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Snake venomics and antivenomics of *Bothrops colombiensis*, a medically important pitviper of the *Bothrops atrox-asper* complex endemic to Venezuela: Contributing to its taxonomy and snakebite management

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ABSTRACT

The taxonomic status of the medically important pitviper of the *Bothrops atrox-asper* complex endemic to Venezuela, which has been classified as *Bothrops colombiensis*, remains *incertae cedis*. To help resolving this question, the venom proteome of *B. colombiensis* was characterized by reverse-phase HPLC fractionation followed by analysis of each chromatographic fraction by SDS-PAGE, N-terminal sequencing, MALDI-TOF mass fingerprinting, and collision-induced dissociation tandem mass spectrometry of tryptic peptides. The venom contained proteins belonging to 8 types of families. PI Zn^{2+}-metalloproteinases and K49 PLA_2 molecules comprise over 65% of the venom proteins. Other venom protein families comprised PIII Zn^{2+}-metalloproteinases (11.3%), D49 PLA_2s (10.2%), L-amino acid oxidase (5.7%), the medium-sized disintegrin colombistatin (5.6%), serine proteinases (1%), bradykinin-potentiating peptides (0.8%), a DC-fragment (0.5%), and a CRISP protein (0.1%). A comparison of the venom proteomes of *B. colombiensis* and *B. atrox* did not support the suggested synonymy between these two species. The closest homologues to *B. colombiensis* venom proteins appeared to be toxins from *B. asper*. A rough estimation of the similarity between the venoms of *B. colombiensis* and *B. asper* indicated that these species share approximately 65–70% of their venom proteomes. The close kinship of *B. colombiensis* and *B. asper* points at the ancestor of *B. colombiensis* as the founding Central American *B. asper* ancestor. This finding may be relevant for reconstructing the natural history and cladogenesis of Bothrops. Further, the virtually indistinguishable immunological crossreactivity of a Venezuelan ABC antiserum (raised against a mixture

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of *B. colombiensis* and *Crotalus durissus cumanensis* venoms) and the Costa Rican ICP polyvalent antivenom (generated against a mixture of *B. asper*, *Crotalus simus*, and *Lachesis stenophrys* venoms) towards the venoms of *B. colombiensis* and *B. asper*, supports this view and suggests the possibility of indistinctly using these antivenoms for the management of snakebites by any of these Bothrops species. However, our analyses also evidenced the limited recognition capability or avidity of these antivenoms towards a number of *B. colombiensis* and *B. asper* venom components, most notably medium-size disintegrins, bradykinin-potentiating peptides, PLA_2 proteins, and PI Zn^{2+}-metalloproteinases.

1. Introduction

The genus Bothrops (subfamily Crotalinae of Viperidae) comprises 32 (http://www.reptile-database.org) or 37 species [1] of pitvipers, commonly referred as lanceheads, which are widely distributed in tropical Latin America, from north-eastern Mexico to Argentina, and the southern parts of the lower Caribbean islands [1]. Among the South American pitviper fauna, genus Bothrops shows the greatest diversity with respect to number of species, morphology, and natural history traits [1,2]. Sequence divergence analysis suggests that the common ancestor of all Bothrops was the first viperid to colonize South America, sometime during the Miocene, 10–23 million years ago (mya) [2]. Rapid dispersal and diversification of Bothrops across South America may have occurred through adaptive radiations into habitats devoid of viperid competitors [2]. A single species, the ancestor of *B. asper*, reinvaded Central America, where it remains the only widespread species of Bothrops [2]. The subsequent uplift of the northern Andes and the mountains of lower Central America in the late Miocene or early Pliocene (8–5 mya), which culminated with the closure of the Isthmus of Panama 3.5 mya [3], may have further fragmented the Central and northern South American herpetofauna into allopatric populations, thus influencing the cladogenesis of Bothrops [2–5].

The phylogeny of Bothrops has undergone several taxonomic revisions but it remains still incompletely understood [1,2,6,7]. Bothrops (*sensu lato*) is a paraphyletic clade composed of at least five separate lineages [1,2,6]. The *Bothrops asper-atrox* complex represents a monophyletic clade of medium to large-sized pitvipers widely spread throughout the tropical parts of Central and South America [1,2]. The status and phylogenetic alliances of many of the conventionally recognised species within the *asper-atrox* group (*B. asper*, *B. atrox*, *B. colombiensis*, *B. isabelae*, *B. leucurus*, *B. marajoensis*, *B. moojeni*, and *B. pradoi*) are still open to discussion [2,6,8]. Resolving population identities and species boundaries within the *B. asper-atrox* complex remains a challenging problem requiring further taxonomical revision.

Originally described by Hallowell in 1845 as *Trigonocephalus colombiensis* [9], the taxonomic status of *B. colombiensis* (Manapare Lancehead Viper; type locality, "Republic of Colombia, within two hundred miles of Caracas, Venezuela") has been a matter of debate [10]. The name *B. colombiensis* has been used for the populations of the *B. asper-atrox* group from northern Venezuela. Roze [11] mentioned that Hallowell himself suspected *T. colombiensis* to be identical with *T. lanceolatus* (*B. atrox*). Sandner-Montilla [12] considered the name *B. colombiensis* as *nomen oblitum* and synonymized it to *B. lanceolatus* described by Lacépède in 1789. Johnson and Dixon [10] considered *B. colombiensis* in the synonymy of *B. atrox*. However, mitochondrial DNA sequence analysis showed that *B. colombiensis* is distinct from *B. atrox* from Surinam, and closer to *B. asper* from Belice [2]. *B. asper* was also recognized as conspecific with *B. atrox* before being separated by Hoge [13] based on subtle morphological differences. Most authorities now consider *B. atrox* and *B. asper* as distinct species [1]. The range of *B. atrox* partly encircles the purported range of *B. colombiensis* (western, central and north-eastern Venezuela and north of the Orinoco River [14]), except in north central Venezuela, and references that recognize *B. atrox* and *B. colombiensis* as different do not indicate sympatry of the two species [1]. On the other hand, *B. asper*, another adaptable wide-ranging species, shares distributional relationship with *B. colombiensis* in northern South America. According to Campbell and Lamar [1], the species *B. asper* and *B. atrox* are distributed in Venezuela, but these authors do not recognize *B. colombiensis* as a separate species. Clearly, the evolutionary and taxonomic status of Venezuelan populations of the B. asper-atrox complex known as *B. colombiensis* is uncertain and requires further studies.

In the past decades, significant advances have been made in resolving phylogenies as a direct result of the acceptance and refinement of cladistic methods. In addition, the combined use of molecular features (particularly mitochondrial DNA sequence information and enzymatic activities) with traditional morphological characters has helped to increase the robustness and resolution of a phylogenetic hypotheses. Venom is a shared derived trait of the advanced snakes (Caenophidia). Snake venom composition is inherited rather than environmentally induced, being under a strict genetic control [15] and thus venom protein typification may render valuable molecular markers for taxonomical purposes [16–18].

Beside the evolutionary and ecological importance of venoms, accidental envenomation by snakebite constitutes a highly relevant public health hazard in Central and South America which has an impact in terms of mortality and morbidity [19–24]. Bothrops species inflict ninety percent of snakebites in Central and South America [19]. The prototypic species of the *asper-atrox* complex are amongst the most dangerous venomous snakes in tropical Latin America. *B. asper* is considered the most dangerous snake in Central America. In Costa Rica it is responsible for the majority of snakebites [20–24]. *B. atrox* is the cause of more human fatalities in South America than any other American reptile [22]. In Venezuela, *B. colombiensis* has been reported to account

for over 36% of the more than 5000 snake bite accidents caused yearly by species from the genera *Bothrops*, *Bothriechis*, *Bothriopsis*, *Porthidium*, *Crotalus*, *Lachesis* and *Micrurus* [14,25]. In the period 1980–1990, 586 deaths by snakebite were recorded in Venezuela, representing the main cause of mortality due to animal envenomation in this country [26,27].

Bothropoid venoms induce a complex pathophysiological picture characterized by local and systemic effects such as edema, myonecrosis, blistering, hemorrhage, defibrination, shock and nephrotoxicity [20–22]. A robust understanding of the toxin composition of Bothrops venoms is not only relevant for systematics but also to the fields of medicine, pharmacology, and toxinology. Here, we report a detailed proteomic characterization of the venom of *B. colombiensis*. Comparison of the venom proteomes of *B. colombiensis*, *B. asper* [5] and *B. atrox* [28] suggests a remarkable close kinship between *B. colombiensis* and *B. asper* from the Pacific versant of Costa Rica. Our data support the hypothesis that the population known as *B. colombiensis* is likely to correspond to *B. asper*. Further, a polyvalent antivenom produced at the Instituto Clodomiro Picado (San José, Costa Rica) against a mixture of venoms from *B. asper*, *C. simus*, and *L. stenophrys*, and an antivenom raised in Venezuela against a mixture of *B. colombiensis* and *C. d. cumanensis* venoms showed the same immunoreactivity towards *B. asper* and *B. colombiensis* toxins. The relevance of this observation for the taxonomy and snakebite management of *B. colombiensis* is discussed.

2. Experimental section

2.1. Venoms and antivenoms

The venom of *B. colombiensis* was pooled from snakes captured nearby the Venezuelan towns of Santa Bárbara (Barinas State), San Felipe (Yaracuy State), Barlovento (Miranda State) and Araira (Miranda State), and kept in captivity in the serpentarium of the Universidad de Oriente, Puerto La Cruz, Venezuela. The venom of *Bothrops atrox* was obtained from specimens collected near Pucallpa, Department of Ucayali, Perú, and maintained at the serpentarium of Museum of Natural History, Universidad Nacional Mayor de San Marcos. Venom samples from *B. asper* were obtained from specimens collected in the Caribbean (Distrito Quesada, San Carlos, Alajuela province) and the Pacific (Distrito de Sabanillas, Acosta, San José province) regions of Costa Rica and kept at captivity at the Serpentarium of Instituto Clodomiro Picado (Universidad de Costa Rica, San José) [5]. Crude venoms were centrifuged at low speed to remove cells and debris, lyophilized, weighed on a microbalance, and stored at −20 °C until used. Venom pools were prepared by mixing equal amounts of samples from at least 11 specimens from both sexes from the Caribbean or from the Pacific regions. The proteomic characterization of these venoms has been reported [5].

The anti-bothropic and anti-crotalic (ABC) horse antivenom used for the antivenomic study (Batch L138, expiry date: September 2009) was raised against a mixture of the venoms of *Bothrops colombiensis* and *C.d. cumanensis* in the Centro de Biotecnología, Facultad de Farmacia, Universidad Central de Venezuela (UCV). Inmunoglobulins were purified by ammonium sulphate precipitation and by pepsin digestion. The polyvalent (Crotalinae) antivenom (batch 4201007POLQ, expiry date: October 2010) manufactued at the Instituto Clodomiro Picado (ICP), Universidad de Costa Rica, was produced by immunizing horses with a mixture of equal amounts of the venoms of *Bothrops asper*, *Crotalus simus*, and *Lachesis stenophrys* obtained from adult specimens kept in captivity at the ICP serpentarium [29]. Whole immunoglobulins were purified by caprylic acid precipitation [30]. IgG concentration was determined spectrophotometrically using an extinction coefficient (ε) of 1.4 for a 1 mg/ml IgG concentration at 280 nm using a 1 cm light pathlength cuvette [31].

2.2. Isolation of venom proteins

For reverse-phase HPLC separations, 2–5 mg of crude, lyophilized venom of *B. colombiensis* were dissolved in 100 μL of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation in an Eppendorff centrifuge at 13,000 ×*g* for 10 min at room temperature. Proteins in the soluble material were separated using an ETTAN™ LC HPLC system (Amersham Biosciences) and a Lichrosphere RP100 C_{18} column (250×4 mm, 5 μm particle size) eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B) (5%B for 10 min, followed by 5–15%B over 20 min, 15–45%B over 120 min, and 45–70%B over 20 min). Protein detection was at 215 nm and peaks were collected manually and dried in a Speed-Vac (Savant). The relative abundances (% of the total venom proteins) of the different protein families in the venoms were estimated from the relation of the sum of the areas of the reverse-phase chromatographic peaks containing proteins from the same family to the total area of venom protein peaks. In a strict sense, and according to the Lambert–Beer law, the calculated relative amounts correspond to the "% of total peptide bonds in the sample", which is a good estimate of the % by weight (g/100 g) of a particular venom component. The relative contributions of different proteins eluting in the same chromatographic fraction were estimated by densitometry after SDS-PAGE separation.

2.3. Characterization of HPLC-isolated proteins

Isolated protein fractions were subjected to N-terminal sequence analysis (using a Procise instrument, Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Amino acid sequence similarity searches were performed against the available databanks using the BLAST program [32] implemented in the WU-BLAST2 search engine at http://www.bork.embl-heidelberg.de. The molecular masses of the purified proteins were determined by SDS-PAGE (on 12 or 15% polyacrylamide gels) and by electrospray ionization (ESI) mass spectrometry using an Applied Biosystems QTrap™ 2000 mass spectrometer [33] operated in Enhanced Multiple Charge mode in the range 600–1700 m/z.

2.4. In-gel enzymatic digestion and mass fingerprinting

Protein bands of interest were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to

automated reduction with DTT and alkylation with iodoacetamide, and in-gel digestion with sequencing-grade bovine pancreas trypsin (Roche) using a ProGest digestor (Genomic Solutions) following the manufacturer's instructions. 0.65 μL of the tryptic peptide mixtures (total volume of ~20 μL) were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% TFA, dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes. A tryptic peptide mixture of *Cratylia floribunda* seed lectin (SwissProt accession code P81517) prepared and previously characterized in our laboratory was used as mass calibration standard (mass range, 450–3300 Da).

2.5. Collision-induced dissociation tandem mass spectrometry (CID-MS/MS)

For peptide sequencing, the protein digest mixture was loaded in a nanospray capillary column and subjected to electrospray ionization (ESI) mass spectrometric analysis using a QTrap mass spectrometer (Applied Biosystems) [33] equipped with a nanospray source (Protana, Denmark). Doubly- or triply-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were analyzed in Enhanced Resolution MS mode and the monoisotopic ions were fragmented using the Enhanced Product Ion tool with Q_0 trapping. Enhanced Resolution was performed at 250 amu/s across the entire mass range. Settings for MS/MS experiments were as follows: Q1 — unit resolution; Q1-to-Q2 collision energy — 30–40 eV; Q3 entry barrier — 8 V; LIT (linear ion trap) Q3 fill time — 250 ms; and Q3 scan rate — 1000 amu/s. CID spectra were interpreted manually or using a licensed version of the MASCOT program (http://www.matrixscience.com) against a private database containing 927 viperid protein sequences deposited in the SwissProt/TrEMBL database (UniProtKB/Swiss-Prot Release 56.4 of 04-Nov-2008; UniProtKB/TrEMBL Release 39.4 of 04-Nov-2008; http://us.expasy.org/sprot/; 212 in SwissProt, 715 in TrEMBL) plus the previously assigned peptide ion sequences from snake venomics projects carried out in our laboratory [5,34–38]. MS/MS mass tolerance was set to ±0.6 Da. Carbamidomethyl cysteine and oxidation of methionine were fixed and variable modifications, respectively.

2.6. Antivenomics: immunodepletion of venom proteins by Costa Rican and Venezuelan polyvalent antivenoms

We have coined the term "antivenomics" for the identification of venom proteins bearing epitopes recognized by an antivenom using proteomic techniques [34]. Briefly, two milligrams of whole venom were dissolved in 70 μL of 20 mM phosphate buffer, pH 7.0, mixed with 4 mg of purified polyvalent antivenom IgGs, and incubated with gentle stirring for 1 h at 37 °C. Thereafter, 6 mg of rabbit anti-horse IgG antiserum (Sigma) in 350 μL of 20 mM phosphate buffer, pH 7.0, were added, and the mixture was incubated for another 1 h at 37 °C. Immunocomplexes were precipitated by centrifugation at 13,000 rpm for 30 min in an Eppendorf centrifuge and the supernatant was submitted to reverse-phase separation as described for the isolation of venom proteins. HPLC-fractions were characterized as described above. Control samples were subjected to the same procedure except that antivenom IgGs were not included in the reaction mixture.

2.7. Neutralization of lethality

The ability of ICP polyvalent antivenom to neutralize the lethal activity of *B. colombiensis* venom was assessed as previously described [39]. Briefly, the Median Lethal Dose (LD_{50}) of venom was estimated in CD-1 mice (16–18 g) by injecting intraperitonealy various doses of venom. Deaths were recorded for 48 h and LD_{50} was estimated by probits. For neutralization assays, mixtures of a fixed amount of venom and variable volumes of antivenom were prepared and incubated for 30 min at 37 °C. Aliquots of the mixtures, containing 4 Median Lethal Doses (LD_{50}) of venom, were injected in CD-1 mice by the intraperitoneal route. Control mice received 4 LD_{50}s of venom without antivenom. Deaths were recorded for 48 h and the Median Effective Dose (ED_{50}) was estimated by probits, and defined as the ratio venom/antivenom at which 50% protection was achieved. ED_{50} was expressed as the milligrams of venom neutralized per milliliter of antivenom [39].

2.8. Two-dimensional gel electrophoresis (2-DE)

2-DE was performed essentially according to the manufacturer's (GE Healthcare Amersham Biosciences) instructions unless otherwise indicated. For isoelectric focusing (IEF), 40 μg of *B. colombiensis*, *B. atrox* and *B. asper* venoms were dissolved in DeStreak™ rehydratation solution with 1% carrier ampholytes pH 3–10 and applied onto 7-cm pH 3–10 linear immobilized pH gradient (IPG) strips. IEF was carried out with an Ettan-IPGphor isoelectric focusing unit at 20 °C applying the following conditions: 300 V (0.5 h), ramping to 1000 V (0.5 h), ramping to 5000 (1.3 h) and 5000 V (0.5 h). After IEF, the IPG strips were kept at −70 °C until use. For the second (SDS-PAGE) dimension, the IPGs were equilibrated for 15 min with gentle shaking at room temperature in equilibration buffer (6 M urea, 2% [w/v] SDS, 30% [v/v] glycerol, 50 mM Tris–HCl [pH 8.8]) containing 1% (w/v) DTT, followed by 15 min equilibration in buffer containing 3% (w/v) iodoacetamide. IPG strips were then placed on top of 8–15% SDS-PAGE gels and run in a Protean II (Bio-Rad) electrophoresis unit at room temperature. Protein spots were visualized by staining using the PlusOne™ silver staining protocol.

3. Results and discussion

3.1. Characterization of the venom proteome of *B. colombiensis*

Despite its medical importance in Venezuela, literature on the toxin composition and biological activities of the venom of *B. colombiensis* is scarce. Indeed, only 6 references are listed in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) [40–45], and no venom protein sequence from this species is available in the SwissProt/TrEMBL database. To address the need

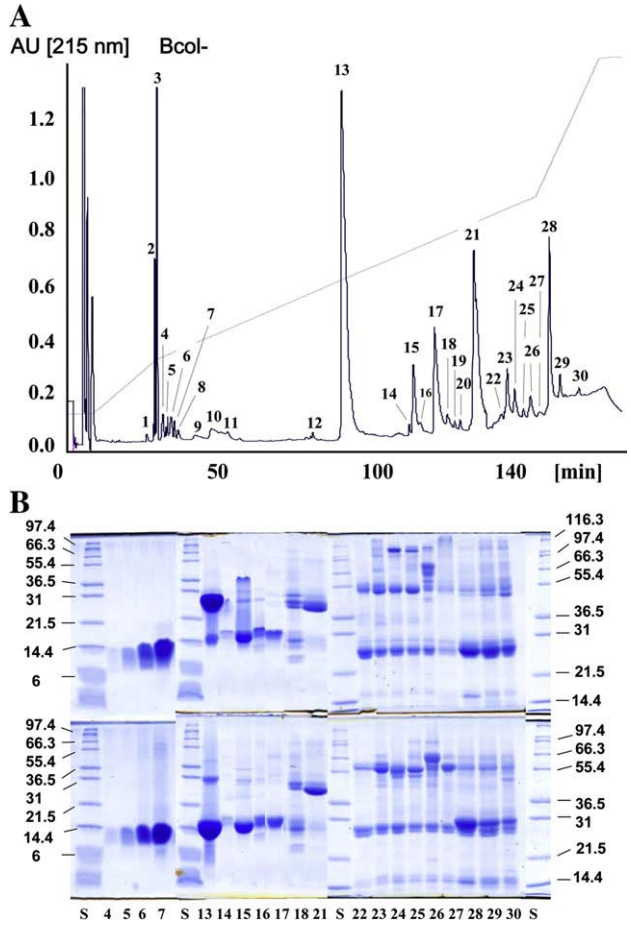

Fig. 1 – Characterization of the venom proteome of *B. colombiensis*. (A) Reverse-phase HPLC separation of the proteins from the venom of *B. colombiensis*. Two milligrams of total venom proteins were applied to a Lichrosphere RP100 C_{18} column, which was then developed with the following chromatographic conditions: isocratically (5% B) for 10 min, followed by 5–15% B for 20 min, 15–45% B for 120 min, and 45–70% B for 20 min. Fractions were collected manually and characterized by N-terminal sequencing, ESI mass spectrometry, tryptic peptide mass fingerprinting, and CID-MS/MS of selected doubly- or triply-charged peptide ions. The results are shown in Table 1. (B) SDS-PAGE showing the protein composition of the reverse-phase HPLC separated venom protein fractions displayed in panel A and run under non-reduced (upper panels) and reduced (lower panels) conditions. Molecular mass markers (in kDa) are indicated at the side of each gel. Protein bands were excised and characterized by mass fingerprinting and CID-MS/MS. The results are shown in Table 1.

for detailed studies of the venom proteome of *B. colombiensis*, we sought to characterize the complement of secreted proteins, which may contribute to a deeper understanding of the biology, ecology, and phylogenetics of this snake population, and may also serve as a starting point for studying structure-function correlations of individual toxins. To this end, pooled crude venom was fractionated by reverse-phase HPLC (Fig. 1A), followed by analysis of each chromatographic fraction by SDS-PAGE (Fig. 1B), N-terminal sequencing, and MALDI-TOF mass spectrometry (Table 1). Protein fractions showing single electrophoretic band, molecular mass, and N-terminal sequence were straightforwardly assigned by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) to a known protein family. Protein fractions showing heterogeneous or blocked N-termini were analyzed by SDS-PAGE and the bands of interest were subjected to automated reduction, carbamidomethylation, and in-gel tryptic digestion. The resulting tryptic peptides were then analyzed by MALDI-TOF mass fingerprinting followed by amino acid sequence determination of selected doubly- and triply-charged peptide ions by collision-induced dissociation tandem mass spectrometry. Product ion spectra were manually interpreted for *de novo* sequencing and the CID-MS/MS-deduced peptide ion sequences (Table 1 and Table 2) were submitted to BLAST similarity searches. The 30 fractions isolated by reverse-phase HPLC (Fig. 1) comprised at least 20 different proteins (Table 3), which belong to 8 different groups of toxins (Fig. 2), distributed into 2 major protein families, snake venom metalloproteinase (SVMP) and phospholipase (PLA_2), representing 86% of the total venom proteins, and 6 minor protein families (bradykinin-potentiating peptides, disintegrin, DC-fragment, cysteine-rich secretory protein, serine proteinase, and L-amino acid oxidase (LAO)).

Sánchez and coworkers have reported the isolation and characterization of a disintegrin (colombistatin) from the venom of *Bothrops colombiensis* [45]. Colombistatin contains 73 amino acids (isotope-averaged molecular mass of 7.778 kDa), including an RGD motif, and inhibited ADP-induced platelet aggregation (IC_{50} of 210 nM), human urinary (T24) and skin melanoma (SK-Mel-28) cancer cell adhesion to fibronectin, and cell migration [45]. The N-terminal sequence and molecular mass of colombistatin are identical to those of the medium-sized disintegrin eluted in fraction 5 of Fig. 1 (Table 1). According to their molecular masses, the disintegrin molecules found in fractions 4 and 6–8 may correspond to N- and C-terminal truncated forms of colombistatin (Table 1).

The venomics results revealed that the major toxin families of *B. colombiensis* are SVMP (42% of the total venom proteins) and PLA_2 (44%), providing clues for rationalizing the distinct signs of envenomation caused by this Bothrops species. A study of the fibrino(geno)lytic, procoagulant, hemorrhagic and lethal activities of crude venom and fractions from *B. colombiensis* venom has been only recently published [44]. This report provides biochemical evidence that metalloproteinases harbour the non-hemorrhagic and coagulant activities reported by Gabrijelcic et al. [40] to be associated with a protein of around 92 kDa under nonreducing conditions and 67 kDa in reduced SDS-PAGE. The PIII-SVMP isolated in fractions 24–25 (Table 1) exhibits the molecular features of the reported [44] α-fibrinogenase enzyme. Other metalloproteinases (<50 kDa, i.e. the PI-SVMP and the PIII-SVMP eluting in fractions 26–30) (Fig. 1 and Table 1) may be responsible for the fibrinolytic and hemorrhagic activities present in *B. colombiensis* venom [44]. On the other hand, the crude venom showed amidolytic (kallikrein-like and t-PA-like) activity [44] probably linked to the presence of serine proteinases. Serine proteinases may

Table 1 – Assignment of the reverse-phase isolated fractions of *Bothrops colombiensis* venom (Fig. 1A) to protein families by N-terminal Edman sequencing, mass spectrometry, and collision-induced fragmentation by nESI-MS/MS of selected peptide ions from in-gel digested protein bands

HPLC fraction	N-terminal sequencing	Isotope-averaged molecular mass	Peptide ion		MS/MS-derived sequence	Protein/protein family
			m/z	z		
Bcol-						
1	n.p.					
4	GEECDCGAPENPCCDAA	7204 Da	684.6	3	XRPGAQCAEGXCCDQCR*	Disintegrin colombistatin 3–70 [39]
			575.8	2	CTGQSADCPR*	
5	EAGEECDCGAPENPC	7778 Da				Disintegrin colombistatin 1–73 [39]
6	EAGEECDCGAPENPC	7555 Da				Disintegrin colombistatin 1–71 [39]
7	EAGEECDCGAPENPCCD	7408 Da				Disintegrin colombistatin 1–70 [39]
8	EECDCGAPENPCCDAAT	7462 Da				Disintegrin colombistatin 4–72 [39]
9–11	Blocked	1371.1	685.9	2	ZGGWPRPGPEIPP	Bradykinin-potentiating peptide
		1385.1	693.1	2	ZKWPRPGPEIPP	Bradykinin-potentiating peptide
12	SPPVCGNYFVEVGEE*	23 kDa	502.1	2	GQGTYYCR*	DC-fragment [~Q7T1T5]
			898.6	3	SECDIAESCTGQSPECPTDDFHR*	
13	SLFELGKMILQETGK*	13721 Da	766.9	2	SYGAYGCNCGVXGR*	K49-PLA_2 [P24605 L114d]
			868.6	2	EXCECDKAVAXCXR*	
			542.6	2	YYXKPXCK*	
			665.3	2	MILQETGKNPAK*	
			697.9	2	TXVCGENNSCXK*	
			738.8	2	KDRYSYSWKDK*	
			721.9	2	ENXNTYNKKYR*	
14–16	SLIEFAKMILEETKR*	13936 Da	523.8	2	TDRYSYSR*	D49-PLA_2 [P20474]
			637.3	2	YMAYPDXXCK*	
			753.1	2	CCFVHDCCYGK*	
			864.6	2	QXCECDKAAAVCFR*	
			992.5	2	TYKKRYMAYPDFLCK	
			919.6	3	RLPFPYYTTYGCYCGWGGQGQPK*	
17	NLWQFEMLIMKIAKTSGFMFYS	13862 Da	753.1	2	CCFVHDCCYGK*	D49-PLA_2 [~Q8AXY1, P81243]
			726.6	2	NLWQFEMLIMK	
			490.3	2	QXCECDR*	
			882.6	2	NLWQFEMLIMKIAK	
			682.3	3	DNKDTYDMKYWFYGAK	
18	VIGGDECDINEHPFL	28 kDa▼/■				Serine proteinase
	SVDFDSESPRKPNEIQ*	26 kDa▼/■	569.6	2	SVDFDSESPR*	CRISP
			768.9	2		MEWYPEAAANAER*
			635.6	3		KPNEIQNEIVDLHNSLR*
	N.D.	24 kDa▼/■	548.3	2	TXTSFGEWR*	PI-metalloproteinase [~P83512]
			790.6	2	VHEMLNTVNGFYR*	
			883.6	2	SVDVHALXANXEVWSK*	
			610.9	2	HSVGVVRDHSK*	
	NLWQFEMLIMKIAKTSGF	14 kDa▼/■	882.9	2	NLWQFEMLIMKIAK	D49-PLA_2 [~Q8AXY1, P81243]
19, 20	NLWQFEMLIMKIAKTSGF	14 kDa▼/■	753.1	2	CCFVHDCCYGK*	D49-PLA_2 [~Q8AXY1, P81243]
			490.3	2	QXCECDR*	
21–30	Blocked	23255 Da	548.3	2	TXTSFGEWR*	PI-metalloproteinase [~P83512]
			790.6	2	VHEMLNTVNGFYR*	
			883.6	2	SVDVHALXANXEVWSK*	
			547.3	2	YNSNXNTXR*	
			838.6	2	YIELAVVADHGIFTK*	
			610.9	2	HSVGVVRDHSK*	
22–25	ADDRNPLEECFRETD*	52 kDa▼/■	532.6	2	NPLEECFR*	L-amino acid oxidase [~Q6TGQ9]
			630.3	2	FWEDDGXHGGK*	
			647.3	2	EGWYANXPGMR*	
			676.6	2	SAGQXYEESXQK*	
			743.8	2	ETDYEEFXEXAK*	
			857.3	2	DPGVXEYPVKPSEVGK*	
			509.3	2	NGXSTTSNPK*	
			569.8	2	HDDXFAYEK*	
			761.8	2	ADDRNPLEECFR*	
	SLPQFAKMILQET	16 kDa▼/■				PLA_2
	VIGGDECNINEHRSL	27 kDa▼/■				Serine proteinase
24	N.D.	98▼/48■ kDa	902.3	2	YFVEVGEECDCGSPR*	PIII-metalloproteinase
			810.9	2	XYEXVNXXNVXYR*	

Table 1 (*continued*)

HPLC fraction Bcol-	N-terminal sequencing	Isotope-averaged molecular mass	Peptide ion *m/z*	z	MS/MS-derived sequence	Protein/protein family
26	N.D.	66 kDa▼	590.8	2	ZXVVTPEQQR*	PIII-metalloproteinase
			688.3	2	YVEFVVVXDHR	
27–30	N.D.	56 kDa▼	590.8	2	ZXVVTPEQQR*	PIII-metalloproteinase
			810.9	2	XYEXVNXXNVXYR*	
28–30	TPEQQRYVILVIV	26 kDa▼/■	548.3	2	TXTSFGEWR*	PI-metalloproteinase [~P83512]
			790.6	2	VHEMLNTVNGFYR*	
			610.9	2	HSVGVVRDHSK*	
30	N.D.	48 kDa▼	526.3	2	GNYYGYCR*	PIII-metalloproteinase
	N.D.	16 kDa▼	766.9	2	SYGAYGCNCGVXGR	PLA_2 [Q9PVE3]

X, Ile or Leu. Unless other stated, for N-terminal sequencing and MS/MS analyses, cysteine residues were pyridylethylated and carbamidomethylated, respectively; Molecular masses of the native proteins were determined by electrospray-ionization mass spectrometry, MALDI-TOF (*) or by SDS-PAGE before (▼) or after (■) sample reduction with β-mercaptoethanol; n.p., non peptidic material found. N.D., not determined. Proteins sharing tryptic peptide sequences with homologous *B. asper* venom proteins are in boldface and underlined and the common N-terminal and internal tryptic peptide sequences are labeled with asterisks.

also include thrombin-like coagulant enzymes, although their concentration in this venom seems to be very low. Though the PLA_2-associated effects of the venom of *B. colombiensis* have not been investigated, the large content of K49-PLA_2 in the venom supports the hypothesis that the toxic activities of *B. colombiensis* venom may include potent local myotoxicity and edema-forming activities [46,47].

3.2. *The protein profile of B. colombiensis venom argue for a close phylogenetic relationship with B. asper. A hypothetical evolutionary trend*

All the identified *B. colombiensis* venom proteins display strong similarity with entries from Bothrops species (Table 1), highlighting the close phylogenetic relationship of *B. colombiensis*

Table 2 – Identification by MS/MS analysis of non-toxin components present in the reverse-phase HPLC separations of the supernatants of immunodepleted samples shown in panels A, B, and C of Fig. 6. C, carbamidomethyl cysteine, respectively

HPLC fraction			Molecular mass	*m/z*	z	Peptide sequence	Protein
Bcol		Bas(P)					
ABS/AS	ICP-AS	ABC/AS					
	a	a, b	33, 31 kDa	690.6	2	TEKDAFGNANSAR	Horse radish peroxidase [1WAW_A]
				818.9	2	VVSTLPIAHQDWLR	Rabbit IgG Fc fragment [2VUO_A]
			28 kDa	601.4	2	LSVPTSEWQR	Rabbit IgG Fc fragment [2VUO_A]
				818.9	2	VVSTLPIAHQDWLR	
				621.6	3	TTPAVLDSDGSYFLYSK	
h–l	g–j	g–n	50 kDa	887.9	2	VPQVYVLAPHPDELAK	IgG Heavy Chain Horse [AAG01011]
				601.4	2	LSVPTSEWQR	Rabbit IgG Fc fragment [2VUO_A]
				818.9	2	VVSTLPIAHQDWLR	
			48 kDa	480.1	2	AAVESACPR	Horse radish peroxidase [1WAW_A]
				511.3	2	DAFGNANSAR	
				794.1	2	GLCPLNGNLSALVDFDLR	
				690.6	2	TEKDAFGNANSAR	
				601.4	2	LSVPTSEWQR	Rabbit IgG Fc fragment [2VUO_A]
				818.9	2	VVSTLPIAHQDWLR	
			28 kDa	601.4	2	LSVPTSEWQR	Rabbit IgG Fc fragment [2VUO_A]
				818.9	2	VVSTLPIAHQDWLR	
				621.6	3	TTPAVLDSDGSYFLYSK	
			26 kDa	516.4	2	YAASSYLTR	IgG light chain Horse [AAA50975]
				762.6	2	VTQGTTSVVQSFNR	Ig kappa chain b4 rabbit [AAB59259]
				601.4	2	LSVPTSEWQR	Rabbit IgG Fc fragment [2VUO_A]
				818.9	2	VVSTLPIAHQDWLR	

Molecular masses were estimated by SDS-PAGE after reduction with β-mercaptoethanol.

Table 3 – Overview of the relative occurrence of proteins from different toxin families in the venom of *Bothrops colombiensis*

Protein family	% of total venom proteins		
	B. colombiensis	*B. asper*	
		Pacific	Caribbean
BPP/C-NP	0.8	–	–
Medium disintegrin	5.6	1.4	2.1
DC-fragment	0.5	<0.1	<0.1
CRISP	0.1	0.1	0.1
PLA_2	44.3	45.1	28.8
• K49	34.1	36.0	18.8
• D49	10.2	9.1	10.0
Serine proteinase	<1	4.4	18.2
L-amino acid oxidase	5.7	4.6	9.2
C-type lectin-like	–	0.5	0.5
Zn^{2+}-metalloproteinase	42.1	44.0	41.0
• PIII	11.3	13.5	8.8
• PI	30.8	30.5	32.2

For comparison, the venom toxin composition of adult *B. asper* from the Pacific and the Caribbean regions of Costa Rica reported in [5] are listed.

with the New World South American bothropoid genera [6]. In particular, the closest homologues to *B. colombiensis* proteins appear to be venom proteins from *B. asper* [5]. Fig. 3 displays the close correspondence between the reverse-phase elution profiles of the venom proteins from these Bothrops species. *B. colombiensis* proteins exhibiting high homology to *B. asper* toxins are underlined in Table 1 and their common protein and peptide sequences are labeled with asterisks.

In Costa Rica, *B. asper* is distributed in two geographically isolated populations separated since the late Miocene or early Pliocene (8–5 Mya ago) by the high mountain ridge extending diagonally across the center through the entire length of the country. Geographic venom composition variations have been reported between the Caribbean and the Pacific *B. asper* populations [5]. The reverse-phase HPLC profiles (Fig. 3) and the overall venom toxin composition of *B. colombiensis* (Table 3) have a closer resemblance to the venom of *B. asper* specimens from the Pacific (P) versant than from those of the Caribbean (C) region of Costa Rica. For a rough estimation of the similarity in protein composition between the venoms of *B. colombiensis* and *B. asper*, we calculated the relative figure of common structural features. The two species share 55 out of 77 amino acid sequences listed in Table 1, indicating that *B. colombiensis* and *B. asper* (P) share approximately 70% of their venom proteome. The similarity between *B. colombiensis* and *B. asper* (C) was about 65%. The high resemblance between these venoms is not restricted to any particular toxin family but extends to disintegrins, DC-fragment, PLA_2s, serine proteinases, LAO, and PI- and PIII-metalloproteinases (Fig. 3, Table 1). On the other hand, our venomics study clearly contradicts the synonymization of *B. colombiensis* with *B. atrox*

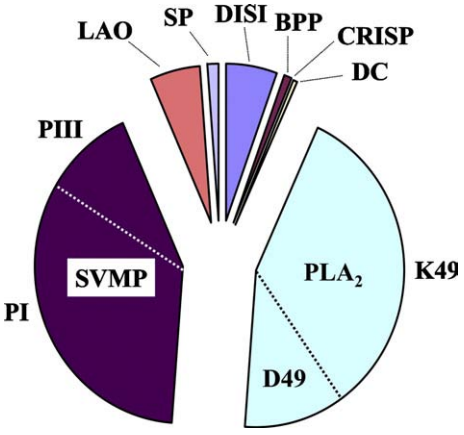

Fig. 2 – Overall protein composition of *B. colombiensis* venom. BPP, bradykinin-potentiating peptide; DC, disintegrin/cysteine-rich fragment from PIII snake venom Zn^{2+}-metalloproteinase (SVMPs); DISI, disintegrin; SP, serine proteinase; LAO, L-amino acid oxidase; PLA_2, phospholipase A_2; CRISP, cysteine-rich secretory protein. Details of the individual proteins characterized are shown in Table 1, and the percentages of the different toxin families in the venom are listed in Table 3.

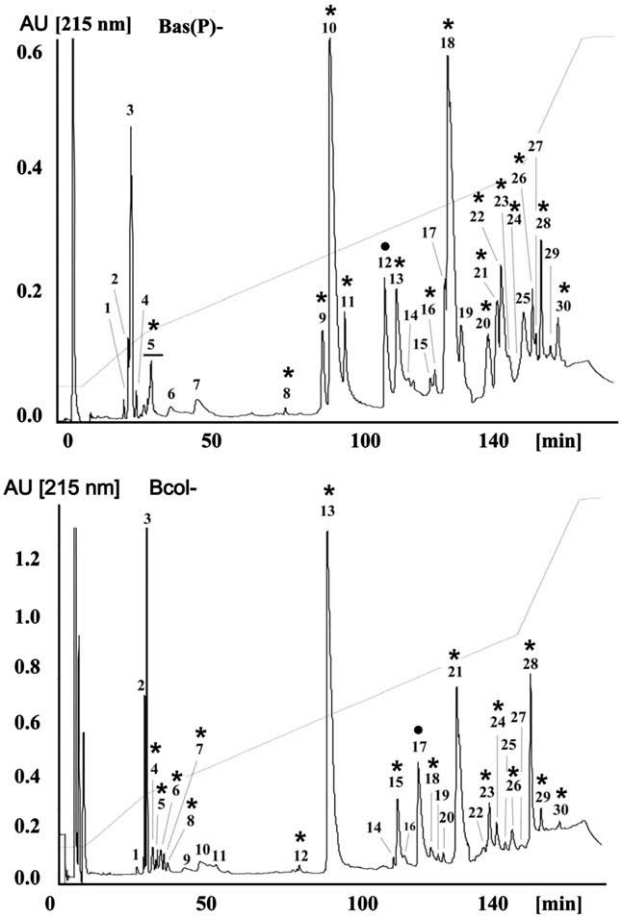

Fig. 3 – Comparison of the reverse-phase HPLC separations of the venom proteins of *B. asper* (upper panel) and *B. colombiensis* (lower panel). Proteins exhibiting identical chromatographic elution time and containing common tryptic peptide sequences are labeled with asterisks. PLA_2 molecules Bas(P)-12 and Bcol-17, labeled with closed circles, share peptide ion sequences but elute at different times. For detailed information see Table 1.

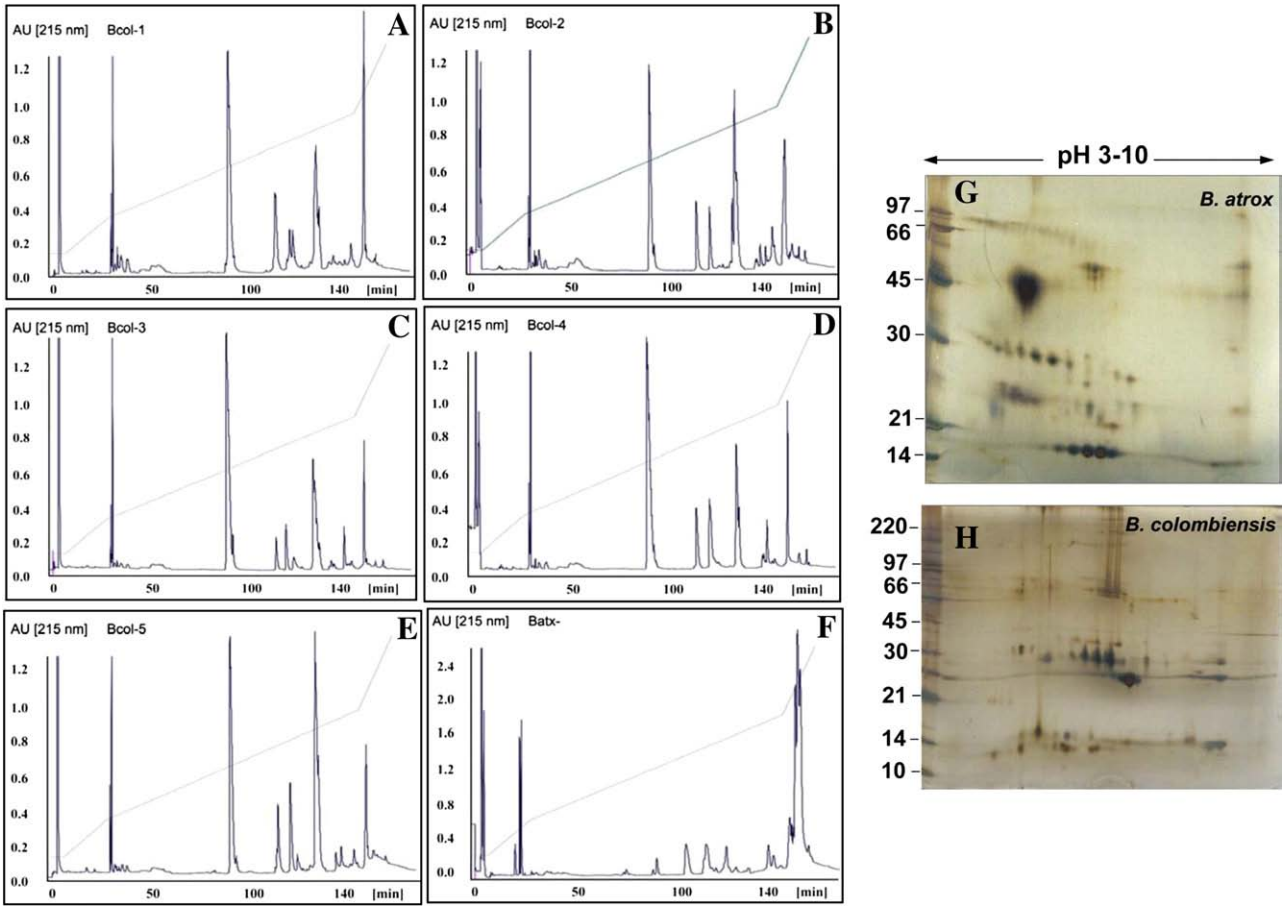

Fig. 4 – Comparison of the reverse-phase HPLC chromatograms and 2DE separations of *B. colombiensis* and *B. atrox* venom proteins. Panels A–E show the venom protein profiles of the venoms of *B. colombiensis* collected from different locations in Venezuela (see Fig. 5A): Bcol-1 (Sabana de Uchire, Anzoátegui State), Bcol-2 (San Felipe, Yaracuy State), Bcol-3 (Araira, Miranda State), Bcol-4 (Barlovento, Miranda State), and Bcol-5 (Santa Bárbara, Barinas State). Panel F, reverse-phase HPLC separation of the venom proteins of *B. atrox* (Brazil). A detailed characterization of geographical variations observed in the venoms from *B. colombiensis* will be reported elsewhere. Panels H and G, two-dimensional electrophoretic separations of the venom proteins of *B. colombiensis* (H) and *B. atrox* (G) venoms.

[10]. A comparison of the venom proteomes of *B. colombiensis* captured in different locations in Venezuela with that of *B. atrox* evidences small geographical variations in the composition of the former and little similarity between the reverse-phase HPLC profiles of the *B. colombiensis* and *B. atrox* venoms (Fig. 4, panels A–F). This point is particularly evident when the two-dimensional electrophoretic separations of the venom proteins are compared (Fig. 4, panels H vs. G). In addition, proteomics data (Table 1 in this work versus Table 1 in [28]) show that only 9 peptides (2 from an L-amino acid oxidase, 1 from a PIII-SVMP, 3 from a CRISP molecule, and 3 from PLA_2 P20474) out of approximately 200 ion sequences reported [28] appear to be conserved in the two species. Further, C-type lectin-like proteins represent abundant toxins in adult *B. atrox* venom (from the Brazilian Amazon region) whereas these molecules have not been detected in the *B. colombiensis* venom proteome. In addition, peptide sequences from the major PI-metalloproteinase found in *B. atrox* venom (Fig. 1 in [28]) depart from the corresponding polypeptide stretches of the hemorrhagic metalloproteinase BaP1 (P83512), which is strongly conserved in *B. colombiensis* and *B. asper* venoms, being the closest homologs the PI-metalloproteinases Q8QG89 from *B. insularis* followed by the PII-metalloproteinase Q98SP2 from *B. jararaca*.

As a whole, our results indicate a closer kinship of *B. colombiensis* and *B. asper* than any of these species with *B. atrox*. This conclusion, in the context of the hypothesis that *B. asper* emerged by dispersal of a single South American bothropoid species into Central America [2], points at the ancestor of *B. colombiensis* as the founding *B. asper* ancestor species (Fig. 5). Arrival of the Central American colonists may have predated the uplift of the mountains of lower Central America (8–5 Mya), which fragmented the original homogeneous lowland Costa Rican herpetofauna into allopatric Caribbean and Pacific populations [3,4].

3.3. *Antivenomics of B. colombiensis: venom components immunodepleted by polyvalent antivenoms*

Accidental envenomation by Bothrops species constitutes a relevant public health issue in Central and South America

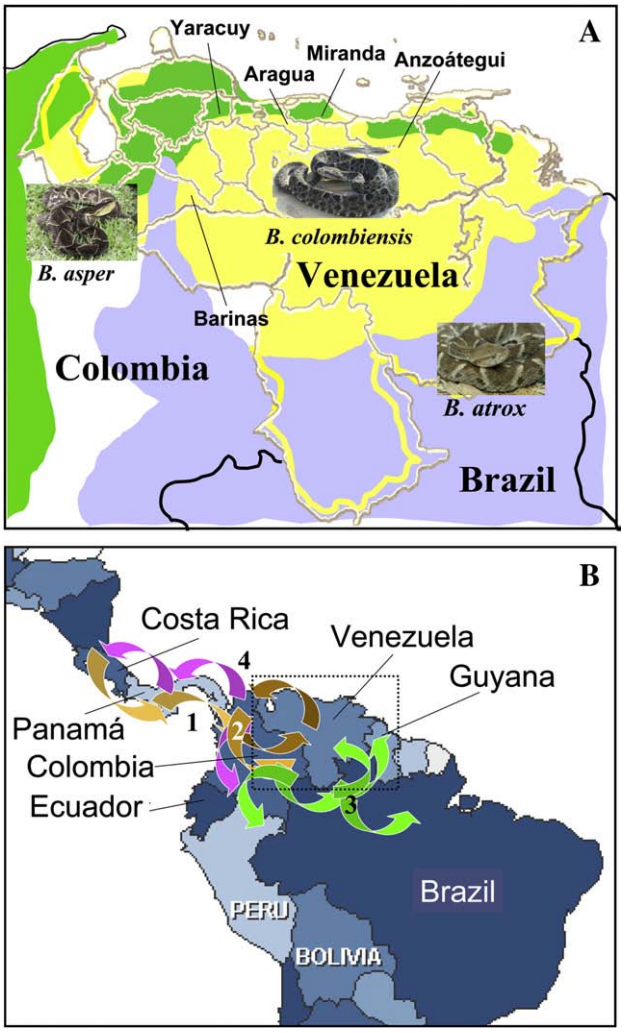

Fig. 5 – Geographical distribution of *B. colombiensis*, *B. asper*, and *B. atrox* (A) and the biogeographical scenario for the bidirectional Bothrops colonization suggested by the venomics data (B). Panel A, approximate geographical distribution of *B. colombiensis* (yellow), *B. asper* (green), and *B. atrox* (gray) in northern South America within the range highlighted in panel B (adapted from Campbell and Lamar [1]). The location of states where *B. colombiensis* specimens were collected for this study are indicated in the physical map of Venezuela. Panel B, detail of Central America and the northern part of South America. The ocher arrows on the map show the hypothesized directions of colonization of South America by the common Bothrops ancestor sometime during the Miocene, 10–23 mya [2] (1), the radiation and diversification of Bothrops across South America (2 and 3) establishing the present-day patterns of distribution of *B. colombiensis* (brown) and *B. atrox* (green) populations, and the reinvasion of Central America by a single species, the ancestor of *B. asper*, (4) (magenta arrows). Arrows do not point to the exact geographical distribution of the snakes. Comparison of the venom proteomes of species within the *asper-atrox* group points at the ancestor of *B. colombiensis* as the founding *B. asper* ancestor species.

[19–22]. Adequate treatment of snake envenoming is critically dependent on the ability of antivenoms to reverse venom-induced coagulopathy, haemorrhage, hypotensive shock and other signs of systemic envenoming. Several antivenoms are produced in Latin America using different venoms in the immunization schemes [48]. Each of these antivenoms is effective against envenomations by snake venoms not included in the immunization protocol, demonstrating the high degree of immunological cross reactivity between Central and South American crotaline snake venoms. A practical consequence of this fortunate circumstance is the possibility of using these heterologous antivenoms to circumvent the restricted availability of species-specific antivenoms in some regions. However, before testing in clinical trials, antivenoms need to be evaluated experimentally by assessing their neutralizing ability against the most relevant toxic and enzymatic activities of snake venoms. Simple laboratory tests have been adapted for the evaluation of antivenoms [49 and references cited]. Our antivenomics approach [33,50] is simple and easy to implement in a protein chemistry laboratory, and may thus represent another useful protocol for investigating the immunoreactivity, and thus the potential therapeutic usefulness, of antivenoms towards homologous and heterologous venoms.

Following recommendations of international workshops pointing to the need of strengthening antivenom production and distribution in Central and South America, and in other regions of the world [51–53], we have investigated the spectrum of toxin recognition and neutralization by two antivenoms targeting the venoms of the phylogenetically close and medically-important snakes, *B. asper* and *B. colombiensis*, to assess their degree of crossreactivity. Fig. 6 displays a reverse-phase separation of *B. colombiensis* venom proteins recovered in the soluble fraction after incubation of the venom with the Venezuelan ABC antivenom (panel A) or the ICP polyvalent antivenom (panel B) followed by immunoprecipitation with rabbit anti-horse IgG antiserum. Both antivenoms showed essentially the same immunoreactivity. They essentially immunoprecipitated all DC-fragments, PIII-SVMPs (except the 65 kDa Bcol-26/Bas-25), serine proteinases, CRISP, and L-amino acid oxidase, but displayed limited immunoreactivity towards the medium-sized disintegrins (Bcol 4–8), PLA_2 molecules (Bcol 13–17) and PI-SVMPs (Bcol 21–26 and Bcol 28–29). We estimate that 25–35% of the latter toxins were only partially immunodepleted from *B. colombiensis* venom by the antivenoms. Similarly, the ABC antivenom immunodepleted PIII-SVMPs, serine proteinases, CRISP, L-amino acid oxidase, and C-type lectin-like molecules from the venom of *B. asper* (Fig. 6C). On the other hand, the medium-sized disintegrin (BasP5), DC-fragment (BasP8), PLA_2 molecules (BasP9-13), and the PI-SVMPs BasP18, BasP22, and BasP27 were only partially (~35–40%) immunoprecipitated from the venom of *B. asper* from the Pacific versant of Costa Rica.

Their immunological profiles further evidence the close evolutionary kinship between *B. colombiensis* and *B. asper*. Our results also reveal a limitation of the two antivenoms tested in precipitating, and thus probably neutralizing, the low Mr venom protein components of both venoms. The failure of snake antivenoms to recognize low molecular mass toxins (6 kDa to <32 kDa, predominantly PLA_2 proteins and α-neurotoxins) has been previously reported [34,50,54,55]. Our

present and previous antivenomic studies [34,50] revealed the existence in viperid venoms of all three possible classes of toxins with regard to their immunoreactivity towards antivenoms: C-toxins, completely immunodepletable toxins; P-toxins, partly immunodepleted toxins; and N-toxins, non-inmunoprecipitable proteins. Further, class P toxins could not be completely removed from the venom by incrementing up to five times the antivenom/venom ratio, suggeting that these toxins are poorly immunogenic and trigger the generation of low-affinity antibodies in the horse. Assuming a link between the *in vitro* toxin immunodepletion capability of an antivenom and its *in vivo* neutralizing activity towards the same toxin molecules, future immunization protocols should make use of mixtures of immunogens to improve the generation of high-affinity antibodies against class P and class N toxins.

The effectiveness of ICP antivenom in the neutralization of *B. colombiensis* venom was evidenced by the abrogation of the lethal effect. The Median Lethal Dose of *B. colombiensis* venom, by the intraperitoneal route, was 7.76 mg/kg in mice. Neutralization assays with the polyvalent ICP antivenom indicated that the Median Effective Dose (ED_{50}) was 2.16 mg venom neutralized per mL antivenom (95% confidence limits: 1.56–2.99 mg venom per mL antivenom). For comparative purposes, the ED_{50} of this antivenom against the venom of *B. asper* has been reported as 3 mg venom/mL antivenom [56].

3.4. *Concluding remarks and perspectives*

The phylogeny of the *B. atrox-asper* complex is still far from being resolved, and the identity of several taxa remains unclear. From examination of 374 Middle American specimens, Sasa [57] concluded that "the taxonomic value of some characters commonly employed in *B. atrox-asper* keys [1] must be regarded with caution, especially because several of the traits greatly overlap between species or are influenced by gender, locality, or life stage". Our venomic and antivenomic study of the pooled venom of specimens of the Venezuelan Manapare lancehead pitviper, *B. colombiensis*, captured in different areas of Venezuela, ranging from Barinas (southwest), Yaracuy (northwest), to Araraira and Barlovento (north-central), provides strong support for its closer phylogenetic relationship to *B. asper* than to *B. atrox*. However, though our data would be in line with the suggested synonymy of *B. colombiensis* and *B. asper*, the taxonomic status and species boundaries of the Venezuelan Bothrops populations remain a challenging problem. A subspecies usually arise as a consequence of geographical isolation within a species, represents a

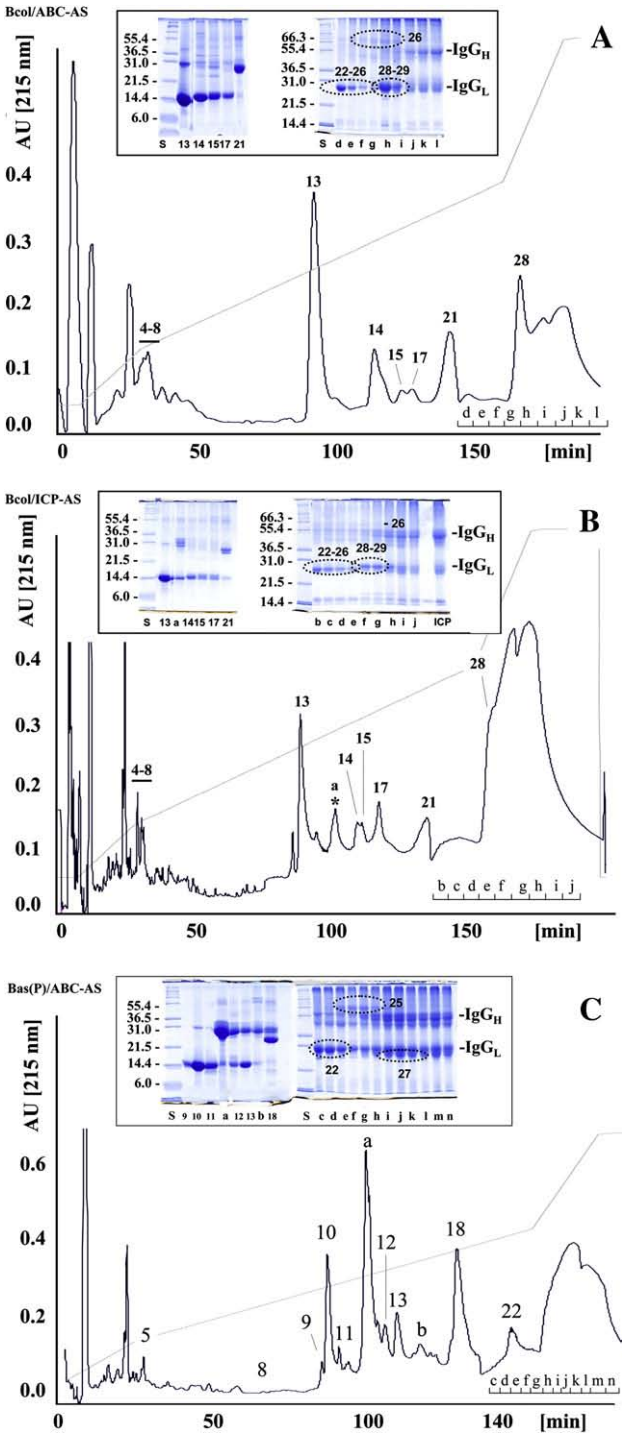

Fig. 6 – Immunodepletion of venom proteins by the polyvalent antivenoms. Panels A and B show, respectively, reverse-phase separations of *B. colombiensis* venom proteins recovered after incubation of the crude venom with the anti-bothropic and anti-crotalic Venezuelan antivenom and with the polyvalent (Crotalinae) Costa Rican antivenom, followed by rabbit anti-horse IgG antiserum and immunoprecipitation. The inserts shows an SDS-PAGE analysis of β-mercaptoethanol-reduced fractions labelled in the chromatograms. Protein fraction numbering is as in Table 1. Panel C, reverse-phase HPLC separation of *B. asper* venom proteins non immunoprecipitated by incubating the crude venom with the anti-bothropic and anti-crotalic Venezuelan antivenom, followed by rabbit anti-horse IgG antiserum and immunoprecipitation. Protein numbering as in [5]. Peak labeled "a" and "b" in panels B and C correspond to IgG fragments with N-terminal sequence LLGGPSVFIIPPKPK. CID-MS/MS of tryptic peptide ions from the 50 kDa and the 25 kDa protein bands in fractions h–l (panel A), g–j (panel B), and g–n (panel C) were identified by MS/MS as IgG fragments of the primary and secondary antibodies used for immunoprecipitation (Table 2).

taxonomic subdivision that ranks just below a species, and comprises a geographically separated group of genetically distinct individuals whose members can interbreed. Hence, classification of *B. colombiensis* and *B. asper* as geographical variations of the same species, as subspecies, or as distinct species requires considerable additional effort, including detailed genomic and field analyses. Nonetheless, our study may be relevant for reconstructing the natural history and cladogenesis of Bothrops. The well-documented geographical and individual venom variability among Bothrops species [5], and the overlap of the distribution ranges of Colombian *B. asper* and Amazonian *B. atrox* with *B. colombiensis* [1,14], call for additional comparative proteomic analyses of venoms from specimens of isolated populations and from specimens inhabiting areas of suspected sympatry to investigate the degree of intraspecific and intrapopulational variability and interspecific relationships.

A thorough characterization of the venom proteomes of *B. colombiensis* may also contribute to a deeper understanding of the pathophysiology of envenomings by this snake. A major aim of our combined venomic and antivenomic studies is the understanding of antivenom specificity as a first step towards the development of improved antivenoms with a more complete toxin neutralization profile. Hence, knowledge of the relative contributions of different venom toxin families to the composition of the venom might be relevant for generating immunization protocols that elicit the production of toxin-specific antibodies showing greater specificity and effectiveness than conventional antivenoms raised by immunizing horses with whole venom. On the other hand, the remarkable similarity of the venoms of *B. colombiensis* and *B. asper* may be regarded as a fortunate circumstance for the management of snakebites by any of these Bothrops species.

Envenomations by *B. colombiensis* (LD_{50} intraperitoneal in mice = 5.8–11.6 μg/g) [44] and *B. asper* (LD_{50} intraperitoneal in mice = 2.8–3.7 μg/g) [58; http://www.venomdoc.com/LD50/ld50ip.html] are characterized by local tissue damage (dermonecrosis, blistering, edema, local hemorrhage and myonecrosis), and in severe cases by systemic alterations such as defibrin(ogen)ation, thrombocytopenia, platelet hypoaggregation, bleeding distant from the bite site, disseminated intravascular coagulation, cardiovascular shock and acute renal failure [59–61]. Mortality rate in Costa Rica has remained at low levels (below 0.2 per 100,000 population and, in half of the years, below 0.1 per 100,000 population) for the period 1993–2006. This is likely a consequence of confronting snakebite envenomation as a public health problem along with continuous improvement of the local production of antivenoms and their widespread distribution to hospitals and clinics [62]. The polyvalent antivenom manufactured at the Instituto Clodomiro Picado (ICP, University of Costa Rica) using a mixture of three snake (*B. asper*, *C. simus*, *L. stenophrys*) venoms has been reported to neutralize the lethality and the hemorrhagic, caseinolytic, coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of a number of venoms of Central and South American snakes [39,63,64]. Though the spectrum of neutralizing activities of the Venezuelan ABC antivenom raised against a mixture of the venoms of *B. colombiensis* and *C. d. cumanensis* remains to be investigated, administration of this antivenom reduces the mortality rate due to *B. colombiensis* envenomation from 8% (no treatment) to 0.7% [59,65]. The indistinguishable immunological crossreactivity of the Venezuelan ABC and the Costa Rican ICP polyvalent antibodies towards the venoms of *B. colombiensis* and *B. asper* strongly suggest the possibility of indistinctly using any of these antivenin for the management of snakebites by any of these Bothrops species (i.e. if antivenom availability is compromised). Though this hypothesis needs to be further tested by more detailed preclinical neutralization studies, characterization of the venom using our antivenomic approach provides a ground for the presumed protection of the ICP polyvalent antivenom against the lethal, hemorrhagic, coagulant, defibrinating, fibrin(ogen)olytic and caseinolytic activities of *B. colombiensis* venom, which are associated with serine proteinases and/or PIII-SVMPs [66]. Our study also predicts the limited neutralization capability of the ABC and the ICP antivenoms towards a number of Bothrops venom components, most notably BPPs, medium-sized disintegrins, PLA_2s, and some PI-SVMP molecules.

Antivenoms should ideally neutralize all venom components playing a role in the pathophysiology of envenomation. However, the venom-immunisation protocols have been based on injection of crude venom since first generation antivenoms were described over 100 years ago [67,68], and make no attempt to direct the immune response to the most devasting venom proteins (many venom proteins are not toxic and many low molecular mass venom proteins are highly toxic but weakly immunogenic). Consequently, the dose-efficacy of antivenoms is likely to suffer from the presence of redundant antibodies to non-toxic molecules and a lack of potent neutralizing antibodies to small molecular mass toxins. This in turn results in the need for high volumes to effect treatment and a consequent increase in the risk of serum-sickness and early adverse reactions (anaphylactic and anaphylactoid effects). Antivenoms can be improved by using proteomic and molecular approaches for selecting candidate epitopes for structure-based design of high affinity antibodies [69], including those targeting P- and N-toxins. Structure-based designed DNA constructs encoding a string of relevant and widespread distributed epitopes have been successfully used for immunization [70]. These new approaches to design antibodies *a la carte* are critically dependent upon a detailed knowledge of the venom toxin composition and immunological profile. Our venomic and antivenomic protocols may become useful for a more precise knowledge of the most relevant and abundant venom components and for the identification of toxins not recognized by antivenoms, thus paving the way for the design of more effective immunotherapeutics.

Acknowledgements

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