las principales familias de toxinas; y 3) los árboles filogenéticos reconstruidos nos permitieron detectar eventos putativos de duplicación y pérdida que ocurren en las tres familias de toxinas analizadas, sugiriendo un modelo de evolución *birth and death* en el cual genes que han sufrido duplicaciones se mantienen (*birth*) o pueden ser eliminados (*death*) en diferentes especies. Como recomendación general, la inclusión de análisis proteómicos es necesaria a futuro para determinar el perfil final del veneno de cada especie. Del mismo modo, se recomienda incluir especímenes de diferentes localidades para analizar con detalle una posible influencia de la distribución geográfica en el perfil transcriptómico del veneno.

KEYWORDS: *Bothrocophias,* transcriptómica, toxinas, veneno.

UNIVERSIDAD TECNOLÓGICA INDOAMÉRICA
DIRECCIÓN DE POSGRADO
MAESTRÍA EN BIODIVERSIDAD Y CAMBIO CLIMÁTICO

THEME: Transcriptomic analyses for the characterization of venom toxin diversity in Toadheaded pitvipers (*Bothrocophias:* Viperidae: Serpentes).

AUTHOR: Mauricio Mejía Guerrero
TUTOR: David Salazar Valenzuela, Ph.D.

ABSTRACT

Bothrocophias (Toadheaded vipers) is a genus of snakes in the family Viperidae. While the composition and venom function have been extensively studied in *Bothrops* (phylogenetically sister genus), only the venom of a few *Bothrocophias* species has been analyzed by proteomic studies. Here, we aim to characterize the venom toxins diversity in four species of toadheaded pitvipers from Ecuador using transcriptomic analyses. Transcriptomics is a technique that allows to study the genes that are transcribed in a certain tissue; in our case, the venom glands. In this way, get an overview of the toxins that will potentially be found in the final venom of each species. The record and detection of toxins was carried out by bioinformatics analysis. Also we inferred phylogenetic trees for each major family to determine the evolutionary relationship between the transcripts. There were obtained three main results: 1) There were two different types of venom identified: one dominated by Phospholipases A2 (PLA2s) and a second type where Metalloproteinases (SVMPs) it belongs to the most expressed family of toxins; 2) ontogenetic variation was identified in one species (*B. lojanus*) for the composition between the main families of toxins; and 3) reconstructed phylogenetic trees allowed us to detect putative duplication and loss events occurring in the three families of toxins analyzed, suggesting a model of birth and death evolution in which genes that have undergone duplication are maintained (*birth*) or can be eliminated (*death*) in different species. As a general recommendation, the inclusion of proteomic analyses is necessary in the future to determine the final venom profile of each species. Likewise, it is recommended to include specimens from different localities to analyze in detail a possible influence of geographical distribution on the venom transcriptomic profile.

KEYWORDS: *Bothrocophias,* transcriptomics, toxins, venom.

CHAPTER 1

INTRODUCTION

Toxic secretion production has been reported in different organisms including plants, fungi, microorganisms, and animals (Mackessy, 2021; Sunagar et al., 2016). Venoms are mixture compounds of distinct bioactive molecules like proteins, peptides, or inorganic products that produce adverse effects in organisms that are exposed to them (Abd El-Aziz et al., 2020; Brahma et al., 2015; Mackessy, 2021). Such substances are secreted and produced in a specialized gland (i.e., the venom gland) reaching their destination through a specialized envenomation system (Mackessy, 2021; Sunagar et al., 2016). Venoms primarily have predation and defensive purposes and their study has increased in recent years becoming a formal and specialized discipline with different fields and approaches due to the development of new sample processing and analysis techniques that allow their study (Dong & Chen, 2013; Mackessy, 2021)

Within vertebrates, the most numerous venomous lineage constitute squamate reptiles (i.e., snakes, lizards). In particular, snake venoms represent a complex evolutionary adaptation that provides an ecological advantage in different scenarios (e.g., feeding of highly mobile prey such as birds) (Almeida et al., 2021). Venom has been reported in various snake families, among which the most important are Viperidae, Elapidae, and the rear-fanged Colubridae. Their toxins have evolved from normal physiological proteins that have undergone expedited point mutation processes in coding regions, as well as gene duplications, which in turn has triggered protein neofunctionalization (Almeida et al., 2021; Brahma et al., 2015; Mackessy, 2021). The mechanism of venom evolution has several pathways, including predator-prey interactions that influence venom composition and differentiation. These interactions have been demonstrated even at the molecular level (Brahma et al., 2015; Davies & Arbuckle, 2019).

In recent years, there have been significant advances in the study of snake venoms (snake venomics), both in analytical techniques and bioinformatics, which have helped to

develop complex and informative venom catalogs (Abd El-Aziz et al., 2020). Part of these advances refers to detailed analyses of proteins and peptides present in venoms (proteomics) and have made it possible to describe composition differences between snake families, genera, and even within species. According to Mackessy (2021), in general, the enzymatic proteins metalloproteinases (Snake Venom Metalloproteinases, SVMPs) and serine proteinases (Snake Venom Serine Proteinases, SVSPs) are principal constituents of venoms in Viperidae snakes. In New World pitvipers, two venom types have been described:

a) Type I: with dominating SVMPs, which have an essential role in the hydrolysis of many structural proteins (Mackessy, 2021). This venom type has been described in several species of the Americas: *Bothrops bilineatus* (Sanz et al., 2020), *Bothrops brazili* from Perú (Rodrigues et al., 2020), small individuals of *Bothrops jararacussu* (Freitas-De-sousa et al., 2020), *Bothrops moojeni* (Amorim et al., 2018), *Bothriechis aurifer* and *Bothriechis thalassinus* (Pla et al., 2017), and *Porthidium porrasi* (Méndez et al., 2019). The SVMPs are involved in the hydrolysis of many structural proteins and may cause hemorrhage, myonecrosis, or prey predigestion (Mackessy, 2021).

b) Type II: SVMP in lower or no amounts, and a dominant presence of other molecules such as Phospholipases -PLA- (Mackessy, 2021). This venom type has been described in New World species like *Agkistrodon bilineatus* (Lomonte et al., 2014), *Bothrops cotiara* from Argentina (de Roodt et al., 2018), *Bothriechis bicolor* (Pla et al., 2017), *Bothrocophias campbelli* (Salazar-Valenzuela et al., 2014), *Bothrocophias myersi* (Pereañez et al., 2020). The PLA2 can be divided in two different groups and can cause myotoxicity, myonecrosis, lipid membrane damage, or prey immobilization due to its potent neurotoxicity (Mackessy, 2021)

Venom in Elapidae snakes (e.g., cobras and coral snakes) is similar to Type II venom of New World snakes where SVMPs and SVSPs are found in very low amounts and are dominated by PLAs and 3FTxs -three-finger toxins- (Mackessy, 2021). Some examples include species like *Hemachatus haemachatus* where 3FTxs are expressed in 63.3% of the proteome (Sánchez et al., 2018), western Indian *Naja naja* where 3FTxs comprise 68.5% of toxins (Chanda et al., 2019), and *Micrurus surinamensis* where the

3FTxs toxins are highly dominant comprising 95% of venom (Sanz, de Freitas-Lima, et al., 2019). Moreover, some species show dominance in PLA expression like *Micrurus frontalis* (Sanz, de Freitas-Lima, et al., 2019), *Micrurus lemniscatus* (Sanz, Quesada-Bernat, et al., 2019), *Micrurus yatesi* (Mena et al., 2022) or *Pseudechis* spp. (Goldenberg et al., 2018). On the other hand, rear-fanged Colubridae snakes have been less studied and, consequently, less characterized. However, their venoms show different patterns with some species with a high abundance of SVMP and others with 3FTxs dominance (Calvete et al., 2020; Dashevsky et al., 2018; Mackessy, 2021; Mackessy et al., 2020).

Transcriptomic analyses have advanced significantly in recent years. In contrast to proteomics, transcriptomics uses tissues from the venom glands instead of the venom itself (Mackessy, 2021). Transcriptomic analyses use cDNA (complementary DNA), a single strand obtained from RNA transcripts, to create a library of genes expressed in venom glands (Brahma et al., 2015; Mackessy, 2021). Transcriptomic analyses have also been used as the first description of several toxins (Brahma et al., 2015) and have established as another powerful tool for understanding venom profiling (Brahma et al., 2015). Qualitative and quantitative characterization of venom help understands the main elements (divided into toxin families or classes) that compose venom and their abundance at a general level. This way of cataloging toxins in the venom gland has been used in some species of different families of snakes. Nevertheless, their use has been more restricted than proteomic studies due to being a more recent technique, requiring a more complex sample processing system (specifically in Next- Generation Sequencing, NGS), and using bioinformatics tools for data analysis (Damm et al., 2021; Dong & Chen, 2013; Rao et al., 2022). The use of transcriptomic analysis has high potential in venom reconstruction and could play an essential role in future biomedical applications, but also for the understanding of evolutionary processes and diversification in several species and could be an important step in reconstructing their natural history.

Transcriptomics increase their veracity and complexity when used in conjunction with other analyses such as proteomics, orthology inference, or phylogenetic trees (Nachtigall et al., 2022). The use of proteomics allows having a complete catalog of the toxins present during the transcription and post-translation process. Using transcriptomics and proteomics together allows us to observe genes that are active in a tissue but for some reason are not transcribed (as modular toxin expression) into proteins that comprise the

final venom phenotype (Nachtigall et al., 2022). Orthology and phylogenetic analyses allow the reconstruction of the evolutionary relationships between genes. For instance, a group of genes (two or more) from different species that come from a common ancestor (Emms & Kelly, 2019; Wen et al., 2015), and how these genes have been maintained or disappeared among different species. This is important because we can reconstruct the evolutionary history among toxins, and thus have an idea of possible divergence and diversification events. However, these two analyses (orthology inference and phylogenetic reconstructions) have some differences, so their combined use increases the credibility of the results. The principal difference is the way of presenting the reconstruction of phylogenetic trees. Orthology analyses infer the principal orthogroups (groups of genes that come from one common ancestor) and their trees (per orthogroup) are represented in different results (Emms & Kelly, 2019); while phylogenetic trees allow comparison of larger datasets in a single final tree. These larger trees as well allow visualization of putative gene duplication events. Gene duplications seem to be the principal evolutionary force that influences the diversification of venoms (Wong & Belov, 2012), these events are well supported in multigene families (as PLA2s, SVMPs, Serine proteases) and are a clear explanation that evolutionary *birth and death* model is driving diversification in species instead of other models as divergent evolution or concerted evolution. In the *birth and death* model, there are gene *duplication* events that generate new genes (*birth*), and some of these duplicated genes may be eliminated in some species -*loss*- (*death*) (Frankel, 2006).

As with other biological groups, venomous snakes in Ecuador display high levels of diversity (Cartay, 2020; Dupérré & Tapia, 2020; Mestanza-Ramón et al., 2020). To date, 36 species have been reported in two families: Viperidae with 17 species and five genera, and Elapidae with 19 species and two genera (Ochoa-Avilés et al., 2020; Torres-Carvajal, O., Pazmiño-Otamendi, G., Ayala-Varela, F. and Salazar-Valenzuela, 2022; Yañez-Arenas et al., 2018). In the country, most snakebite accidents are caused by pitviper species, while the incidence of coral snakebites is significantly lower (Ochoa-Avilés et al., 2020). Between 2014 and 2019, the number of accidents in Ecuador averaged 1500 per year with a mortality rate of 0.07 per 100 000 inhabitants (Chippaux, 2017; Ochoa-Avilés et al., 2020), especially involving adult males (Ochoa-Andrade et al., 2020). The areas of major ophidian risk are the central and northern Coastal zone and the Amazon Region, both with a high concentration of rural communities (Ochoa-Andrade

et al., 2020; Ochoa-Avilés et al., 2020; Yañez-Arenas et al., 2018). It should be noted that the Amazon region has a higher risk of bites and death than the Coastal zone, where high-risk areas have been located, such as Palora Metzera, Sangay, and Shell (Yañez-Arenas et al., 2018). Snakebite accidents are mainly caused by the following species: *Bothrops asper, B. atrox, B. bilineatus,* and *Lachesis muta* (Ochoa-Avilés et al., 2020; Yañez-Arenas et al., 2018), although it has been suggested that *Bothrocophias microphthalmus* could be a medically important species in southeastern Ecuador (Kuch et al., 2005; Salazar-Valenzuela et al., 2014).

Bothrocophias (Toadheaded pitvipers) is a genus belonging to the Viperidae family. It was separated from *Bothrops* in 2001 by Gutberlet & Campbell, and originally included four species: *Bothrocophias campbelli, B. hyoprorus* (posteriorly *B. hyoprora*), *B. microphthalmus,* and *B. myersi* (Pereañez et al., 2020). More recently, other South American species have been included in the genus which is currently composed of seven species. Snakes of the genus *Bothrocophias* are terrestrial with stout bodies and large heads (Salazar-Valenzuela et al., 2014). In Ecuador, these species occur on both sides of the Andes mountain range between near sea level and 2800 m.a.s.l. Three species were recognized in Ecuador until 2019. Nevertheless, Hamdan et al. (2020) proposed the inclusion of *Bothrops lojanus* (posteriorly *Bothrocophias lojanus*) in the genus. With this change, *Bothrocophias* is represented in the country by four species: *B. campbelli*, *B. hyoprora*, *B. lojanus,* and *B. microphthalmus* (Torres-Carvajal, O., Pazmiño-Otamendi, G., Ayala-Varela, F. and Salazar-Valenzuela, 2022). Some species of toadheaded pitvipers, such as *B. campbelli* or *B. hyoprora,* seem to be less abundant than the more common *B. microphthalmus* (Salazar-Valenzuela et al., 2014; Torres-Carvajal, O., Pazmiño-Otamendi, G., Ayala-Varela, F. and Salazar-Valenzuela, 2022).

Venom composition and function is poorly known in *Bothrocophias*. Of the seven species present in this genus, only two venoms (*B. campbelli* and *B. myersi*) have been studied using proteomic analyses (Calvete et al., 2021; Pereañez et al., 2020; Salazar-Valenzuela et al., 2014). Increasing the knowledge of venom toxin diversity could help understand its composition and action mechanisms. A first characterization of the toxin diversity of *Bothrocophias* venom by performing transcriptomic analyses is proposed in this research. These results could also serve as a reference for further and more specific analyses of evolutionary ecology, biotechnology, and therapeutic applications. We expect

to find similarities with previous proteomic studies on *Bothrocophias*. Thus, to find the two types of venom that have been previously described in New World Vipers.

OBJECTIVES

General objective:

To characterize the venom toxin diversity in four species of Toadheaded pitvipers (genus *Bothrocophias*) of Ecuador using transcriptomic analyses.

Specific objectives:

To determine the main toxin gene families expressed in *Bothrocophias* venom glands.

To characterize qualitative and quantitatively the toxin diversity present in *Bothrocophias* venom glands.

To identify inter and intraspecific differences in the toxin diversity found in four species of *Bothrocophias*.

CHAPTER II

MATERIALS AND METHODS

SAMPLES AND DATA COLLECTION

Collection and storage of samples

We analyzed molecular data from eight specimens from four species of the genus *Bothrocophias* present in Ecuador (*B. campbelli,* n = 1; *B. hyoprora,* n = 1; *B. lojanus,* n = 3; *B. microphthalmus,* n = 3). Voucher specimens are housed in the MZUTI (Museo de Zoología, Universidad Tecnológica Indoamérica) collection. The *B. campbelli* specimen (MZUTI 5638) was collected in Mindo-Pichincha (-0.045402 latitude, -78.760756 longitude, 1463 m above sea level; hereafter asl), Ecuador in 2019. The *B. hyoprora* specimen (MZUTI 5873) was collected in Pacayacu-Sucumbíos, Ecuador (-0.026915, -76.504441, 245 m asl). All samples of *B. lojanus* are from Azuay, Ecuador. The three specimens were collected from different locations and years: MZUTI 5403 from San Marcos (-3.3206, -79.09615, 2767 m asl) in 2016; MZUTI 5404 from Las Nieves (-3.33078, -79.10054, 2613 m asl) in 2016, and MZUTI 5421 from Poetate (-3.422747, -79.223164, 2750 m asl) in 2018; finally, all *B. microphthalmus* samples (MZUTI 5528, 5635, and 5637) have the same data and data collection; they were collected in Mera-Pastaza-Ecuador (-1.370698, -78.048107, 1133 m asl) in 2018. Sampling efforts were conducted predominantly during the night. Upon visually locating an individual, the collection and handling process was performed using snake hooks and tongs for safety reasons. Specialized bags and containers were used for transportation. Some of the collected snakes were handed to us by local people or herpetologist colleagues

For the tissue extraction process, live specimens were taken to *Centro de Investigación de la Biodiversidad y Cambio Climático (BioCamb).* For each venom extraction, a collection cup was prepared using a sterile beaker with the top covered with parafilm. The specimen of interest was immobilized using specialized restraining tubes that allowed a safe handling. After securing a safe grip of the snake's head, the collection cup was presented, and biting for the injection of venom was allowed.

Venom glands and other tissues were surgically excised four days after venom extraction due to maximal mRNA concentrations have been observed at this time (Brahma et al., 2015). Immediately after the extraction, tissues were transferred to RNAlater® and stored briefly at 4 °C (24 hours) prior to long term storage at −20 °C.

RNA extraction and cDNA library construction

RNA extraction was developed by *Laboratorio de Toxinología Aplicada (LETA)* in Butantan Institute, Brazil. Tissues were pulverized in a Precellys® 24 homogenizer and RNA was extracted with TRIZOL® (Invitrogen) following the modification of the method described by Chomczynski and Sacchi (1987), based on the use of guanidine isothiocyanate followed by phenolic extraction. Total RNA was quantified by Quant-iTTMRiboGreen® RNA reagent and Kit (Invitrogen, Life Technologies Corp.). Quality control of the extracted RNA was then performed in an Agilent 2100 Bioanalyzer using Agilent RNA 6000 Nano kit, to verify the integrity of total RNA, through band discrimination corresponding to fractions 18S and 28S of total RNA. All procedures with RNA were made with RNAse-free tubes and filter tips, as well as using water treated with diethylpyrocarbonate (DEPC, Sigma).

Libraries were prepared for each individual sample. One μg of total RNA was used with Illumina TruSeq Stranded RNA HT kit consisting of: TruSeq Stranded RNA HT / cDNA Synthesis PCR, TruSeq Stranded RNA HT / Adapter Plate Box and TruSeq Stranded HT mRNA. Fragment Size Distribution was evaluated by microfluidic gel electrophoresis in the Bioanalyzer device (Agilent 2100), using the Agilent DNA 1000 kit, according to the manufacturer's protocol. Quantification of each library was then performed by Real-Time PCR using the KAPA SYBR FAST Universal qPCR kit, according to the manufacturer's protocol, using the StepOnePlusTM Real-Time PCR System. Aliquots of each cDNA library were diluted to a concentration of 2 nM. Next, a pool of all samples (5 μL of each library) was prepared and the concentration of the pool was again determined by Real Time PCR. The cDNA libraries were sequenced on an Illumina HiSeq 1500 System in Rapid Run mode, using a paired-end flowcell for 300 cycles of 2*151bp.

ASSEMBLY AND ANNOTATION OF TRANSCRIPTOME

We developed *de novo* assemblies of transcriptome data using five different assemblers: Bridger (Chang et al., 2015) with a k-mer length set to 30, Externer (Rokyta et al., 2012) with three different parameters (default, and two overlaps: 150, and 2000), NGen (DNAStar INC, 2021) with k-mer length set to 21, rnaSPAdes (Bushmanova et al., 2019) using three different k-mer parameters (21, 75, and 127), and Trinity (Haas et al., 2013) with a standard k-mer set to 31. After obtaining the nine assemblies, we combined them into a general transcriptome FASTA file.

Toxin annotation and curation:

We used the final assembly FASTA file to annotate toxic and non-toxic proteins and peptides; the annotations were carried out using ToxCodAn (Nachtigall, Rautsaw, et al., 2021) as described in the user program section. The resulting coding sequences (CDS) files (toxins and putative-toxins) with redundancy filter were manually curated using Geneious Prime (v 2022.1.1) and annotated using BlastX (https://blast.ncbi.nlm.nih.gov). Toxins and putative toxin files were merged into a single file to remove chimeric sequences with ChimeraKiller (v0.7.3; https://github.com/masonaj157/ChimeraKiller); output files were manually curated and the "good" sequences were merged into a preliminary file. Then, we performed CD-HIT (Fu et al., 2012) to cluster sequences with 99% of identity.

Non-toxin annotation:

The Non-toxin FASTA file resulting from ToxCodAn (Nachtigall, Rautsaw, et al., 2021) was used to annotate and curate all the other sequences that were not recognized as toxins from venom glands. The first annotation was carried out with CodAn (Nachtigall, Kashiwabara, et al., 2021) and compared with a general vertebrate database to obtain the CDS. Then, the Non-Toxin Annotation script provided in ToxCodAn (Nachtigall, Rautsaw, et al., 2021) was performed to annotate the predicted CDS. Next, chimeric sequences were removed using ChimeraKiller (v0.7.3; https://github.com/masonaj157/ChimeraKiller) and curated manually to obtain the

"good" sequences. Finally, CD-HIT (Fu et al., 2012) was performed to cluster sequences with 98% identity to remove data redundancy.

INTRA AND INTERSPECIFIC ANALYSES

Assessing the expression of toxins

After obtaining the final toxins and non-toxins files, they were merged into the final transcriptome with all curated data. A cleaning script was used to remove non-necessary information in the file. The resulting FASTA file was used to assess the expression level of toxins (specific expression per toxin) and non-toxins (non-specific levels) with RSEM (Li & Dewey, 2011). The TPM (Transcripts Per Million) field was employed to analyze the level per particular toxin, family toxin unified, and non-toxin expression as a united set. These analyses were developed by individuals and by species. The graphics to visualize the expression levels were developed with the *autoplot* script provided by Nachtigall et al. (2021) guide.

Orthology analyses

To analyze interspecific variation, we used OrthoFinder v.2.4.0 (Emms & Kelly, 2019) to establish the orthology relationship (sequences derived from the same common gene) between toxins expressed within each species. We also estimated a phylogenetic tree per main toxin family to compare to the orthology results; the protein sequence alignment was performed using MAFFT with default parameters (Rozewicki et al., 2019), and IQTree (Nguyen et al., 2015) was used to assemble the maximum likelihood tree with the best-fit model automatically selected and bootstrap with 1000 replicates parameter. The resulting trees were modified with FigTree v1.4.4 (https://github.com/rambaut/figtree/).

CHAPTER III

RESULTS

Transcriptomic venom characterization

The first step in assessing the toxin composition of the venom gland in each species was to assemble and characterize an average mixed transcriptome. For *B. campbelli* (Fig. 1), 51 toxins were recovered in 1377 curated transcripts; of the total of the transcripts recovered from the venom gland, toxins are expressed in 88.95% whereas non-toxins represent 11.05%. This species' venom profiling is highly dominated by Phospholipases A2 (PLA2) with 60.95% expression. Snake Venom Metalloproteinases, as a whole (SVMPs), and C-type lectin (CTL) are also abundant at 15.1% and 14.01% respectively. SVMPs are divided into two subgroups: SVMPs type II (7.86%) and SVMPs type III (7.24%), while Snake Venom Serine Proteases (SVSPs) are less abundant at 5.99%; other toxins such as CRIPS, LAAO, and others represent 3.95%.

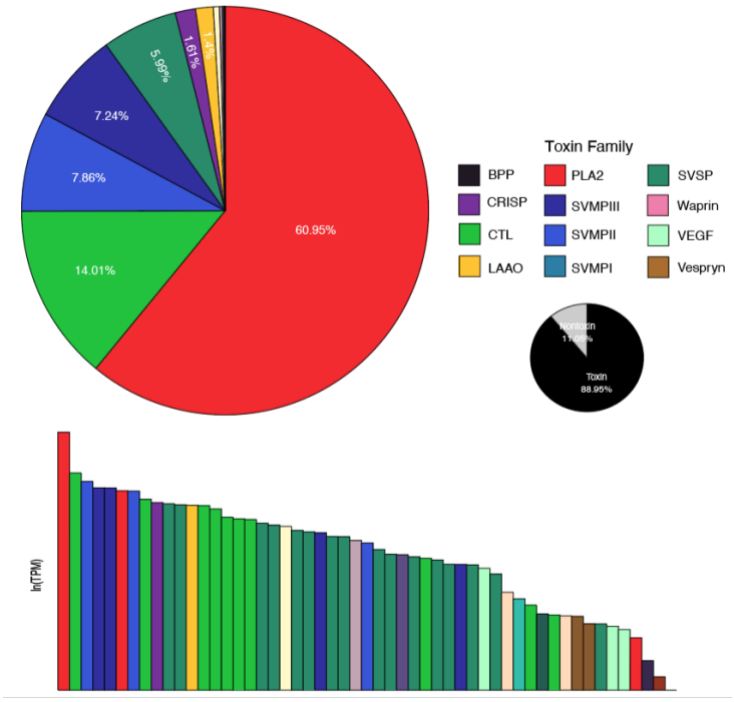

Figure 1 *Bothrocophias campbelli* venom composition reconstructed from a venom gland. At the top left, the pie chart represents the whole expression per toxin family as it can be observed, PLA2 represents the most abundant family. At the top right, the per toxin family colors are shown to interpret graphics properly, and the pie chart shows the percentage of expression between toxins and non-toxins. At the bottom, the barplot represents the expression level in Transcripts Per Million (TPM), regularized in natural logarithm (ln) per toxin.

For *B. hyoprora* (Fig. 2), 52 toxins were recovered in 2239 curated transcripts. Toxins represent 69.05% of transcripts expressed for this species, whereas non-toxins represent 31.95%. PLA2s are the most noticed toxins at 39.38%; nonetheless, it should be noted that SVMPs as a whole group represent 36.45%, a similar percentage to PLA2s. SVMPs type III are more common than SVMPs II (31.39% versus 5.06%, respectively). CTLs represent important toxins as well, expressed at 9.03%. Other toxins represent 15.14%.

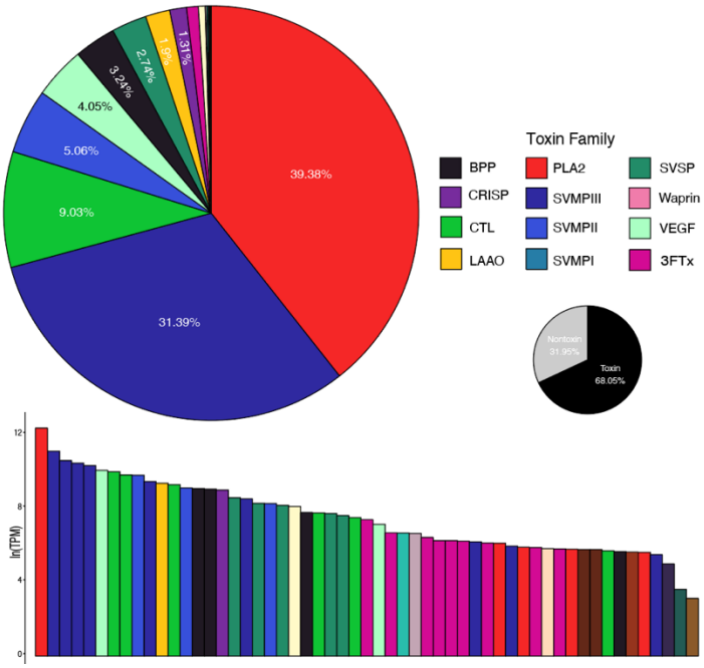

Figure 2. *Bothrocophias hyoprora* venom composition reconstructed from a venom gland. At the top left, in the pie chart PLA2 represents the most abundant family. At the bottom, the barplot represents the level of expression, in Transcripts Per Million (TPM), regularized in natural logarithm (ln) per toxin.

In *B. lojanus* (Fig. 3), 49 toxins were recovered in 1676 curated transcripts. Toxins represent 75.4% of transcripts expressed in the venom gland for this species, while non-toxins represent 24.6%; the most abundant toxins are SVPMs at 56.31%, divided into SVMPs I (2.92%), SVMPs II (28.46%), and SVMPs III (24.93%). CTL represents 17.12%; another common toxin family is SVSPs with 12.26% of toxin transcripts, whereas PLA2s are expressed in 8.79%. Other toxins represent 5.52%.

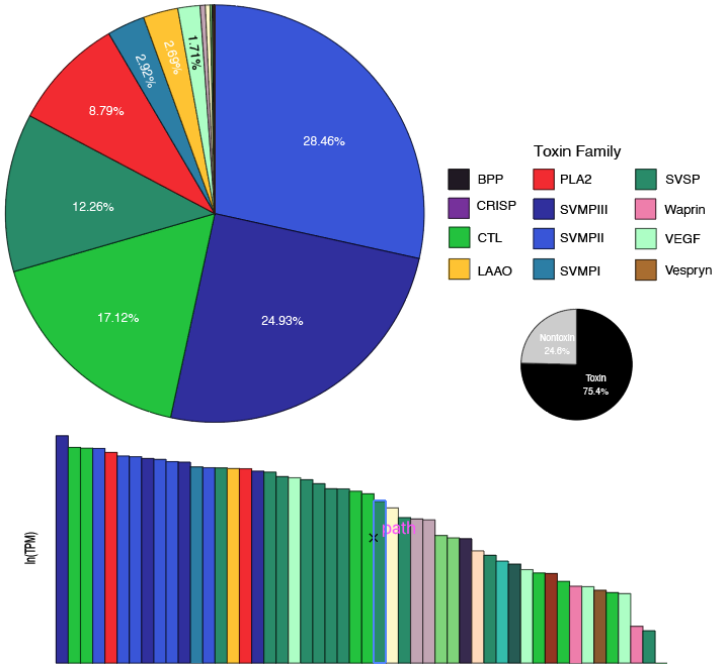

Figure 3. *Bothrocophias lojanus* venom composition reconstructed from a venom gland. At the top left, SVMPII represents the most abundant class followed by SVMPIII. At the top right, the figure shows the per toxin family colors to interpret graphics properly, and the pie chart shows the percentage of expression between toxins and non-toxins. At the bottom, the barplot represents the level of expression, in Transcripts Per Million (TPM), regularized in natural logarithm (ln) per toxin.

For *B. microphthalmus* (Fig. 4), 89 toxins were recovered in 2173 curated transcripts. Toxins represent 80.88% of transcripts expressed for this species, whereas non-toxins represent 19.12%. SVMPs are the most expressed toxins at 44.85%: SVMPs III are more abundant (35.47%) compared to SVMPs II (2.23%) and SVMPs I (7.15%). On the other hand, CTL and PLA2s are also highly abundant families and described in 21.96% and 15.74%, respectively, whereas SVSPs are expressed in 8.54%. Other toxins together represent 8.91%. All the toxin families and their percentage per species are shown in Table 1.

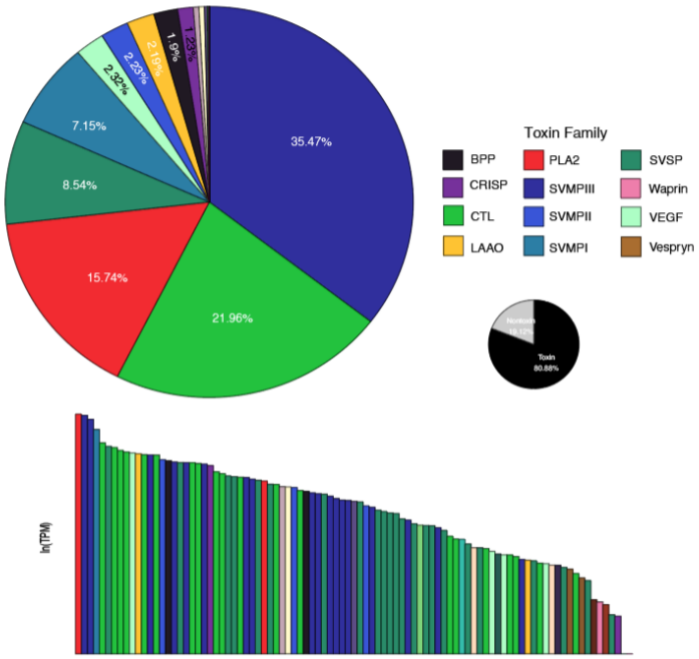

Figure 4. *Bothrocophias microphthalmus* venom composition reconstructed from a venom gland. At the top left, SVMPIII represents the most abundant class followed by CTL. At the top right, the figure shows the per toxin family colors to interpret graphics properly, and the pie chart shows the percentage of expression between toxins and non-toxins. At the bottom, the barplot represents the level of expression, in Transcripts Per Million (TPM), regularized in natural logarithm (ln) per toxin.

Table 1. Toxin families characterization among the four analyzed species

Toxin family	***B. campbelli***	***B. hyoprora***	***B. lojanus***	***B. microphthalmus***
3FTx	-	0.91	-	-
Bradykinin-potentiating peptide (BPP)	-	3.24	-	1.9
Cysteine-rich secretory proteins (CRISP)	1.61	1.31	-	1.23
C-type Lectin (CTL)	14.01	9.03	17.12	21.97
Cystatin	0.01	-	0.01	0.01
Hyaluronidase (HYAL)	0.01	0.12	0.02	0.03
Kunitz-type protease inhibitors (KUN)	0.02	0.15	0.04	0.03

L-amino acid oxidase (LAAO)	1.4	1.9	2.69	2.19
Lipases (LIPA)	0.01	0.04	0.02	0.02
Nerve growth factors (NGF)	0.48	0.53	0.36	0.43
Nucleotidases (NUC)	0.11	-	-	0.21
Phosphodiesterase (PDE)	-	-	0.17	0.07
Phospholipases A (PLA)	**60.95**	**39.4**	8.79	15.75
Phospholipases B (PLB)	0.23	0.12	0.4	0.44
Metalloproteinases (SVMP)	15.1	36.46	**56.31**	**44.86**
Serino proteinases (SVSP)	5.99	2.74	12.26	8.54
Translationally-controlled tumor protein (TCTP)	-	-	0.08	-
Vascular endothelial growth factor (VEGF)	0.06	4.05	1.71	2.32
Waprin	-	-	0.01	-

Main family of toxins per species is shown in bold, "-" represents absence of toxin family. The results are presented in percentage of family per species (%).

Intraspecific variation

Intraspecific variation analysis was performed in three species. In *B. lojanus* (Fig. 5), there is no change in qualitative venom composition on the three analyzed individuals. However, there is some variation in the quantitative composition of principal toxin families. For instance, in MZUTI 5404, PLA2s are expressed at 14.43% of total toxins; almost twice as much as in MZUTI 5421 and more than twice as much as in MZUTI 5403. In the case of CTLs, MZUTI 5404 shows approximately half of the expression found in MZUTI 5403 and MZUTI 5421 (20.25% and 19.99%, accordingly). On the other hand, SVMPs is the most important family in the three individuals, yet SVMP III and SVMP II are expressed in different quantities. In MZUTI 5404, SVMP III is the major group, expressed at 36.79% (vs SVMP II at 16.27%), whereas SVMP II is the principal

group in MZUTI 5403 and 5421 (31.65% and 35.39%, respectively). Finally, in MZUTI 5403 the two principal toxins (individually represented in barplots) are an SVMP III and a CTL, in MZUTI 5404 an SVMP III and a PLA2; while in MZUTI 5421 an SVMP II and an SVMP III. All the toxin families and their percentage per specimen are shown in Table 2.

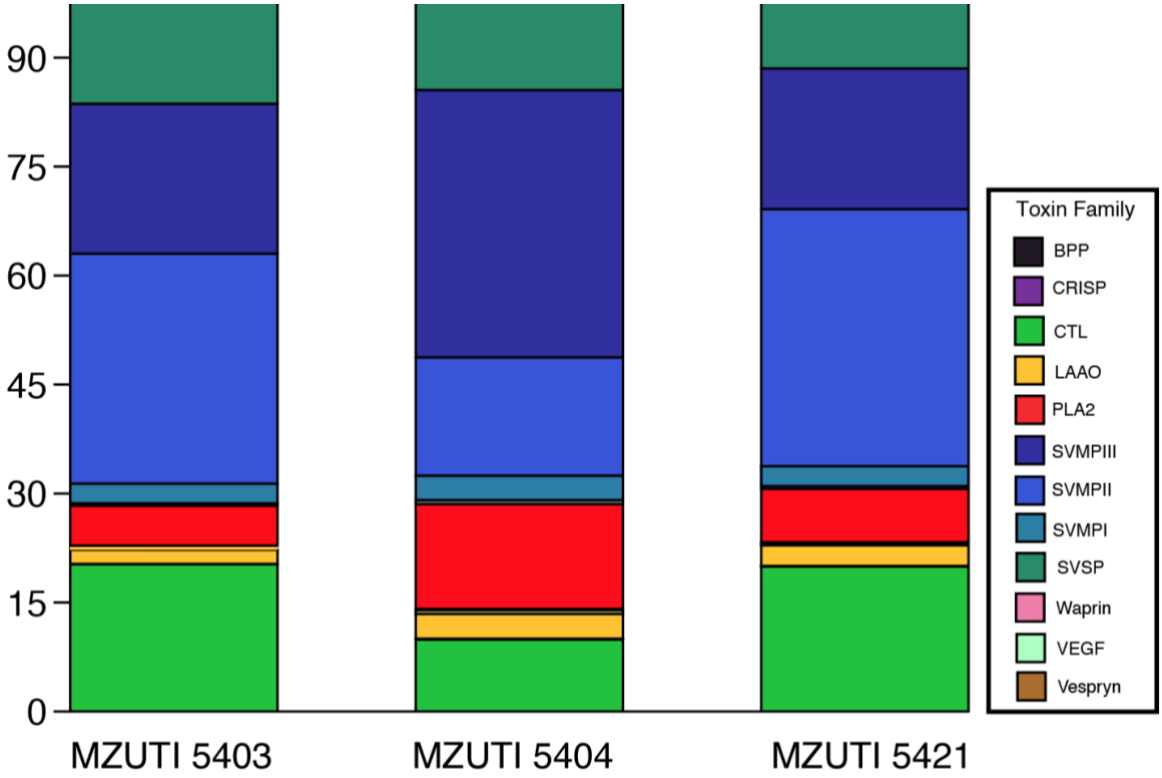

Figure 5. *Bothrocophias lojanus* intraspecific variation. MZUTI 5404 and MZUTI 5421 are male adults while MZUTI 5404 is a male juvenile. Barplots show the expression percentage per toxin family.

Table 2. Toxin families percentage and intraspecific variation in *B. lojanus* specimens

Toxin family	MZUTI 5403	MZUTI 5404	MZUTI 5421
CTL	20.26	9.92	19.99
Cystatin	0.00	0.01	0.00
HYAL	0.01	0.04	0.02
KUN	0.04	0.05	0.03
LAAO	1.93	3.40	2.86
LIPA	0.02	0.03	0.02
NGF	0.38	0.49	0.24
PDE	0.19	0.17	0.15
PLA2	5.50	14.43	7.35
PLB	0.32	0.55	0.36
SVMPI	2.72	3.37	2.75

SVMPII	**31.65**	16.28	**35.39**
SVMPIII	20.63	**36.79**	19.35
SVSP	14.70	12.15	9.98
TCTP	0.06	0.09	0.08
VEGF	1.57	2.20	1.43
Waprin	0.01	0.01	0.00

For *B. microphthalmus* (Fig. 6), a change in the quantitative composition of principal toxin families is evident. In MZUTI 5528 the most abundant toxin class is SVMP III, representing 34.6% of total toxin expression; followed by PLA2 at 22.11%, while CTLs are expressed at 18.78%. However, in MZUTI 5635 the more common toxin class is CTL, expressed at 35.63% of the total, followed by SVMP III at 31.8% and SVMP I at 14.19%, whereas PLA2 represents 6.36%. In addition, in MZUTI 5637, the first toxin class is SVMP III with a total amount of 40.12%; the second one is PLA2 at 19.3%; while CTLs are expressed at 10.87%. As previously described, CTLs, SVMPs, and PLA2s show different patterns between all samples. For instance, in MZUTI 5635, CTLs are expressed almost double and more than three times than in MZUTI 5528 and 5637 (35.63% vs 18.78% and 10.87%, respectively). Another important change is evident in PLA2s, in which MZUTI 5528 is expressed similarly to MZUTI 5637 (22.11% and 19.3%, respectively) but almost 3.4 more than that in MZUTI 5635 (6.38%). Lastly, for MZUTI 5528 the two principal individual toxins (represented in barplots) are an SVMP III and a PLA2, in MZUTI 5635 an SVMP III and an SVMP I, while in MZUTI 5637 a PLA2 and an SVMP III. All the toxin families and their percentage per specimen are shown in Table 3.

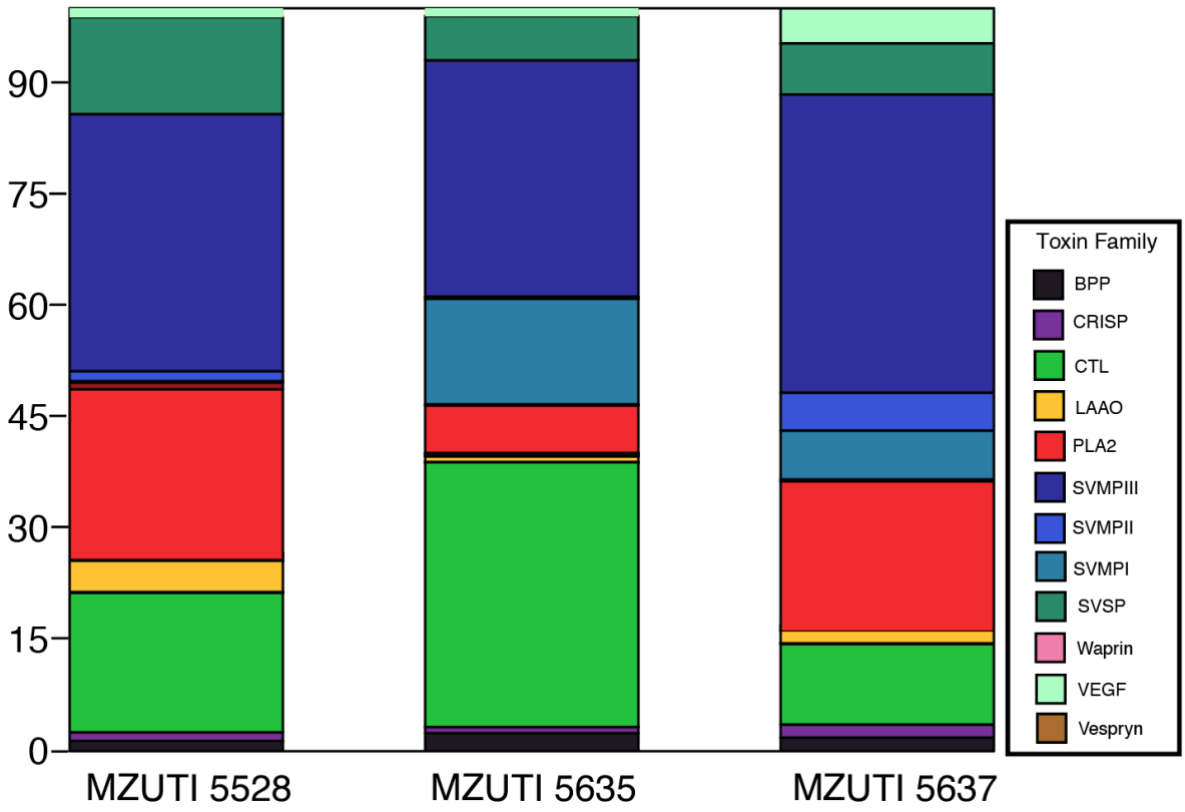

Figure 6. *Bothrocophias microphthalmus* intraspecific variation. MZUTI 5528 and MZUTI 5635 are adults while MZUTI 5637 is a juvenile. Barplots show the expression percentage per toxin family.

Table 3. Toxin families percentage and intraspecific variation among *B. microphthalmus* specimens

Toxin family	MZUTI 5528	MZUTI 5635	MZUTI 5637
BPP	1.39	2.44	1.84
CRISP	1.16	0.81	1.74
CTL	18.78	**35.64**	10.87
Cystatin	0.02	0.01	0.01
HYAL	0.03	0.02	0.05
KUN	0.05	0.02	0.02
LAAO	4.20	0.75	1.74
LIPA	0.02	0.01	0.02
NGF	0.56	0.30	0.45
NUC	0.30	0.12	0.22
PDE	0.10	0.03	0.07
PLA2	22.11	6.38	19.31
PLB	0.91	0.17	0.25
SVMPI	0.19	14.19	6.58
SVMPII	1.33	0.31	5.10
SVMPIII	**34.61**	31.80	**40.13**

SVSP	13.04	5.94	6.88
VEGF	1.19	1.05	4.72
Waprin	0.00	0.00	0.00

Orthology analyses

Following venom gland characterizations, we inferred orthologs in the three most important toxin families: CTLs, PLAs, and SVMPs. Thirteen orthogroups were identified and divided into four CTLs, one PLA, and eight SVMPs. From the four CTL orthogroups, two (CTL-OGs 1-2) include isoforms by the four species analyzed (33 isoforms), one orthogroup (CTL-OG4) is formed by isoforms from three species (four isoforms) and one includes isoforms from only one species (*B. microphthalmus*- CTL-OG3; five isoforms); while, three sequences were recognized as non-assigned orthogroup. For PLAs, all 11 isoforms from the four species are part of the same orthogroup. In the case of SVMPs, five orthogroups (SVMP-OGs 1-5) are composed of isoforms from the four species; one (SVMP-OG6) is formed by isoforms of three species (excluding *B. campbelli*), and two (SVMP-OGs 7-8) are composed by isoforms from two (*B. lojanus* and *B. microphthalmus*) species, while two sequences were recognized as non-assigned orthogroup. All the orthogroups and the number of isoforms present in each one are detailed in Table 4.

Table 4. Isoforms present per each orthogroup in *Bothrocophias* species

Orthogroup	***B. campbelli***	***B. hyoprora***	***B. lojanus***	***B. microphthalmus***	**Total**
CTL-OG1	5	4	4	5	18
CTL-OG2	4	2	2	7	15
CTL-OG3	0	0	0	5	5
CTL-OG4	1	0	1	2	4
PLA-OG1	2	5	2	2	11
SVMP-OG1	2	3	1	5	11
SVMP-OG2	1	2	1	4	8
SVMP-OG3	1	1	1	5	8
SVMP-OG4	1	2	2	2	7
SVMP-OG5	2	1	3	1	7
SVMP-OG6	0	1	1	2	4
SVMP-OG7	0	0	1	2	3

SVMP-OG8	0	0	1	1	2

The three reconstructed phylogenetic trees are presented in Figures 7-9. The results from the orthology analyses were used to compare these putative orthologs with the evolutionary relationships in the major toxin families. For CTLs, the orthogroups are consistent with the phylogenetic relationships and each orthogroup forms a consistent clade except for the "B.lojanus_Toxin20952" sequence (CTL OG1) that is part of the CTL OG2 clade. In addition, the CTL OG3 clade is part of the CTL OG1 clade. For PLA2s, the phylogenetic tree recovered three clades; considering that OrthoFinder found only one orthogroup, the analyses among transcripts were made for phospholipase type instead of orthogroups. The first and second clades are formed by basic PLA2s, except for the "B.microphthalmus_Toxin328" sequence that corresponds to an acidic PLA2; whereas the third clade is all formed by acidic PLA2s.

For SVMPs, in most cases, the orthogroups recovered in previous steps are well supported by the phylogenetic relations. Orthogroups 3, 4, 6, 7, and 8 are formed by well-structured and supported clades with all monophyletic groups. However, the other three orthogroups include clades with unresolved relationships; OG 1 includes a partially resolved polytomy and a sequence from another clade; OGs 2 and 5 included isoforms from different clades as an overlap as well as the orthology non-assigned sequences ("B.hyprora_Toxin489").

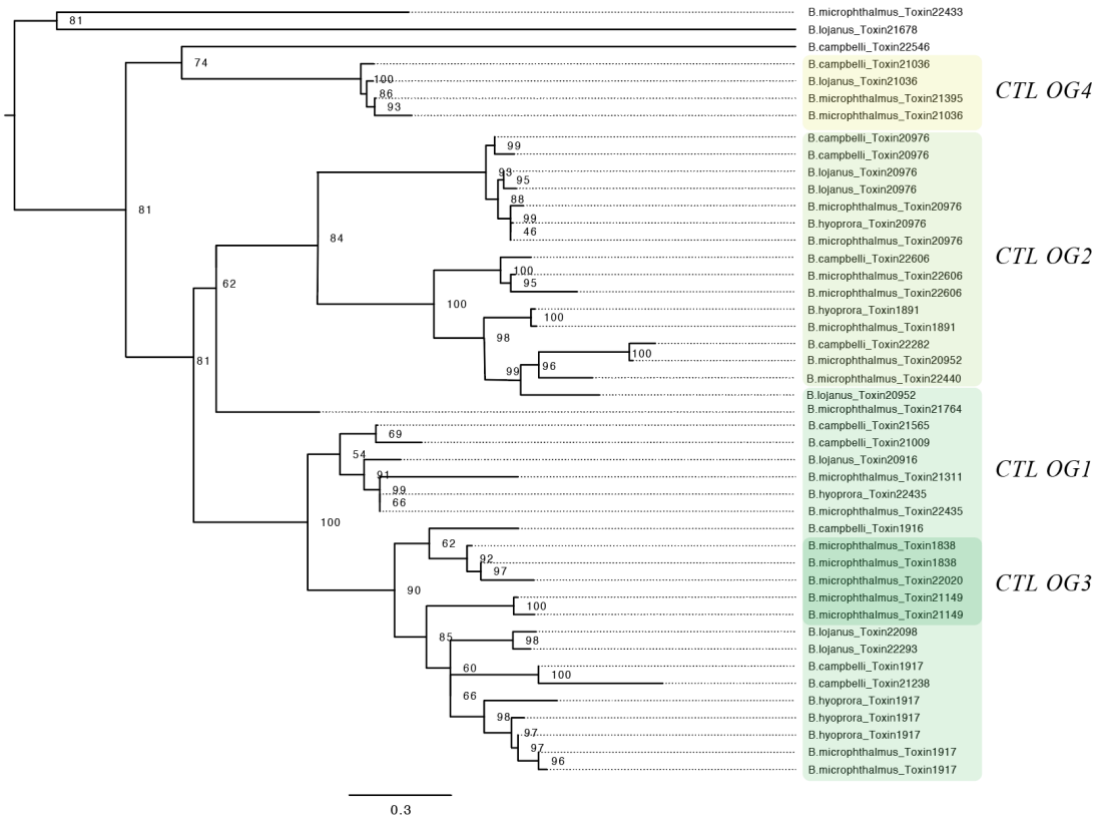

Figure 7. Maximum likelihood CTLs transcripts phylogeny. Colors in names represent the putative orthogroups recovered in orthology analyses. The transcripts without highlighted color represent unassigned groups.

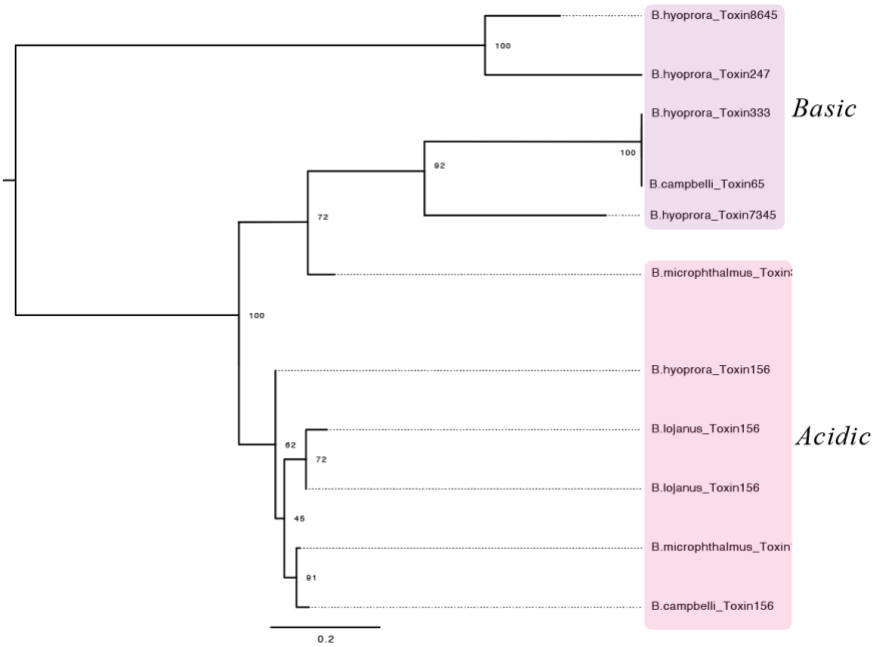

Figure 8. Maximum likelihood PLA2s transcripts phylogeny. Colors in names represent two PLA2 type: acidic and basic. All the PLA2s correspond to the same putative recovered orthogroup from orthology analyses.

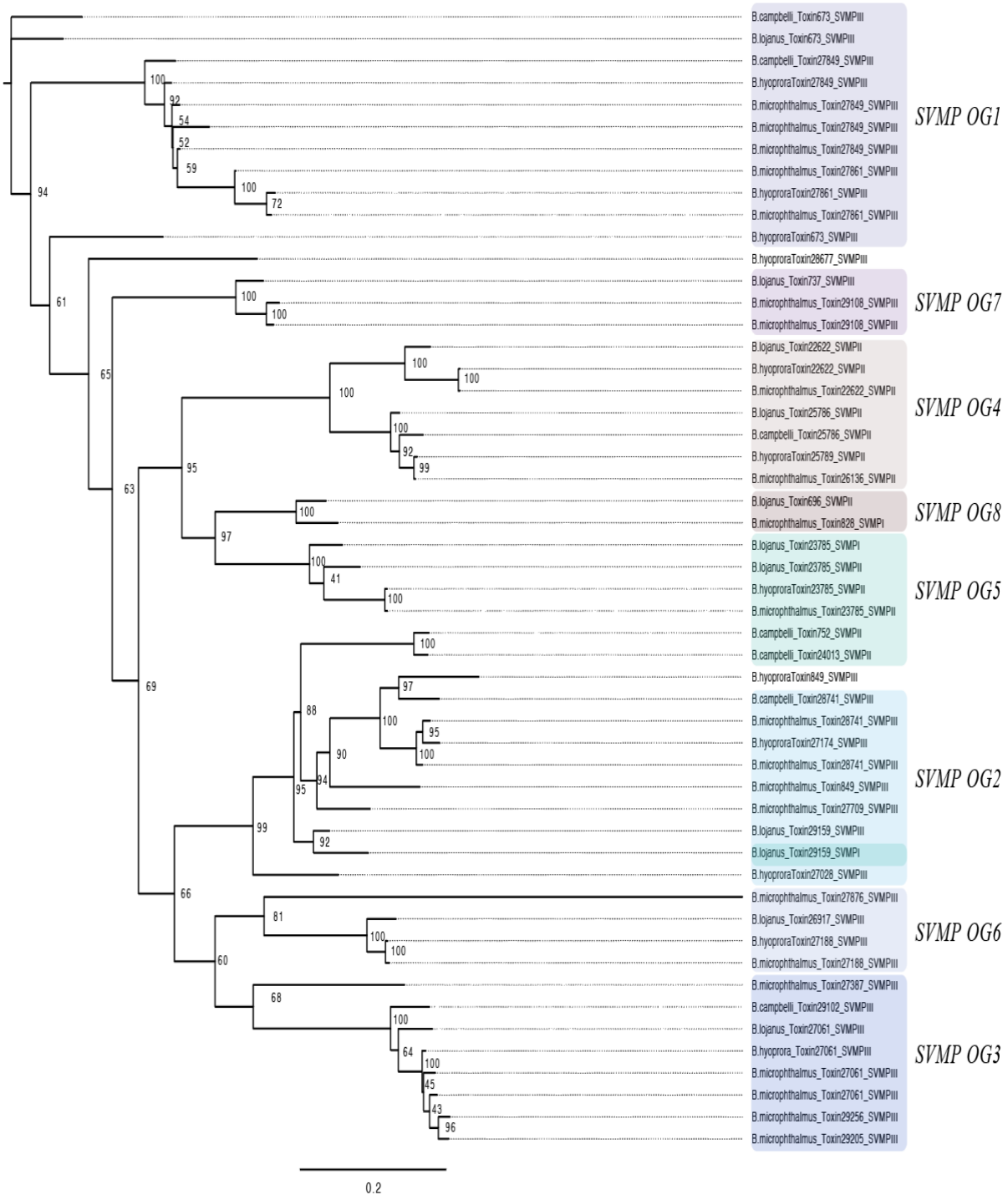

Figure 9. Maximum likelihood SVMPs transcripts phylogeny. Colors in names represent the putative orthogroups recovered in orthology analyses. The transcripts without highlighted color represent unassigned group

CHAPTER IV

DISCUSSION

Transcriptomic expression profiling

Within New World pitvipers, *Bothrops* snakes represent the sister lineage of *Bothrocophias* (Hamdan et al., 2020) and some of its species have been used for venom transcriptome characterization. For instance, Nachtigall et al. (2022) described the venom composition of two species from Brazil: *Bothrops cotiara* and *B. fonsecai*. Likewise, the first venom gland transcriptome analysis of *B. moojeni* was used to describe novel toxins sequences (Amorim et al., 2017). Other examples include *B. insularis* (Junqueira-de-Azevedo & Ho, 2002) and *B. jararacussu* (Kashima et al., 2004) from Brazil. Nevertheless, for the genus *Bothrocophias* there are no previous venom gland transcriptomic analyses. Nevertheless, some venom proteomic analyses have been published: one for Ecuadorian specimens of *B. campbelli* (Salazar-Valenzuela et al., 2014), another for Colombian specimens of *B. myersi* (Pereañez et al., 2020), and another for Peruvian samples of *B. andianus, B. hyoprora,* and *B. microphthalmus* (Lomonte et al., 2020). Our transcriptomic results for *B. campbelli* agree with the venom profiling described by Salazar-Valenzuela et al. (2014): a venom rich in PLA2s, with SVMPs and CTLs in lower amounts. In this venom, PLA2s are highly dominant in venom composition with more than three times as much expression as other toxins in both cases. This highly dominated PLA2 pattern is also present in its congener *B. myersi* where the difference is more than double (54.04% of PLAs vs 21.50% of SVMPs; Pereañez et al., 2020). Additionally, our results of SVMPs expression are similar to that present in *Atropoides mexicanus* (18.2%), a Central American species closely related to the South American *Bothrops-Bothrocophias* clade (Angulo et al., 2008; Hamdan et al., 2020).

Our findings for *Bothrocophias hyoprora* show the same venom type; however, it does not present the huge dominance of PLA2s as *B. campbelli*, and SVMPs are almost as expressed as PLA2s. Also, our results agree with the profile proposed by Lomonte et al. (2020) who found high peaks of PLA2s in *B. hyoprora* venom, suggesting a high abundance of these toxins. In addition, our PLA2s result expression is similar to *Bothriechis nigroviridis* (38.3%; Fernández et al., 2010) and *Agkistrodon laticinctus* (39.2%; Lomonte et al., 2014). Besides, SVMPs' total amount is similar to *A. taylori* and

A. bilineatus as well described by Lomonte et al. (2014). As previously discussed, these two species (*B. campbelli* and *B. hyoprora*) correspond to Type II New World pitviper venom described by Mackessy (2021) and are less common than the SVMPs rich venom type.

On the other hand, the other two species (*B. lojanus* and *B. microphtahlmus*) used in this research belong to Type I venom, where the venom is dominated by SVMPs (Mackessy, 2021). This venom type has been widely reported in *Bothrops*, the sister clade of *Bothrocophias*, and it is known as a more common venom type than the other found in *B. campbelli* and *B. hyoprora.* The results for *B. lojanus* show a high dominance of SVMPs where more than half of the toxin expression corresponds to this family (56.31%), being comparable to the composition reported in the venom gland transcriptome of *Bothrops jararaca*'s newborns (53.2%; Zelanis et al., 2012), as well as its southern populations (52.8%; Gonçalves-Machado et al., 2016). It could be also compared with *Bothrops ayerbei* (53.7%; Mora-Obando et al., 2014) and *Bothrops insularis* (43.2%; Valente et al., 2009) venoms.

Finally, findings in the venom gland transcriptome of *B. microphthalmus* show a higher abundance of toxins than those generally reported in *Bothrops* (Nachtigall et al., 2022). Despite this, this species' venom is similar in composition to *B. lojanus* where SVMPs correspond to the major toxin family, followed by CTLs and PLAs. Our results agree with previously characterized venom for *B. microphthalmus* where PLA2s show scarce presence, suggesting low myotoxic activity (Lomonte et al., 2020). However, a more-than-a-half SVMPs dominance is not evidenced and its composition resembles *Bothrops bilineatus* (43.7%; Sanz et al., 2020), Peruvian *Bothrops brazili* (33.05%; Rodrigues et al., 2020), *Bothrops jararaca*'s adults (25.4%- 33.1%; Zelanis et al., 2012) and its southeastern populations (34.4%; Gonçalves-Machado et al., 2016). This venom composition can also be compared to species from other genera of American Vipers, such as *Cerrophidion godmani* (32.8%; (Lomonte et al., 2012), *Porthidium lansbergii* (35.5%; (Jiménez-Charris et al., 2014), *Porthidium porrasi* (36.5%; Méndez et al., 2019), and *Porthidium volcanicum* (38.9%; Ruiz-Campos et al., 2021).

Intraspecific variation

Variability among snake venoms has been observed in different families or genera, as well as within the same species (Brahma et al., 2015); consequently, it is of great importance to obtain several samples of the same species to provide a better understanding of intraspecific diversity in venom composition (Nachtigall et al., 2022), and can provide a better understanding not only of biomedical approach such as the development of more effective anti-venom therapy but also of the natural history that influences the development of different venom compositions. As explained earlier, we carried out intraspecific analyses in two of the four species (*B. lojanus* and *B. microphthalmus*).

B. lojanus venom variation shows the highest compositional variability in CTLs, PLAs, SVMPs II, and SVMPs III in one of the three individuals analyzed; this variation occurs in a male juvenile (MZUTI 5404), suggesting an ontogenetic variation that agrees with previous *Bothrops* variability studies that show differences in venom composition between newborns, juveniles and adults (Alape-Girón et al., 2008; Machado Braga et al., 2020). For instance, *B. jararaca* transcriptome analyses reveal variation among newborns and adults in, mainly, the SVMPs and PLA2s expression levels (Zelanis et al., 2012). Likewise, higher SVMP III levels in juveniles than in adults, as in our results, have been reported in the Costa Rican *Bothrops asper* (Alape-Girón et al., 2008) and the Amazon *Bothrops atrox* (Monteiro et al., 2020). It should be noted that ontogenetic variability has also been demonstrated by the change in diet of individuals (Freitas-De-sousa et al., 2020). Thus, we may hypothesize that transcriptome ontogenetic variation in this species may be influenced by change in diet among juveniles and adults; nevertheless, to corroborate this hypothesis, the stomach contents of the individuals used should be analyzed. Finally, venom variability throughout a species distribution has been reported in other South American pitvipers. However, we were not able to assess it in *Bothrocophias lojanus* because the collection distances between the three specimens were too small, ranging only from ~1.6 km to ~16 km.

In contrast to *Bothrocophias lojanus,* a difference between juveniles and adults is not evident in *B. microphthalmus*. The juvenile MZUTI 5637 is similar in composition to the adult MZUTI 5528 which does not suggest an ontogenetic variation. Despite this, one adult (MZUTI 5635) shows different composition from the other adult MZUTI 5528, and the juvenile MZUTI 5637. In addition, distribution-composition differences cannot be

explored since the three individuals have the same collection data. Therefore, we can suggest an intrapopulation variability in the composition instead of an ontogenetic variation.

Orthology analyses

Inferring orthogroups by orthology analyses allowed us to understand the main evolutionary relationships for the venom composition of species. However, these orthogroups only draw putative relations and need to be contrasted with phylogenetic trees analyses between the main toxin families to better understand their evolutionary relationship. Thus, the three reconstructed phylogenetic trees allowed us to better discern the putative duplication and loss events in toxin families analyses (Nachtigall et al., 2022). In this research, by having four species, the one-to-one orthologues are not the only relation detected between isoforms, and consequently, several many-to-one orthologues were detected. However, we focused on the one-to-one orthologues to ease the description and discussion of results.

For CTLs, two strict-sister-terminals one-to-one orthologues were identified, whereas several many-to-one relations were also detected. For instance, in the CTL OG4 clade, the "B.campbelli_Toxin21036" transcript belongs to a many-to-one orthology relationship for the rest of the clade. In addition, this orthogroup is supported by a monophyletic relation suggesting that its transcripts came from an ancestral common gene and clustered in a true orthogroup. Nevertheless, in this clade, three of the four species have a representative sequence except for *B. hyoprora*, while *B. microphthalmus* retained two transcripts, suggesting a putative duplication/loss event occurred within the clade. The other putative orthogroups show paraphyletic relationships that cannot be resolved in this research. Our results could also be explained by putative allelic variations in sequences that were not clustered by CD-HIT. For instance, CTL OG3 is formed by five sequences from the same species, and two of them have the same sequence identification with low genetic distances; these allelic variations have been observed more frequently in toxins than in non-toxins and suggest a heterozygote advantage in venom evolution, protein neofunctionalization, and consequently venom phenotype diversification (Otto & Yong, 2002).

The least abundant group, PLA2s, show three well-supported clades. In the first clade there are two different *B. hyoprora* toxins that could represent a putative duplication event or even an allelic variation. The second clade is well supported, where three species have a representative sequence, except for *B. lojanus*. Moreover, in this clade, a subclade is formed by two sequences from *B. hyoprora* and one from *B. campbelli*, suggesting the occurrence of two separate events: a putative loss in *B. lojanus*, and a putative duplication/loss in *B. hyoprora-B.campbelli* that resulted in a one-to-one orthologue. Interestingly, all basic forms of PLA2s correspond to two phylogenetically non-sister species (based on Arteaga et al., 2016 phylogeny) which may suggest that there is a positive selection in *B. hyoprora* that favors the maintenance of basics forms. On the other hand, the third clade is exclusively formed by acidic PLA2s with only one identified one-to-one orthologue and a putative duplication event in *B. lojanus*. This clade is also constructed by transcripts from all species, suggesting that acidic PLA2s from *Bothrocophias* are more conserved across species than basic ones. It is known that conserved regions exist in PLA2s from several viper species (Moura-da-Silva et al., 1995); however high mutation rate in nonsynonymous sites (as well as duplication and loss events) and consequently diversification of PLA2s among species have been observed in *Bothrops* and *Crotalus* snakes (Dowell et al., 2016; Moura-da-Silva et al., 1995; Nachtigall et al., 2022), which led us to propose that a similar process is occurring in Toadheaded pitvipers too.

For SVMPs, eight strict-sister-terminals one-to-one orthologues were identified; whereas several many-to-one relations were observed. As detected in CTLs, not all the putative orthogroups were well supported and monophyletic. Instead, a few putative OGs show paraphyletic relations. For instance, SVMP OG2 is part of a clade with all the OG2 sequences plus one non-assigned sequence and two SVMP OG5 transcripts. In this case, the non-assigned transcript forms a one-to-one orthologue with an orthogroup sequence ("B.campbelli_Toxin28741_SVMPIII") suggesting they are true one-to-one orthologues and may be included in the orthogroup. In addition, in this clade, there is a transcript previously recognized as part of the OG 5 ("B.lojanus_Toxin29159_SVMPI"); therefore, we hypothesize three likely scenarios: 1) the SVMP I sequence corresponds to an SVMP III chimeric sequence that was not identified by ChimeraKiller, and lost the C-terminal part of the complete transcript (disintegrin, and cysteine-rich domains); it should be noted that its sister transcript is a *B. lojanus* sequence with the same identification and that the

protease domain is a highly conserved region with low levels of variation (Brust et al., 2013); 2) an alternative splicing process occurred in this gene, triggering a novel sequence that is real and is being transcribed; these alternative splicing events have been reported in different genera, such as *Crotalus*, *Protobothrops*, and *Sistrurus* showing the potential of alternative splicing to develop novel transcripts and consequently different proteins (Ogawa et al., 2019; Pahari et al., 2007; Rokyta et al., 2012); and 3) duplication or allelic variation events occurred causing these paralogues transcripts. Other putative orthogroups appear to be well supported and could be true orthogroups.

Our overall results suggest that duplication and loss events are occurring in the three analyzed toxin families which can potentially trigger diversification of the venom. These gene duplications play an important role in venom evolution and seem to be the main event that causes neofunctionalization (as well as subfunctionalization) of genes (Casewell et al., 2011; Hargreaves et al., 2014; Wong & Belov, 2012). As previously discussed, the orthology analyses provided a good first step to deciphering the relation between toxin genes. Nevertheless, in this research, a few putative orthogroups were better understood using phylogenetic relations, showing the importance of performing joint analyses. In addition, all the putative duplication/loss events evidenced in this research allowed us to suggest that all three analyzed toxin classes are evolving under a *birth and death* evolution model. This model has been proven in toxin studies in some snakes and seems to be a better explanation of the evolution of multigene families (Brahma et al., 2015), and discern of concerted evolution in which members of a multigene family do not evolve independently, but rather a mutation occurring in one of the copies eventually becomes fixed in all copies within a genome (Frankel, 2006). However, to have more robust support and a better understanding of present and absent toxin genes and their specific relation with other genera, a bigger phylogenetic study and complete genomic study is needed to confirm our hypotheses as suggested by Nachtigall et al. (2022).

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Analyzed venom gland transcriptomes allowed us to characterize the RNA expression profile finding three principal toxin families in *Bothrocophias* species: CTLs, PLA2s, and SVMPs.

B. hyoprora is the only species that shows the presence of 3FTxs; however, their expression levels are lower and are not considered relevant toxins in its transcriptome.

Of 19 toxin families, 11 families are expressed in all species. On the other hand, eight families are not expressed in at least one species; however, not all of them show a similar expression pattern among the species.

Our results support two different compositional patterns among the four sampled species. The first pattern is dominated by PLA2s (*B. campbelli* and *B. hyoprora*) and the other one is dominated by SVMPs (*B. lojanus* and *B. microphthalmus*).

The main interspecific differences in transcriptomic profiling are influenced by the PLA2s (according to previous proteomic reports) and SVMPs levels of expression; other toxins (except for CTLs) do not show an important expression pattern to influence the venom profiling.

We also determined that just one species (*B. lojanus*) contains ontogenetic variation in venom composition. The other species (*B. microphthalmus*) probably variation within population variation. The main intraspecific differences in *B. lojanus* and *B. microphthalmus* are influenced by SVMP III, PLA2s and CTLs. We recommend increasing juvenile, adult, male, and female samples in all species.

Even though *Bothrocophias* specimens can be difficult to locate, we recommend including specimens from different localities to analyze the distribution influence in venom transcriptomic profiling in detail.

Gene duplication plays a relevant role in venom composition in all species; these events (as well as loss events) seem to be the main evolutionary force influencing the differences among species.

Our orthology analyses indicate that *birth and death* evolution is probably occurring in CTLs and SVMPs, especially within the *B. hyoprora* clade.

To sum up, as a general recommendation, the inclusion of proteomic analyzes is necessary to determine the complete final venom profile for each species. Additionally, it is recommended to do genomic studies to discern the evolutionary relationships of genes and their putative duplication/loss events.

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