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# Proteomic and toxicological profiling of the venom of *Bothrocophias campbelli*, a pitviper species from Ecuador and Colombia

David Salazar-Valenzuela [a, b], Diana Mora-Obando [c], María Laura Fernández [c], Amaru Loaiza-Lange [b], H. Lisle Gibbs [a], Bruno Lomonte [c, *]

[a] *Department of Evolution, Ecology and Organismal Biology, The Ohio State University, 300 Aronoff Laboratory, 318 W. 12th Ave., Columbus, OH 43210-1293, USA*
[b] *Escuela de Biología, Pontificia Universidad Católica del Ecuador, Avenida 12 de Octubre y Roca, Apartado 17-01-2184, Quito, Ecuador*
[c] *Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José 11501, Costa Rica*

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ABSTRACT

Detailed snake venom proteomes for nearly a hundred species in different pitviper genera have accumulated using 'venomics' methodologies. However, venom composition for some lineages remains poorly known. *Bothrocophias* (toad-headed pitvipers) is a genus restricted to the northwestern portion of South America for which information on venom composition is lacking. Here, we describe the protein composition, toxicological profiling, and antivenom neutralization of the venom of *Bothrocophias campbelli*, a species distributed in Colombia and Ecuador. Our analyses show that its venom mainly consists of phospholipases $A_2$ (43.1%), serine proteinases (21.3%), and metalloproteinases (15.8%). The low proportion of metalloproteinases and high amount of a Lys49 phospholipase $A_2$ homologue correlate well with the low hemorrhagic and high myotoxic effects found. Overall, *B. campbelli* venom showed a simpler composition compared to other crotalines in the region. A polyvalent antivenom prepared with a mixture of *Bothrops asper*, *Crotalus simus*, and *Lachesis stenophrys* venoms cross-recognized *B. campbelli* venom and neutralized its lethal effect in mice, albeit with a lower potency than for *B. asper* venom. Additional work comparing *B. campbelli* venom properties with those of related species could help understand the evolution of different venom protein families during the South American radiation of New World pitvipers.



## 1. Introduction

The genus *Bothrocophias* (Toadheaded pitvipers) consists of six species of South American pitvipers, whose distribution includes cis-/trans-versants of the Northern and Central Andes, as well as the upper Amazon Basin (Gutberlet and Campbell, 2001; Campbell and Lamar, 2004; Carrasco et al., 2012). They are restricted to primary or slightly disturbed forests; therefore some members (e.g. *Bothrocophias campbelli*) appear to be rare in nature (Campbell and Lamar, 2004; Arteaga, 2013). Nevertheless, some species (e.g. *Bothrops hyoprora* and *Bothrops microphthalmus*) may be more common, at least in parts of their distribution (Kuch and Freire, 1995; Cisneros-Heredia et al., 2006). Traditionally, these species were included in the genus *Bothrops* with the recognition of the *Bothrocophias* lineage occurring rather recently (see review in Gutberlet and Harvey, 2004). Phylogenetic studies based on morphological and molecular data have found support for

* Corresponding author.
*E-mail address:* bruno.lomonte@ucr.ac.cr (B. Lomonte).

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recognizing the genus *Bothrocophias* as a monophyletic lineage, and the available evidence suggests it constitutes the sister clade of the remaining lineages of South American pitvipers (Fenwick et al., 2009; Jadin et al., 2011; Carrasco et al., 2012). Toadheaded pitvipers are terrestrial snakes of moderate length, a relatively stout body, and a large head (Gutberlet and Campbell, 2001; Campbell and Lamar, 2004).

The Ecuadorian toadheaded pitviper, *B. campbelli* (Freire-Lascano, 1991) is a medium sized snake (mean adult total length: 101.6 cm, mean adult weight: 580.5 g) known from mid-elevation localities (1000–2250 m above sea level) on the western versant of the Cordillera Occidental of the Andes in Ecuador and Colombia (Campbell and Lamar, 2004; Castro et al., 2005; Cisneros-Heredia et al., 2006; Valencia et al., 2008; Arteaga, 2013; personal observations). Its diet appears to be generalist with prey reports based on rodents, caecilians, and snakes (Freire and Kuch, 2000; Zuffi, 2004; Cisneros-Heredia et al., 2006; Rojas-Rivera et al., 2013). Although no snakebite accidents have been reported for this species, envenomation reports for other species in the genus *Bothrocophias* include local signs and symptoms such as severe pain, swelling, and necrosis, whereas systemic effects include coagulopathies, hemorrhages, haematuria, thrombosis, renal failure, and hypovolemic shock (Campbell and Lamar, 2004; Warrell, 2004).

Snake venoms and their constituent toxins have evolved as a result of the actions of different evolutionary and ecological forces (Mebs, 2001; Mackessy, 2010; Calvete, 2013). Comparative studies of snake venom protein evolution (e.g. Gibbs et al., 2013) are hindered without information of venom composition present in different lineages. The genus *Bothrocophias* represents a lineage of early divergence in the South American pitviper radiation, which occurred throughout much of the Caenozoic when this landmass was isolated from North and Central America (Gutberlet and Harvey, 2004; Werman, 2005). To our knowledge, no venom proteomic studies exist for members of the genus. The present work describes the venom composition of *B. campbelli* analysed by the 'snake venomics' methodology (Calvete et al., 2007), and provides an assessment of some of its toxicological properties and its neutralization by antivenom.

## 2. Materials and methods

### 2.1. Venom samples

Venom was obtained from three adult *B. campbelli* specimens. Two males were found in primary forest near Mindo river, Mindo, Pichincha Province (0°4′,46″N, 78°45′45″W; datum = WGS84; 1208 m above sea level [asl]). A female individual kept in captivity by local people was provided to us at El Cristal, Esmeraldas Province (0°51′52″N, 78°28′52″W; datum = WGS84; 1116 m asl). Animals were constrained using a set of telescoping clear plastic tubes and then induced to bite the top of a 50 ml glass beaker covered with Parafilm®. The secretion was immediately pipetted into a 1.5 ml tube and dried in a vacuum container containing anhydrous calcium sulphate (Drierite desiccant). Dried venom was stored in a −80 °C freezer. For comparative purposes in functional assays, the recently characterized venom of *Bothrops asper* from the Cauca Department, Colombia (Mora-Obando et al., 2014) was used in some experiments.

### 2.2. Reverse-phase HPLC and venomic characterization

A venom pool was prepared by mixing equal parts of venom from the three specimens. Approximately 1 mg was dissolved in 200 μL of water containing 0.1% trifluoroacetic acid (TFA), centrifuged at 15,000 × *g* for 5 min to remove debris, and fractionated by RP-HPLC on a $C_{18}$ column (Teknokroma; 4.6 × 250 mm, 5 μm particle) using an Agilent 1200 chromatograph. To assess individual variability, each of the three venom samples was also analysed separately under identical conditions as was the pooled venom. Elution was performed at 1 mL/min by applying a gradient towards solution B (0.1% TFA in acetonitrile), as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min. Absorbance of the eluent was recorded at 215 nm and the peak areas of the chromatogram were integrated using ChemStation B.04.01 software (Agilent). Venom composition was analysed following the 'snake venomics' strategy (Calvete et al., 2007) with slight modifications as described in Lomonte et al. (2014a). HPLC fractions were manually collected, dried in a vacuum centrifuge, redissolved in water, and further separated by SDS-PAGE under reducing conditions (5% 2-mercaptoethanol at 100 °C for 5 min) in 12% gels. After Coomassie blue R-250 staining, gel images were recorded using ChemiDoc/Image-Lab (Bio-Rad) and analysed by densitometry, which was combined with the integration of HPLC peak areas to estimate protein relative abundances. Protein bands were then excised and subjected to in-gel reduction (10 mM dithiothreitol), alkylation (50 mM iodacetamide), and overnight digestion with trypsin in a ProGest processor (Digilab), following manufacturer's recommendations. The resulting peptides were extracted and characterized by MALDI-TOF-TOF mass spectrometry on an AB4800-Plus Proteomics Analyzer instrument (Applied Biosystems). Peptides were mixed with an equal volume of saturated α-cyano-hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), spotted (1 μL) onto an Opti-TOF 384-well plate, dried, and analysed in positive reflector mode. Spectra were acquired using a laser intensity of 3000 and 1500 shots/spectrum, using as external standards CalMix-5 (ABSciex) spotted on the same plate. Up to 10 precursor peaks from each MS spectrum were selected for automated collision-induced dissociation MS/MS spectra acquisition at 2 kV, in positive mode (500 shots/spectrum, laser intensity 3000). For the assignment of proteins to known families, the resulting spectra were analysed against the UniProt/SwissProt database (January 2014) using ProteinPilot v.4 (ABSciex) and Paragon® algorithm at a confidence level of ≥95%. A few peptide sequences with lower confidence scores were manually searched using BLAST (http://blast.ncbi.nlm.nih.gov).

### 2.3. Toxicological profiling

#### 2.3.1. Animals

Animal experiments were conducted using CD-1 mice of either sex, following protocols approved by the



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