

Comparative characterization of Viperidae snake venoms from Perú reveals two compositional patterns of phospholipase A₂ expression

Bruno Lomonte^{a,*}, Cecilia Díaz^{a,b}, Fernando Chaves^a, Julián Fernández^a, Marco Ruiz^a, María Salas^c, Alfonso Zavaleta^c, Juan J. Calvete^d, Mahmood Sasa^{a,e}

^a Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

^b Departamento de Bioquímica, Escuela de Medicina, Universidad de Costa Rica, San José, Costa Rica

^c Departamento Académico de Ciencias Celulares y Moleculares, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Peru

^d Laboratorio de Venómica Evolutiva y Traslacional, Instituto de Biomedicina de Valencia, CSIC, Valencia, Spain

^e Escuela de Biología, Universidad de Costa Rica, San José, Costa Rica

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ABSTRACT

Snake species within the *Bothrops* complex (*sensu lato*) are of medical relevance in Latin America, but knowledge on their venom characteristics is limited, or even unavailable, for some taxa. Perú harbors 17 species of pit vipers, within the genera *Bothrops*, *Bothriechis*, *Bothrocophias*, *Porthidium*, *Crotalus*, and *Lachesis*. This study compared the venoms of twelve species, through chromatographic and electrophoretic profiles, as well as proteolytic and phospholipase A₂ (PLA₂) activities. Also, proteomic profiles were analyzed for nine of the venoms using a shotgun approach. Results unveiled conspicuous differences in the expression of venom PLA₂s among species, six of them presenting scarce levels as judged by RP-HPLC profiles. Since most species within the bothropoid lineage possess venoms with high to intermediate abundances of this protein family, our findings suggest the existence of a phenotypic duality in the expression of venom PLA₂s within the *Bothrops* (*sensu lato*) complex. *Bothrops barnetti* and *Bothrocophias andianus* venoms, very scarce in PLA₂s, were shown to lack significant myotoxic activity, highlighting that the observed variability in PLA₂ expression bears toxicological correlations with effects attributed to these proteins. Finally, an attempt to identify phylogenetic relationships of bothropoid species from Perú presenting low- or high-PLA₂ venom phenotypes showed an interspersed pattern, thus precluding a simple phylogenetic interpretation of this venom compositional dichotomy.

1. Introduction

In the Americas, an average of 6.2 snakebites per 100,000 population (about 57,500 accidents) is estimated to occur annually, leading to a fatality rate of 0.04 per 100,000 population (Chippaux, 2017). The genus *Bothrops* (lanceheads), distributed from Mexico to southern Argentina, is responsible for the majority of envenomings in Latin America (Chippaux, 2017). Classified within the Viperidae family, this genus includes 45 species listed in the Reptile Database (<http://www.reptile-database.org>) and has been the subject of dynamic taxonomic reclassifications within the bothropoid lineages in the last two decades (Wüster et al., 2002; Fenwick et al., 2009; Carrasco et al., 2012, 2016; Alencar et al., 2016). Despite the medical relevance of species within the *Bothrops* complex (*sensu lato*), knowledge on the characteristics of their venoms is still limited, or unavailable, for some species.

The growing body of information on the composition of viperid venoms, as provided by “omics” approaches, has revealed the existence of a limited number of protein families that are generally shared among conspecific species, with both qualitative and quantitative variations (Calvete, 2013, 2017; Lomonte et al., 2014; Göçmen et al., 2015; Tan et al., 2015; Viala et al., 2015; Serrano et al., 2018). Knowledge of this variability is essential for the production of broad-coverage therapeutic antivenoms to confront snakebite envenomings. Also, the study of inter- and intraspecific venom variability may shed light on understanding the evolutionary pathways that have shaped the present-day landscape of the snake venom toxin arsenals.

Perú harbors a rich herpetofauna, which includes 34 species or subspecies of venomous snakes distributed in two families, Elapidae and Viperidae. Sixteen coral snakes represent the former, all included in the genus *Micrurus* of subfamily Elapinae, and a single species of sea snake,

* Corresponding author. Universidad de Costa Rica, San José, 11501, Costa Rica.

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

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Hydrophis platurus within subfamily Hydrophiinae. Viperidae includes 17 pitviper species (Carrillo de Espinoza and Icochea, 1995) within the genera *Bothrops*, *Bothriechis*, *Bothrocophias*, *Porthidium*, *Crotalus*, and *Lachesis* (taxonomical nomenclature in the present work follows the proposal of Carrasco et al., [2016] for the *Bothrops* complex, *sensu lato*). Viperidae species are responsible for the vast majority of the estimated 1500–2500 snakebites/year recorded in Perú (Ministerio de Salud, 2017), resulting in a mortality rate of not less than 0.043/100,000 population (Chippaux, 2017).

Previous studies have examined various biochemical aspects and toxic activities of the venoms of Viperidae snakes inhabiting Perú (Sanz et al., 2008; Núñez et al., 2009; Jimenez et al., 2010; Kohlhoff et al., 2012; Guerra-Duarte et al., 2015; Rodrigues et al., 2018), and their neutralization (Laing et al., 2004; Rojas et al., 2005; García et al., 2008; Esteveo-Costa et al., 2016). However, information on most venoms of these species is still partial, and more efforts are needed to gain a broader knowledge of their general properties. In the present study, we have comparatively explored the chromatographic and electrophoretic profiles of the venoms of twelve species of pitvipers, as well as their proteolytic and phospholipase A₂ (PLA₂) activities. Besides, proteomic profiles were analyzed for nine of the venoms using a shotgun approach. These comparative analyses revealed important differences in the expression of PLA₂s among the species, which relate to variations in toxicological activities attributed to these enzymes and their related PLA₂-like homologs.

2. Materials and methods

2.1. Venoms

Venom pools were obtained from adult specimens collected in Perú, from the species and locations indicated in Table 1. In some comparative experiments, venoms of species from other regions of Latin America were included: *Bothrops alternatus* (Coronel Pringles, Argentina), *B. neuwiedii* (Tacuarembó, Uruguay), *B. asper* (Pacific versant, Costa Rica), and *Bothriechis schlegelii* (Costa Rica).

2.2. SDS-PAGE

Venom proteins were separated by SDS-PAGE using pre-cast gradient gels (4–20%; Bio-Rad), either unreduced or after reduction with 2-mercaptoethanol (5 min at 95 °C). Samples (30 µg) were separated at 150 V, along with molecular weight standards (Bio-Rad). Proteins were visualized by Coomassie blue R-250 staining and electrophoretic patterns were recorded with the ImageLab™ software (Bio-Rad).

2.3. Reverse phase-HPLC profiling

Chromatographic profiles of venoms were obtained by RP-HPLC using a C₁₈ column (250 × 4.6 mm, 5 µm particle diameter; Phenomenex) in a model 1220 instrument (Agilent) monitored at 215 nm. Venoms (1.5–2.0 mg) were dissolved in purified water (18 MΩ/cm) containing 0.1% trifluoroacetic acid (TFA; solution A), injected, and eluted with a gradient toward acetonitrile with 0.1% TFA (solution B), at a flow rate of 1 mL/min, for a total time of 94 min: 0% B for 5 min, 0–15% B in 10 min, 15–45% B in 60 min, 45–70% B in 10 min, and 70% B for 9 min (Lomonte and Calvete, 2017).

2.4. Phospholipase A₂ activity

Phospholipase A₂ (PLA₂) activity of the venoms was assayed using the synthetic substrate 4-nitro-3-octanoyloxy-benzoic acid (NOBA), as described (Mora-Obando et al., 2014a). Various amounts of venoms (5, 10, or 20 µg), in 25 µL, were added to 25 µL of NOBA (1 mg/mL acetonitrile) and 200 µL of 10 mM Tris, 10 mM CaCl₂, 0.1 M NaCl (pH 8.0), in 96-well microplates. Blanks in which venom was replaced with buffer were included. The mixtures were incubated at 37 °C for 60 min, and final absorbances at 450 nm were measured in a microplate reader (Multiskan FC, Thermo), using three replicates for each venom concentration.

2.5. Proteolytic activity

Proteolytic activity of the venoms was determined using azocasein (10 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, pH 8.0) as substrate, as previously described (Jiménez-Charris et al., 2015). Various amounts of venoms (5, 10, or 20 µg) were incubated with azocasein at 37 °C for 90 min, in a total volume of 100 µL, and the reaction was stopped by adding 200 µL of 5% trichloroacetic acid (TCA). Blanks in which venom was omitted were included. After centrifugation of the microplates, 150 µL of the supernatant was transferred to clean wells, and 150 µL of 0.5 M NaOH was added for color development. Absorbances were measured at 450 nm in a microplate reader (Multiskan FC, Thermo), using three replicates for each venom concentration.

2.6. Proteomic profiling

Venoms from nine species were analyzed by a bottom-up shotgun proteomic approach. Samples of 15 µg were reduced with 10 mM dithiothreitol for 30 min at 56 °C, alkylated with 50 mM iodoacetamide for 20 min in the dark, and digested with sequencing grade trypsin at 37 °C overnight, in a total volume of 40 µL. After the addition of 0.5 µL of formic acid, the resulting tryptic peptide mixtures were centrifuged and separated by RP-HPLC on a nano-Easy 1200 chromatograph (Thermo)

Table 1
Venoms from Viperidae species of Perú analyzed in the present study, and their localities.

Species ^a	Common name	n ^b	District	Province	Department
<i>Bothrops atrox</i>	jergón de la selva	60	Llata, Chaglia, Puerto Inca, Codo del Pozuzo	Humalíes, Pachitea, Puerto Inca	Huánuco
<i>Bothrops barnetti</i>	macanche	20	Quebrada Honda, Olmos	Talara, Lambayeque	Piura, Lambayeque
<i>Bothrops bilineatus</i>	loro machaco	10	Pichanaki, Vitoc, Chanchamayo	Chanchamayo	Junin
<i>Bothrops taeniata (castelnaudi)</i>	jergón de árbol	nd	Satipo	Jauja	Junin
<i>Bothrops chloromelas</i>	–	8	Huancabamba	Huancabamba	Piura
<i>Bothrops oligolepis (peruvianus)</i>	jergón negro	8	Satipo	Jauja	Junin
<i>Bothrops pictus</i>	jergón de la costa	5	Pachacamac	Lima	Lima
<i>Bothrocophias andianus</i>	jergona	4	Macchu Picchu	Urubamba	Cusco
<i>Bothrocophias hyoprora</i>	jergón shushupe	10	Puerto Pakuy, Imaza	Bagua	Amazonas
<i>Bothrocophias microphthalmus</i>	jergón pudridora	nd	Pichanaki	Chanchamayo	Junin
<i>Bothriechis schlegelii</i>	loro	4	Reserva de Tumbes	Zarumilla	Tumbes
<i>Lachesis muta</i>	shushupe	10	Alto Marañón, Nazareth, Pijuyal	Bagua, Condorcanqui	Amazonas

^a Taxonomical nomenclature used in this work follows the proposal of Carrasco et al. (2016), which synonymizes *Rhinocerocephis*, *Bothropoides*, and *Bothriopsis* with *Bothrops*, while maintaining *Bothrocophias* and assigning *Bothrops andianus* to that genus.

^b n: number of individuals in the venom pool; nd: not determined.

on-line with a Q-Exactive Plus® mass spectrometer (Thermo). Twelve μL of peptide mixture, containing 0.7 μg , were loaded on a C_{18} trap column (75 $\mu\text{m} \times 2 \text{ cm}$, 3 μm particle; PepMap, Thermo), washed with 0.1% formic acid (solution A), and separated at 200 nL/min on a C_{18} Easy-spray® column (75 $\mu\text{m} \times 15 \text{ cm}$, 3 μm particle; PepMap, Thermo). A gradient toward solution B (80% acetonitrile, 0.1% formic acid) was developed in a total of 120 min (1–5% B in 1 min, 5–26% B in 84 min, 26–80% B in 30 min, 80–99% B in 1 min, and 99% B in 4 min). MS spectra were acquired in positive mode at 2.0 kV, with a capillary temperature of 200 °C, using 1 μscan in the range 400–1600 m/z , maximum injection time of 50 msec, AGC target of 1×10^6 , and resolution of 70,000. The top 10 ions with 2–5 positive charges were fragmented with AGC target of 3×10^6 , minimum AGC 2×10^3 , maximum injection time 110 msec, dynamic exclusion time 5 s, and resolution 17,500. MS/MS spectra were processed against protein sequences contained in the UniProt/SwissProt database (Serpentes, October 2019) using Peaks X® (Bioinformatics Solutions) and matches were assigned to known protein families by similarity. Cysteine carbamidomethylation was set as a fixed modification, while deamidation of asparagine or glutamine and methionine oxidation were set as variable modifications, allowing up to 3 missed cleavages by trypsin. Parameters for match acceptance were set to FDR < 0.1%, detection of at least one unique peptide, and -10lgP protein score ≥ 100 .

2.7. Myotoxicity assay

The myotoxic effect of the venoms of *Bothrops barnetti* and

Bothrocophias andianus (selected as examples of venoms nearly devoid of PLA₂s) was tested in mice. The venom of *B. asper* from the Pacific variant of Costa Rica, known to be abundant in PLA₂s (Alape-Girón et al., 2008), was included in this assay as a positive control. Groups of five CD-1 mice (18–20 g body weight) received an intramuscular venom injection (50 $\mu\text{g}/50 \mu\text{L}$) in the gastrocnemius. After 3 h, blood was obtained from the tip of the tail into heparinized capillary tubes, centrifuged, and plasma (4 μL) was assayed for creatine kinase (CK) activity using a kinetic commercial kit (CK-Nac, Biocon Diagnostik). A control group of mice received a similar injection of phosphate-buffered saline (PBS; pH 7.2) instead of venom. This experiment followed the ethical guidelines of the Institutional Committee for the Use and Care of Animals (CICUA) of the University of Costa Rica (No. 021–17). Statistical significance of the differences between groups was determined by ANOVA, followed by Tukey–Kramer post-hoc test, using the InStat v.3 (GraphPad) software.

3. Results

3.1. Electrophoresis

Venom SDS-PAGE patterns, under non-reducing and reducing conditions, are shown in Fig. 1. Although none of the inter-species patterns are entirely identical, significant resemblances are evident between several venoms, for example, *Bothrops atrox*, *B. barnetti*, *B. pictus*, and *B. neuwiedii*, especially after disulfide bond reduction. More differences appear in the unreduced electrophoretic patterns than in reduced ones, suggesting inter-species variations in the oligomeric association of

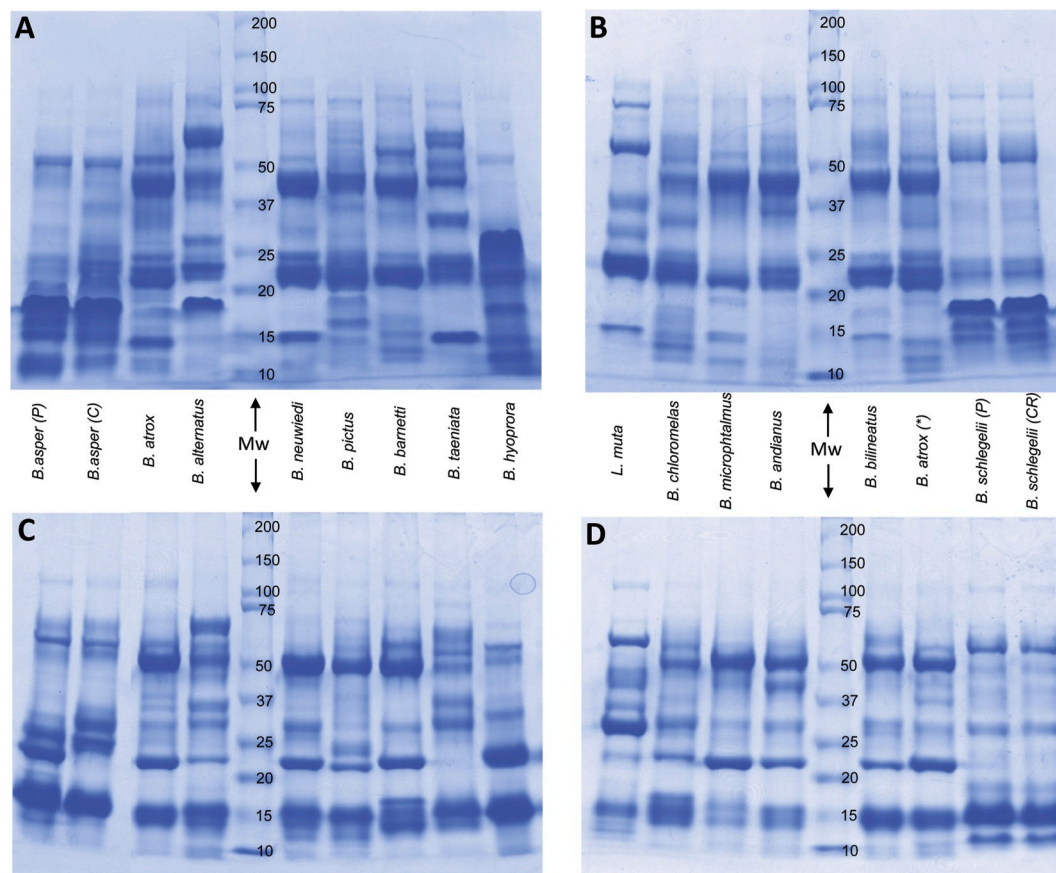


Fig. 1. SDS-PAGE profiles of venoms from twelve viperid species of Perú, unreduced (A, B) or reduced (C, D). Venoms of some species from other locations are included: *Bothrops asper* from the Pacific (P) or Caribbean (C) regions of Costa Rica, *B. alternatus* from Argentina, *B. neuwiedii* from Uruguay, and *B. schlegelii* from Costa Rica (CR). An additional sample of *B. atrox* venom from Perú is indicated with an asterisk. Molecular weight (Mw) markers are indicated in kDa. Proteins were stained with Coomassie brilliant blue G-250. The venom of *B. oligolepis* could not be analyzed in this technique due to insufficient amount. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

subunits of similar molecular mass. Notably, the electrophoretic pattern of *Bothriechis schlegelii* venom from Perú appears indistinguishable from that of the same species originating from Costa Rica, under both conditions (Fig. 1). All major venom proteins distributed below ~100 kDa. The most prominent reduced protein bands migrated at ~50, ~23, and ~15 kDa.

3.2. RP-HPLC profiles

Venoms were analyzed using a previously standardized C₁₈ RP-HPLC separation protocol (Lomonte and Calvete, 2017). Its application in several proteomic studies on snake venoms has shown that monomeric PLA₂s and PLA₂-like homologs elute within the range indicated with a shadowed dotted box in Fig. 2. Venoms varied largely regarding the presence of chromatographic peaks within this region, and can be separated into two types, namely those presenting conspicuous peaks (blue frame in Fig. 2) and those having barely detectable peaks. The venoms of *Bothriechis schlegelii* and *Bothrocophias hyoprora* showed the most intense peaks in this region, comparable to those of *Bothrops asper* venom from Costa Rica, used as a reference for the elution of PLA₂s (Fig. 2).

Since electrophoretic analysis showed a very high similarity of *Bothriechis schlegelii* venoms collected in Perú and Costa Rica (Fig. 1), their RP-HPLC profiles were determined and compared in Fig. 3. As shown, the two profiles are virtually superimposable, confirming their remarkable high similarity.

3.3. Phospholipase A₂ and proteolytic activities

PLA₂ activity of the venoms on the NOBA substrate was highly variable, as presented in Fig. 4. This activity was strikingly low in the case of *Bothrops oligolepis*, *B. barnetti*, *Bothrocophias andianus*, *Bothrocophias hyoprora*, and *Lachesis muta* venoms. In contrast, *Bothrops atrox*, *B. pictus*, *B. taeniata*, and *Bothriechis schlegelii* venoms were highly active in this assay, while low to intermediate activities were recorded for *Bothrops chloromelas*, *B. bilineatus*, and *Bothrocophias microphthalmus* venoms.

On the other hand, proteolytic activity on the azocasein substrate was more similar among the different venoms (Fig. 5), but *Bothriechis schlegelii*, *Bothrops taeniata*, and *Lachesis muta* venoms showed a slightly lower proteolytic activity than the rest.

3.4. Myotoxicity

The venoms of *Bothrops barnetti* and *Bothrocophias andianus* were selected as examples of venoms virtually devoid of PLA₂s in the RP-HPLC profiles, to test whether this phenotypic trait would correlate with toxic activities that are known to depend mainly on these proteins, such as *in vivo* myotoxicity. As shown in Fig. 6, intramuscular injection of these two venoms in mice did not induce a significant increment of plasma CK activity, in contrast to a venom (*Bothrops asper*, used as a reference) known to contain abundant PLA₂ (Asp49) and PLA₂-like (Lys49) myotoxins.

3.5. Shotgun proteomic profiling

Nine of the twelve venoms here studied were subjected to a bottom-up shotgun proteomic profiling. The complete dataset of matches with known proteins in the Uniprot “Serpentes” database is presented in Supplemental Table S1, and the different protein families identified in each venom are summarized in Table 2. Venom protein families detected by this qualitative approach ranged from 9 (*Bothrops barnetti*) to 13 (*B. atrox*, *B. oligolepis*, *B. chloromelas*). The most variable protein family detected corresponded to hyaluronidase (HYA), found in three out nine venoms, and vascular endothelial growth factor (VEGF), found in five out of nine (Table 2). Nerve growth factor (NGF) was present in seven venoms, while phospholipase B (PLB) phosphodiesterase (PDE) and

cysteine-rich secretory proteins (CRISP) were found in eight. Other major protein families commonly found in viperid venoms were detected in all nine species.

3.6. Relationship of the phospholipase A₂ low/high phenotypes to phylogeny

Differences in the expression of PLA₂s in viperids of the bothropoid lineage were mapped onto the partial phylogeny described by Carrasco et al. (2016) in Fig. 7, using results of the present study together with data from the literature (evaluated either by proteomic analyses or by reported isolation of PLA₂s). The obtained cladogram shows that the majority of bothropoid venoms studied to date typically present PLA₂s (indicated with pink dots). One notable exception is *Bothrops cotiara* from Brazil, reported lacking these enzymes (Tashima et al., 2008). However, *B. cotiara* has also been reported to have PLA₂s as the most abundant venom components in the case of specimens collected in Argentina (de Roodt et al., 2018), and was therefore indicated with both pink and blue symbols in Fig. 7.

In addition to *B. cotiara* from Brazil, another bothropoid species with barely detectable amounts of venom PLA₂s (0.7%) is *Bothrops ayerbeii* (Supplemental Fig. S1; Mora-Obando et al., 2014b), a lineage of the *B. asper* species complex (Silva-de-França et al., 2019).

In contrast to the widespread presence of PLA₂s in characterized venoms of most species included in the genus *Bothrops* (*sensu lato*) (Fig. 7), the chromatographic profiles of six out of the twelve venoms of species from Perú here studied revealed a phenotype in which PLA₂s are notably scarce: *Bothrops barnetti*, at the base of the “alternatus” clade (Carrasco et al., 2016), *Bothrocophias andianus* and *B. microphthalmus*, clustered in the “microphthalmus” clade, and *Bothrops chloromelas*, *B. bilineatus*, and *B. oligolepis*, in the “taeniata” clade (Fig. 7). The venom of *Bothrocophias hyoprora* differed sharply in this regard from those of other *Bothrocophias* species (*B. andianus* and *B. microphthalmus*), showing a major protein peak in the PLA₂-eluting region, and rather resembling the venom of *Bothrocophias campbelli* (Supplemental Fig. S1; Salazar-Valenzuela et al., 2014), a species not represented in the phylogenetic trees displayed in Fig. 7.

4. Discussion

Compositional, biochemical, and toxicological characteristics of the venoms of several species of the *Bothrops* complex (*sensu lato*) remain unexplored. In this study, the venoms of twelve viperid species that inhabit Perú were analyzed using electrophoretic, chromatographic, and enzymatic profiling for PLA₂ and proteolytic activities *in vitro*. Also, their proteomic qualitative profiles were determined using a shotgun approach. Results provide additional information to previous studies on biochemical and toxicological aspects of snake venoms from this South American country (Sanz et al., 2008; Núñez et al., 2009; Jimenez et al., 2010; Kohlhoff et al., 2012; Guerra-Duarte et al., 2015).

In general, venom SDS-PAGE profiles did not deviate from the typical patterns observed in viperids by this technique but revealed some resemblances and moderate differences among the species. Remarkably, the venom of *B. schlegelii* from Perú showed identical electrophoretic and chromatographic profiles to that of the same species found in Costa Rica, separated by a distance of more than 2000 km. This was unexpected, considering that a previous comparison of *B. schlegelii* venoms from Costa Rica and Colombia reported significant intra-specific differences in electrophoretic and toxicological parameters (Prezotto-Neto et al., 2016). Also, there are marked differences in external characters, mainly scutellation and color patterns, between the Central American populations and those at the southern extreme of the distribution of this species in Ecuador and Perú (Campbell and Lamar, 2014). Moreover, venoms of *Bothriechis* are known for their extremely high inter-species variability (Fernández et al., 2010; Lomonte et al., 2008, 2012, 2014; Calvete, 2017; Pla et al., 2017).

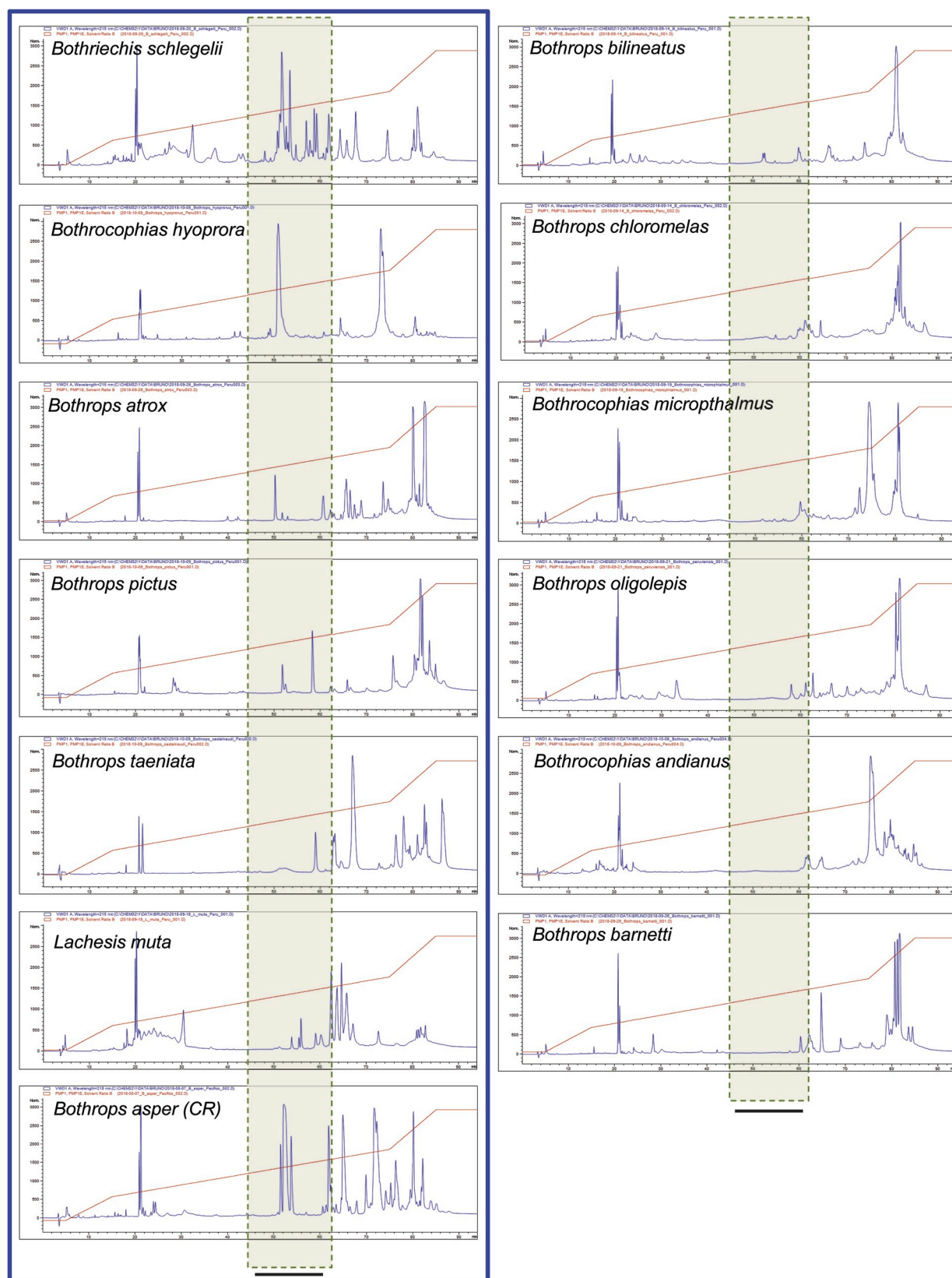


Fig. 2. C₁₈ reverse-phase HPLC profiles of venoms from twelve viperid species of Perú. The venom of *Bothrops asper* from Costa Rica was included as a reference. The dotted light green shaded area indicates the known elution region for PLA₂/PLA₂-like proteins in viperid venoms in this standardized chromatographic gradient. Venoms were divided into two groups, those with evident peaks in such region (enclosed within the blue frame) and those with very minor peaks. For reference to colors, the reader is referred to the web version of this article. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

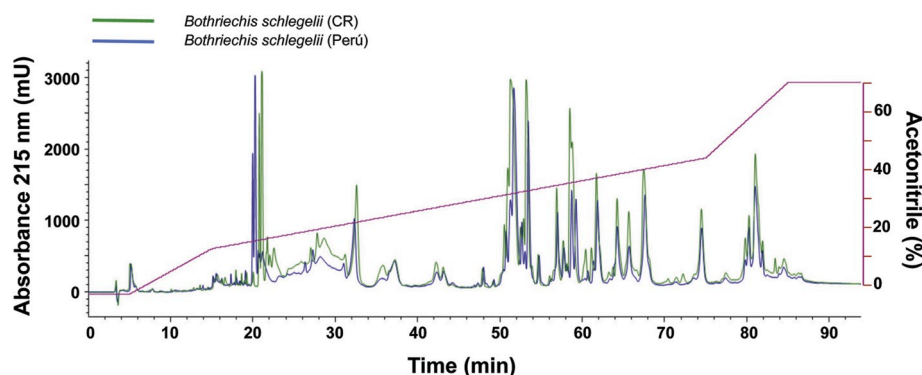


Fig. 3. Superposition of the C_{18} reverse-phase HPLC profiles of the venoms of *Bothriechis schlegelii* from Costa Rica (CR; green trace) and Perú (blue trace). For reference to colors, the reader is referred to the web version of this article. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Comparison of venom RP-HPLC profiles revealed an unexpected feature since six out of the twelve species studied presented only minor protein peaks in the elution region known to correspond to PLA_2 s as observed in several proteomic studies based on this chromatographic protocol (reviewed by Lomonte et al., 2014; Lomonte and Calvete, 2017). This particular characteristic had been observed in venoms from only a few of the bothropoid species studied until now, such as *B. ayerbeii* from Colombia (Mora-Obando et al., 2014b), and *B. cotiara* from Brazil (Tashima et al., 2008). Most commonly, venoms of species within the *Bothrops* complex display prominent to medium-intensity peaks in the PLA_2/PLA_2 -like eluting region. In contrast, venoms from six species of Perú here studied presented a low- PLA_2 phenotypic feature. These observations point to an apparent dichotomy among bothropoid venoms, regarding the occurrence of high to scarce levels of PLA_2 expression, and may bear practical relevance in selecting species whose venoms are used for antivenom production.

Venom compositional dichotomies are not infrequent in other taxonomic groups of venomous snakes. For example, rattlesnakes (*Crotalus* species) display either type I or type II venoms, defined as containing inverse proportions of metalloproteinases and PLA_2 s, respectively (Mackessy, 2008, 2010). Another example relates to venoms of New World coral snakes (*Micrurus* species), which express either ' PLA_2 -rich' or '3FTx (three-finger toxin)-rich' phenotypes (Fernández et al., 2015; Lomonte et al., 2016; Sanz et al., 2019). In contrast to the streamlined 3FTx neurotoxic venoms of non-spitting, particularly African, cobras (*Naja haje*, *N. nivea*, *N. annulifera*) (Malih et al., 2014; Silva de Roodt et al., 2018; Tan et al., 2019a; unpublished results), variable expression of PLA_2 s has also been observed in cytotoxic venom proteomes among species of African (*N. nubiae*, *N. pallida*, *N. nigricollis*, *N. mossambica*) (Petras et al., 2011) and Asian (*N. sumatrana*, *N. siamensis*, *N. philippinensis*) (Chong et al., 2019; Tan et al., 2019a, 2019b) spitting cobras. The emergence of venom spitting in cobras is tightly linked with convergent upregulation of PLA_2 toxins, which potentiate the nociceptive action of their venoms compared to those of their non-spitting counterparts (Kazandjian et al., 2019). Enhanced pain caused by spitting cobras is explicitly associated with the defensive use of venom, rather than for prey subjugation (Kazandjian et al., 2019).

The variable expression of PLA_2/PLA_2 -like proteins in the Peruvian *Bothrops* venoms here studied showed only partial correlation with a functional assay for enzymatic hydrolysis of the NOBA substrate. This result is expected, since protein peaks in the known PLA_2 -eluting RP-HPLC region may correspond to either Asp49 PLA_2 enzymes (catalytically active) or enzymatically inactive (but highly myotoxic) Lys49 PLA_2 -like homologs (Lomonte and Rangel, 2012). Moreover, catalytic efficiency (specific activity) may vary among active PLA_2 s. On the other hand, and in contrast to the highly variable expression of PLA_2 s, proteolytic activity did not vary widely among the studied venoms.

Regardless of their relative abundances, both PLA_2 s and

metalloproteinases were detected by shotgun proteomics in all the studied venoms, together with serine proteinases, C-type lectins/lectin-like, and L-amino acid oxidases, as typical of viperids. Other protein families that appeared common to all venoms were nucleotidases and glutamyl cyclotransferases. In contrast, some differences were observed among them regarding the detection of cysteine-rich secretory proteins, phosphodiesterases, nerve growth factors, phospholipases B, vascular endothelial growth factors, and hyaluronidases.

An inference of present results revealing the low expression of PLA_2 s in several bothropoid venoms from Perú is that they should display low toxicity for effects that depend mainly on this protein family. One of these known effects is myotoxicity (Gutiérrez and Lomonte, 2013). To test this prediction, the venoms of *Bothrops barnetti* and *Bothrocophias andianus* were selected as representatives of the 'low- PLA_2 ' phenotype and assayed *in vivo* for induction of muscle necrosis. Results demonstrated their negligible myotoxic effect. Whether this lack of myotoxicity confers any restriction on the trophic function of snake venoms that express it is still unknown, since information on diet and basic ecology is still very fragmented. Also, it is reasonable to expect that these differences in myotoxicity among bothropoid venoms, shown experimentally in mice, should translate to clinical envenomings. Envenomings by *Bothrops* (*sensu lato*) species are sometimes described in the Latin American medical literature as a single entity having shared general features. However, present results predict that important differences exist depending on the species, e.g., myonecrosis would not be expected in envenomings by species that express minimal amounts of venom PLA_2 s.

An indirect myotoxic action has been reported for some hemorrhagic metalloproteinases (Queiroz et al., 1985; Gutiérrez et al., 1995), but their relative contribution to myonecrosis, in comparison to directly myotoxic PLA_2 s, has not been clearly established. Present results obtained in mice with *B. barnetti* and *B. andianus* venoms suggest that acute muscle damage would be largely driven by PLA_2 s, and are in agreement with studies that showed almost complete inhibition of myonecrosis when the venom of *B. asper* was neutralized by PLA_2 -specific polyclonal (Lomonte et al., 1990) or monoclonal (Lomonte et al., 1992) antibodies.

In addition to its interest from a medical perspective, from a biological standpoint, the study of inter-species variations might contribute to understanding the evolutionary events that shaped the venom proteomes of extant species. In this work, we attempted to visualize the positions and relationships of bothropoid species from Perú presenting low- or high- PLA_2 venom phenotypes, based on the partial phylogeny generated by Carrasco et al. (2016). Although the interspersed pattern of the two phenotypes precludes reaching a simple phylogenetic deduction, it is clear that bothropoid venoms rich in PLA_2 s are more common than those presenting scarce PLA_2 s, and that the latter tend to cluster partially in the "microphthalmus" clade (*Bothrocophias* species), and the "taeniata" clade. The basal phylogenetic position of *Bothrops barnetti*

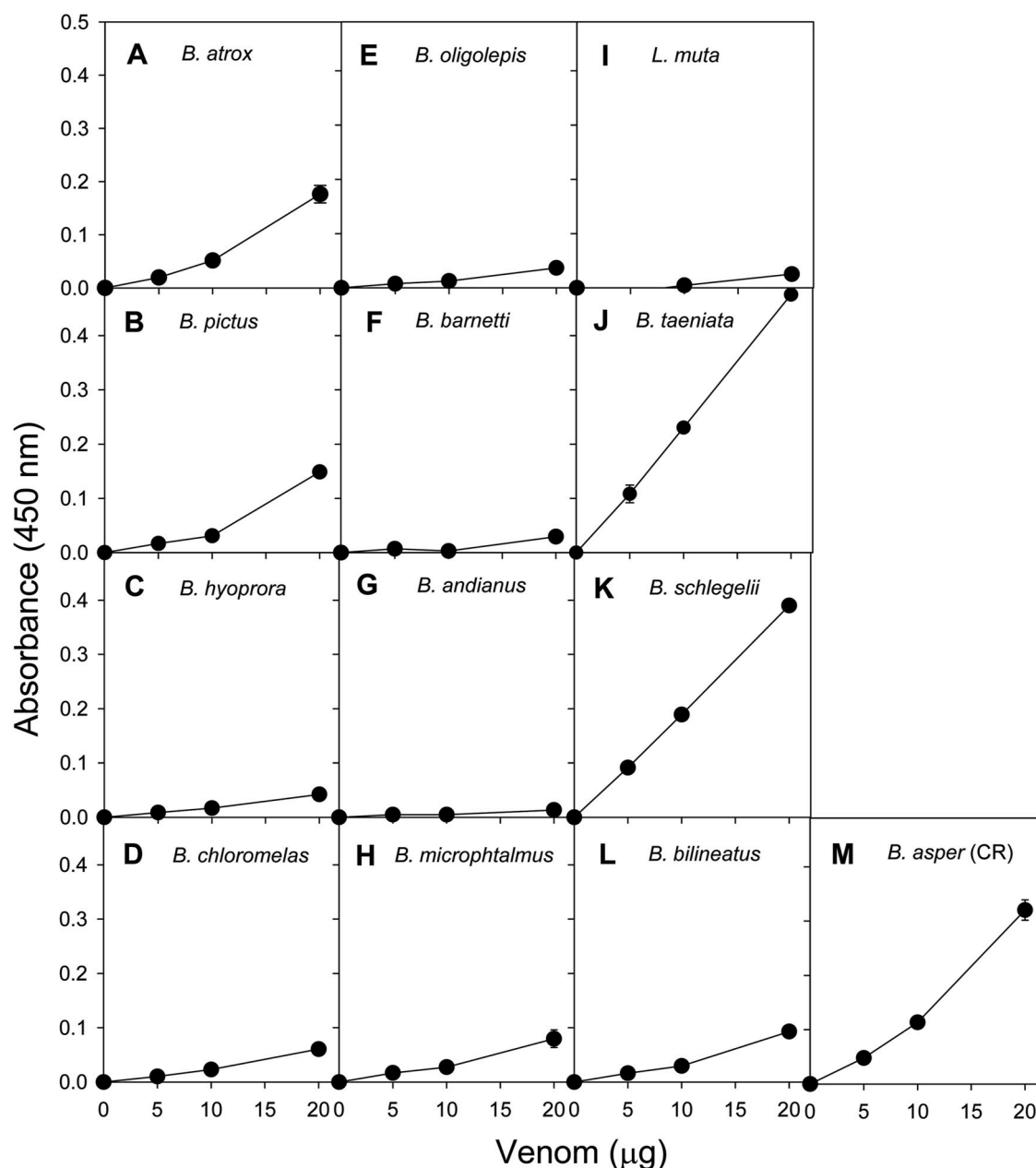


Fig. 4. Phospholipase A₂ activity of venoms from twelve viperid species of Perú on 4-nitro-3-octanoyloxy-benzoic acid. The venom of *Bothrops asper* from Costa Rica was included as a reference. Each point represents the mean \pm SD of three replicates.

(having a “low-PLA₂” venom) for the “alternatus” clade is interesting and leads to note that all clades encompassing a combination of the two phenotypes present a “low-PLA₂” basal species in these phylogenetic trees. If this observation holds after examining a more significant number of species, it would be tempting to suggest that the increased expression of PLA₂s could be an evolutionarily more derived phenotypic feature. However, since the available phylogenetic information is still incomplete and limited in species coverage, further studies will be required to understand the complex relationships between venom phenotypes and phylogeny. Currently enigmatic, understanding the selective forces that originated the highly variable expression of PLA₂s in venoms of the *Bothrops* complex anticipates a challenging research task.

5. Concluding remarks

Venom is an intrinsically ecological trophic trait crucial for the foraging success of the organisms that produce it. The evolution of

venom phenotypes does not appear to be phylogenetically constrained, but more likely shaped by ecological filtering that permits a small number of optimal solutions involving combinations of only a few major toxin families (Barua and Mikheyev, 2019). Of particular note is the occurrence of congeneric, even conspecific, divergent venom phenotypes, such as the 3FTx-rich/PLA₂-predominant venoms described in venoms of the New World genus *Micrurus* (Elapinae) and sea snake (Hydrophiinae) lineages (Sanz et al., 2016; Lomonte et al., 2016); the PIII-SVMP (type-I)/ β -neurotoxic heterodimeric PLA₂ (type-II) venom compositional dichotomy found within *Crotalus* (Mackessy, 2008; Massey et al., 2012; Calvete, 2017; Strickland et al., 2018) and *Bothriechis* (Pla et al., 2017); and the divergent 3FTx/Kunitz-type dendrotoxins venom phenotypes among *Dendroaspis* (Ainsworth et al., 2018). The high/low PLA₂ venom compositional divergence described here among venoms of Peruvian *Bothrops* species represents a new dichotomy. The finding of similar patterns of venom variability among evolutionarily distant snake lineages suggests that dichotomic venom phenotypes may

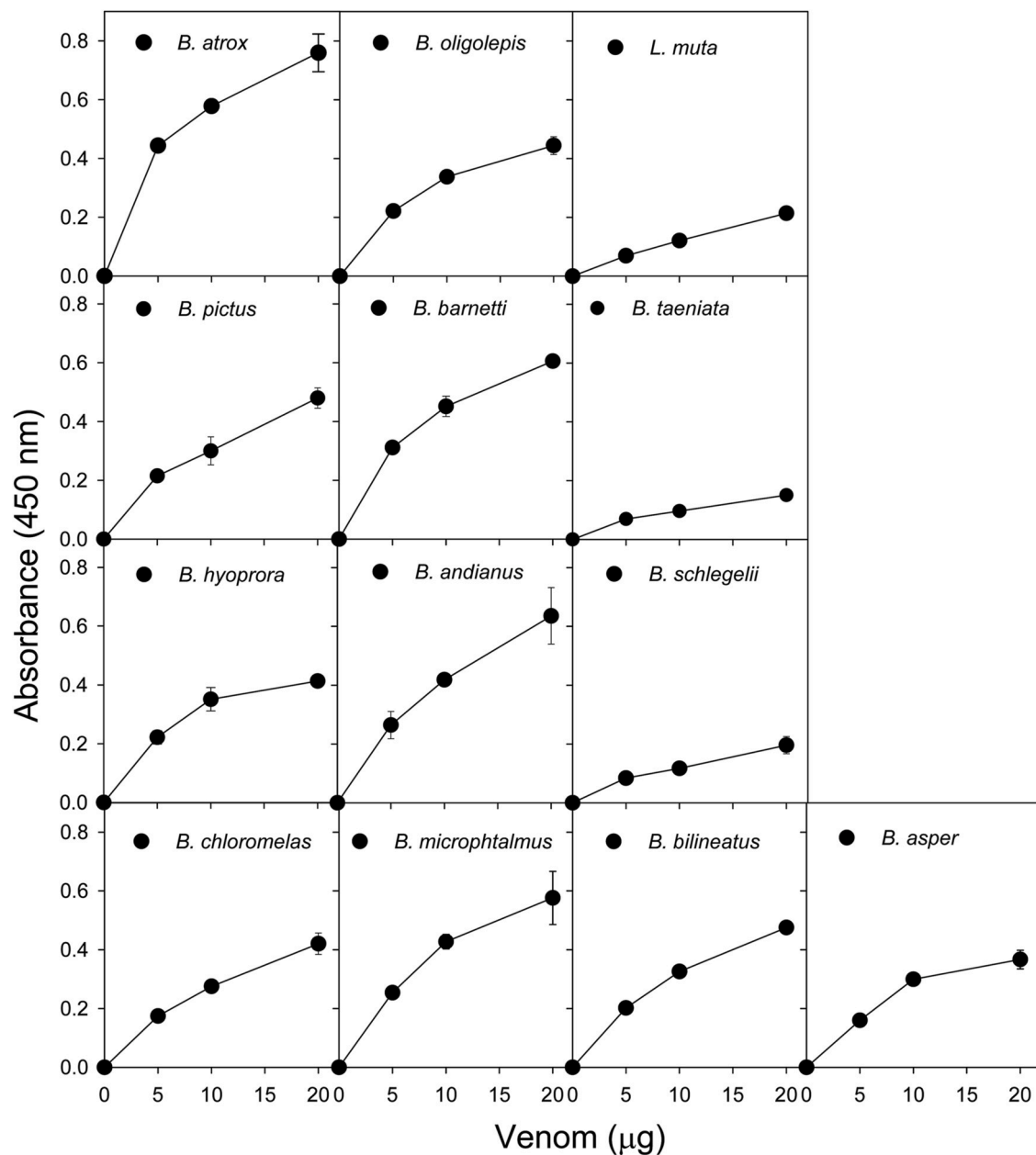


Fig. 5. Proteolytic activity of venoms from twelve viperid species of Perú on azocasein. The venom of *Bothrops asper* from Costa Rica was included as a reference. Each point represents the mean \pm SD of three replicates.

constitute a more general trend than previously thought.

Venom dichotomy has different evolutionary origins in Nearctic, Central American, and South American rattlesnakes. Differential expression of type-I or type-II venom phenotypes in North American rattlesnakes appears to be due to recent lineage-independent losses of the genes coding for the subunits of the heterodimeric β -neurotoxin PLA₂ (Dowell et al., 2016, 2018). In contrast, in Central American *C. simus* it is ontogenetic and post-transcriptionally modulated by miRNAs (Durban et al., 2013). It represents a pedomorphic trait driven by the increased gain of neurotoxicity and lethal venom activity to mammals (rodents) in South American *Crotalus durissus* (Calvete et al., 2010). The 3FTx/PLA₂ dichotomy among micrurine venoms may have been shaped by balancing selection (Sanz et al., 2019), an evolutionary scenario where local adaptation depends on the frequencies of the two toxin classes, with the hetero- (i.e. 3FTx + PLA₂) phenotype having an advantage over the homo-phenotypes (Llaurens et al., 2017; Wang and Mitchell-Olds, 2017). In contrast, the streamlined 3FTx venom of the

aquatic coral snake *M. surinamensis* may have evolved under strong pressure to quickly immobilize aquatic prey (Sanz et al., 2019; Silva et al., 2018). The different evolutionary paths responsible for generating variation in venom composition underscore the importance of considering venom diversification in the proper historical scenario.

Diversification of venoms within *Bothrops* should be understood in the context of the evolution of this clade. The early colonization by the ancestor of all *Bothrops* had its roots in South America during the middle Miocene, 14.07 Mya (CI95% 16.37–11.75 Mya), and was followed much later by *Bothriechis schlegelii* (7–6.6 Mya), *Porthidium* (3.5 Mya), and *Crotalus durissus* (1 Mya) (Wüster et al., 2002; Parkinson et al., 2002; Wüster et al., 2008; Alencar et al., 2016). *Bothrops* represents the most successful South American pitviper radiation, with extant lineages exhibiting extremely diverse morphological and ecological traits (Martins et al., 2002). Except for southwestern South America, the extreme highlands of the Andes, and southernmost Patagonia, extant bothropoid pitvipers are widely distributed in tropical Latin America, from

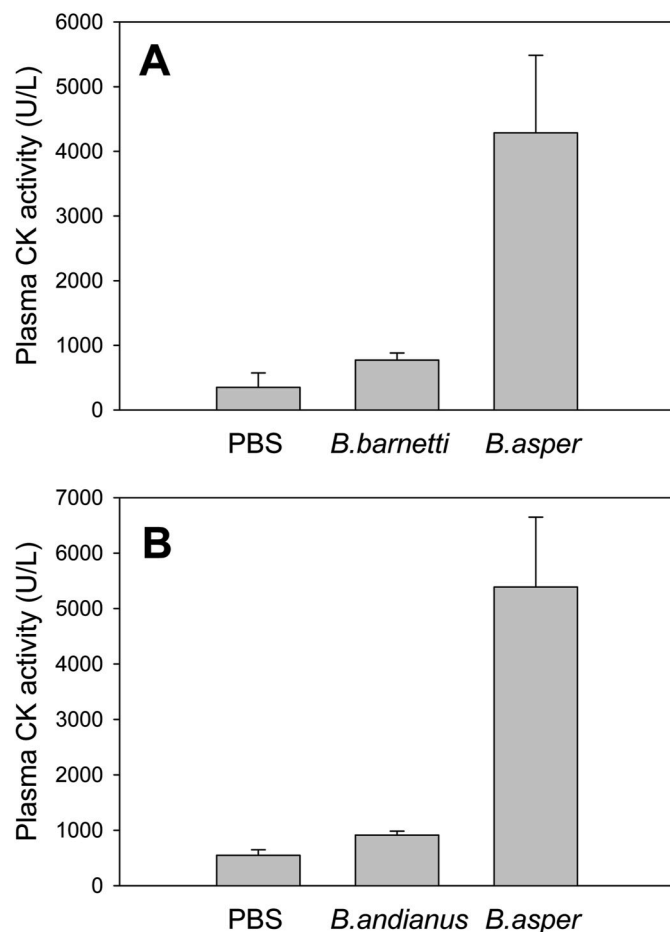


Fig. 6. Comparative evaluation of the myotoxic activity of the venoms of (A) *Bothrops barnetti* and (B) *Bothrocophias andianus* (Perú), compared to *Bothrops asper* (Costa Rica) in mice. Venoms were injected by the intramuscular route into groups of five mice. A control group received an injection of vehicle (PBS) alone. After 3 h the plasma creatine kinase (CK) activity was determined. Each bar represents the mean \pm SD ($n = 5$). No statistical difference ($p > 0.05$) was observed between CK levels of PBS and *B. barnetti* venom, or PBS and *B. andianus* venom. *B. asper* venom was significantly different from both groups, in both independent experiments.

northeastern Mexico to Argentina, and the southern parts of the lower Caribbean islands, where they inhabit a broad spectrum of ecoregions, such as lowland evergreen forests, montane semideciduous forests, savannas, and open montane formations, from sea level to 3000 m of elevation (Campbell and Lamar, 1989; 20104; Carrasco et al., 2012).

Table 2

Protein families identified in nine venoms from Viperidae species of Perú by bottom-up shotgun proteomics.

Snake species	MP*	SP	PLA ₂	LAAO	CTL	NUC	GCY	CRISP	PDE	PLB	NGF	VEGF	HYA	Total N°
<i>Bothrops atrox</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	13
<i>Bothrops oligolepis</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	13
<i>Bothrops chloromelas</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	13
<i>Bothrocophias andianus</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	(–)	12
<i>Bothrops pictus</i>	✓	✓	✓	✓	✓	✓	✓	(–)	✓	✓	✓	✓	(–)	11
<i>Bothrocophias microphthalmus</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	(–)	(–)	11
<i>Bothrocophias hyoprora</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	(–)	✓	(–)	(–)	10
<i>Bothrops bilineatus</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	(–)	(–)	(–)	10
<i>Bothrops barnetti</i>	✓	✓	✓	✓	✓	✓	✓	✓	(–)	✓	(–)	(–)	(–)	9
Family presence	100%	100%	100%	100%	100%	100%	100%	89%	89%	89%	77%	56%	33%	

* Abbreviations: MP: metalloproteinase; SP: serine proteinase; PLA₂: phospholipase A₂; LAAO: L-amino acid oxidase; VEGF: vascular endothelial growth factor; NGF: nerve growth factor; CTL: C-type lectin/lectin-like; NUC: nucleotidase; PDE: phosphodiesterase; GCY: Glutamyl cyclotransferase; PLB: phospholipase B; CRISP: cysteine-rich secretory protein; HYA: hyaluronidase. For a detailed summary of protein matches refer to [Supplemental Table S1](#).
✓: detected; (–) not detected.

Diversification of *Bothrops* should be understood in the context of the unique historical contingencies of the evolution of this clade, the absence of other pitvipers and placental carnivores as potential competitors or predators when their lineage radiated throughout South America (Wüster et al., 2002) and the orogenic processes that created vicariant events (Hamdan et al., 2020; Salazar-Valenzuela et al., 2019). For example, in South America the uplift of the Central Andes mountain range (which began in the late Oligocene to early Miocene (23 Mya) with the formation of the highest peaks during the late Middle Miocene 11–12 Mya and continued until around 2–3 Mya, contemporaneous with deformation in the Santa Bárbara system to the east) (Folguera et al., 2011; Hamdan et al., 2020), the uplift of the Guanacaste, Central, and Talamanca mountain ranges in Mesoamerica since the late Miocene or early Pliocene (8–5 Mya) to the Pliocene, and the closure of the Isthmus of Panama 3.2 Mya (Gregory-Wodzicki, 2000; O'Dea et al., 2016; Bergeoning, 2017), are some of the main vicariance events involved in the cladogenesis of the lancehead vipers as a consequence of the fragmentation of populations and allopatric speciation (Kattan et al., 2004; Saldarriaga-Córdoba et al., 2017; Hamdan et al., 2020). Recent phylogeographic analyses have shown that demographic processes and ecological differentiation in montane habitats would also be reliable drivers implicated in the divergence of different taxa along altitudinal gradients (Salazar-Valenzuela et al., 2019).

An increasing number of studies support the idea that snake venom evolution is driven by diet-related selection pressures leading to local adaptations (Daltry et al., 1996; Barlow et al., 2009; Smiley-Walters et al., 2019; Davies and Arbuckle, 2019; Jackson et al., 2019). Adaptive venom variability represents a common phenomenon at all taxonomic levels among individuals of the same or different geographic locations, dietary habits, genders and across the life history stages of an individual (Calvete, 2013, 2017). Although the mechanisms of venom variation remain elusive, ecological release seemingly promotes increased phenotypic variance (Van Valen, 1965; Bolnick et al., 2007). From a functional perspective, venom from a generalist viperid may have the capability to subdue different prey items, although with varying degrees of effectiveness. However, other dimensions, such as non-consumptive habitat use, or dietary decisions, define the trophic niche of the organism and have significant implications on the individual energy budget and the outcomes of interactions between sympatric taxa (Jackson et al., 2004; Vincent and Brown, 2005; Beermann et al., 2018).

Besides providing insights into the selective pressures that resulted in local adaptation and species-level divergence in venoms, understanding the evolutionary processes and the ecological constraints that moulded snake venoms to their present-day variation may also provide compelling insights into venom clinical toxicology. Thus, toxins that have the highest prey incapacitation activity to a mammalian model are often also the most medically important molecules in the context of a human

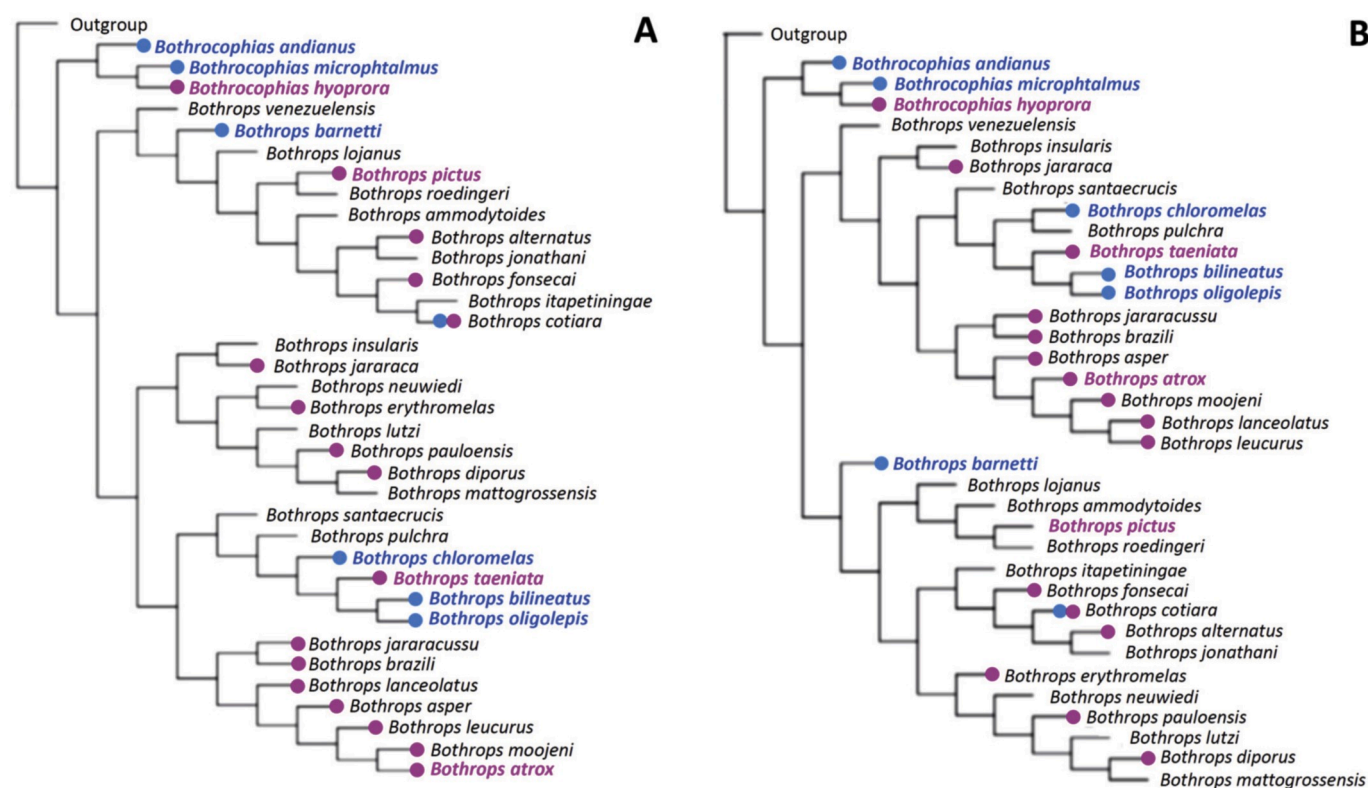


Fig. 7. Partial phylogenies of bothropoid viperids showing the relationships of species reported to produce venoms with high (pink dots) and low (blue dots) phospholipase A₂ contents. The species from Perú analyzed in the present study are indicated by names in corresponding pink or blue colors. The phylogenetic trees were generated by Carrasco et al. (2016) using a total evidence approach from morphological, ecological, and mtDNA sequences (12 S and 16 S rRNA). Phylogenetic reconstruction included (A) or excluded (B) sequences from ND4 and cytb genes. For reference to colors, the reader is referred to the web version of this article. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

envenoming. Identifying the molecular basis of venomous snake adaptation to their natural ecosystems may assist in the identification of those toxins that must be neutralized to reverse the effects of venom, thereby guiding the rational development of next-generation snakebite therapeutics.

Credit author statement

Conceptualization: BL, AZ, MSalas, MSasa. Data curation and Formal analysis: BL, CD, FCH, JF, JJC. Funding acquisition: BL, AZ. Investigation and Methodology: CD, FCH, JF, MR. Resources: AZ, MSalas. Writing and editing: BL, CD, JJC, MS.

Declaration of competing interest

The authors declare that they have no competing interests related to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxcx.2020.100044>.

Ethical statement

The authors declare that they have no competing interests related to this work.

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