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Venom variation in *Bothrops asper* lineages from North-Western South America

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ABSTRACT

Bothrops asper is a venomous pitviper that is widely distributed and of clinical importance in Mesoamerica and northern South America, where it is responsible for 50–80% of all envenomations by Viperidae species. Previous work suggests that *B. asper* has a complex phylogeographic structure, with the existence of multiple evolutionarily distinct lineages, particularly in the inter-Andean valleys of north South America. To explore the impact of the evolutionary history of *B. asper* on venom composition, we have investigated geographic variation in the venom proteome of this species from the populations from the Pacific side of Ecuador and south-western Colombia. Among the 21 classes of venom components identified, proteins from mainly four major toxin families, snake venom metalloproteases (PI- and PII-SVMP), phospholipases A_2 (K49- and D49-PLA_2s), serine proteinases (SVSP), and C-type lectins-like (CTL) proteins are major contributors to the geographic variability in venom. Principal component analyses demonstrate significant differences in venom composition between *B. asper* lineages previously identified through combination of molecular, morphological and geographical data, and provide additional insights into the selection pressures modulating venom phenotypes on a geographic scale. In particular, altitudinal zonation within the Andean mountain range stands out as a key ecological factor promoting diversification in venom. In addition, the pattern of distribution of PLA_2 molecules among *B. asper* venoms complements phylogenetic analysis in the reconstruction of the dispersal events that account for the current biogeographic distribution of the present-day species' phylogroups. Ontogenic variation was also evident among venoms from some Ecuadorian lineages, although this age-related variation was less extreme than reported in *B. asper* venoms from Costa Rica. The results of our study demonstrate a significant impact of phylogenetic history on venom composition in a pitviper and show how analyses of this variation can illuminate the timing of the cladogenesis and ecological events that shaped the current distribution of *B. asper* lineages.

Biological significance: *Bothrops asper*, called "the ultimate pitviper" due to its defensive behavior, large body size, and medical importance, represents a species complex that is widely distributed from southern México southwards across north-western South America to north-western Perú. This work reports the characterization of the venom proteomes of *B. asper* lineages from the Pacific sides of Ecuador and south-western Colombia. Multivariate analyses indicate that variability in venom composition among the *B. asper* lineages is driven by proteins from four major toxin families, presumably in response to selection pressures created by recent and historical ecological conditions created by geological and climatic events from the Pliocene-Pleistocene to the present along the Central and South American Continental Divide. The emerging biogeographic pattern of venom variation, interpreted in the context of the current phylogenetic hypotheses, support and complement previously proposed evolutionary Plio-Pleistocene dispersal events that shaped the present-day distribution range of *B. asper* lineages. In addition, our venomics data indicate the occurrence of genetic exchange between

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Colombian and Pacific Costa Rican populations, which may have occurred during the second wave of *B. asper* migration into Mesoamerica. Our work represents a foundation for a future broader sampling and more complete "-omics" analyses to deepen our understanding of the patterns and causes of venom variation in this medically important pitviper.

1. Introduction

The genus *Bothrops* includes at least 50 species of venomous pitvipers (Viperidae: Crotalinae) which are widely distributed throughout the Americas, from México to southern Argentina [1–3]. *Bothrops* species inhabit diverse habitats, from tropical and subtropical forests to arid and semiarid regions, and from sea level to altitudes of more than 3000 m [4]. They also exhibit diverse morphological and ecological traits, including terrestrial, arboreal and semiarboreal species, feeding habits, and ontogenetic dietary shifts [4]. Although still subject to taxonomic uncertainty [5], all the clades within genus *Bothrops* include species that represent the main medically important venomous snakes in their distributional range [6,7]. Epidemiological data show that around 90% of snakebite envenomations in Central and South America are inflicted by species within genus *Bothrops* [8–10], possibly due to their cryptic coloration and capability to inhabit human-disturbed environments [11].

Bothrops asper (Garman 1884), also called "the ultimate pitviper" due to its defensive behavior, large body size, and great medical importance [10,12,13], are snakes that represent a species complex widely distributed from southern México southwards across north-western South America to north-western Perú [1,11]. Populations of this snake are more frequent in low to middle elevations (0–600 m), inhabiting tropical rainforest and premontane forest, and with lower frequency in dry or deciduous forests where they are mainly restricted to evergreen and riparian vegetation [11,14]. It has crepuscular and nocturnal habits, and can be found in primary forests, as well as among fields, crops (e.g. coffee or banana plantations), human dwellings or even inside houses in rural areas [4,11,14]. It is considered a diet generalist with adults consuming mainly small mammals (essentially rodents), birds and frogs, and juveniles snakes also eating centipedes, lizards, frogs, snakes and fishes [11].

The *B. asper* species complex is phylogenetically nested within the *B. atrox* clade and has undergone several systematic revisions [1,15]. This species was first recognized as a synonym of *B. atrox* [16] and subsequently as the subspecies *B. a. asper* [17]. Its phenotypic variability including cryptic color patterns and overlapping meristic characters with other members of the clade, has complicated the taxonomy of *B. asper* in the last two decades [18,19]. Geographic variants of Colombian *B. asper* distributed in the Cauca and Patia Rivers Valleys were recognized as distinct in 2010, on the basis of subtle morphological differences [20]. However, the taxonomic validity of these Colombian *B. asper* populations as different species (*B. ayerbei* and *B. rhombeatus*) has been unclear due to lack of genetic studies that complement this assessment [21], and problems associated with those nomenclatural acts [22]. More recently, the combination of molecular and morphological analyses has suggested a complex phylogeographic structure for *B. asper* in Central and South America [21,23,24]. Based on the most comprehensive data set available, Salazar-Valenzuela (2016) proposed the existence of nine *B. asper* lineages denominated "México and Nuclear Central America (MNCA)", "Pacific Isthmian Central America (PICA)", "Darien Panamá and Choco (CHOCO)", "Magdalena Valley Colombia and Venezuela (MVV)", "*B. ayerbei* (BAY)", "*B. rhombeatus* (BRH)", "Pacific Ecuador (PEC)", "Highland Ecuador 1 (HEC1)" and "Highland Ecuador 2 (HEC2)" [21]. Some of these groups, particularly found in inter-Andean valleys such as *B. ayerbei* and *B. rhombeatus* from western Colombia and *B. asper* populations from the highlands of Ecuador appear to have diverged only recently, in the Pleistocene within last 1.15–2.1 million years [21,23,24].

Biogeographic studies support *B. asper* as the first species complex to split from the *B. atrox* clade in the Pliocene, around 3.02–2.32 Mya, as the result of geological events [21,25]. In particular, major events that impacted diversification of lancehead (*Bothrops*) vipers [21,26], and other taxonomic groups (e.g birds, amphibians, lizards and mammals) include the uplift of the Guanacaste, Central, and Talamanca mountain ranges in Mesoamerica, which occurred from the late Miocene through the Pliocene [26], the closure of the Isthmus of Panamá at least 3.2 Mya ago [27–32], and the uplift of the Andes mountain range in South America. The resulting habitat fragmentation promoted new ecological niche opportunities and lead to allopatric speciation in these groups [33]. More recent phylogeographic studies have identified demographic processes and ecological differentiation in montane habitats as strong drivers of the divergence of a number of different taxa along altitudinal gradients including *B. asper* populations [24].

Snake venom is an ecological trait crucial to the foraging success of the species producing it [34–36], and as such is subject to different levels of regulation [37,38]. Intraspecific variation in snake venom is common, particularly among species with broad geographic ranges [39]. In this regard, *B. asper* venoms have been extensively analyzed using proteomic [40,41] and transcriptomic [42] methods, and their biological activities investigated using in vivo and in vivo assays [43–45]. Geographic, individual, and ontogenic variation has been observed in venom from specimens from North (México), Central (Guatemala, Honduras, Costa Rica, and Panamá) and South (Colombia, Ecuador) America [40,41,46–51]. However, studies addressing the geographic variation of *B. asper* venoms in the context of inter-lineage genetic variation [21,23] have not been previously reported. Such studies would be of practical importance as biochemical and epidemiological data suggest that the clinical manifestations of envenomation by individuals from different lineages vary across the range of the species [52]. A deep knowledge of the geographic variability of *B. asper* venom would help in interpreting clinical signs and symptoms of envenomation by this snake and offer the possibility of more specific and effective treatments to patients from tailored to specific geographic locations, as well as guiding the design of improved novel taxon-wide antivenoms [53,54].

Using a ecological-evolutionary and clinical framework, we characterized the venom variability of *B. asper* lineages across the species distribution. To this end, we determined the venom proteomes of juvenile and adult snakes from five lineages from south-western Colombia and Ecuador and compared them with previously published data for *B. asper* in North and Central America, from populations in Costa Rica, Panamá, México, and northern Colombia. We also used patterns of venom differentiation across lineages to investigate evolutionary trends, such as the geographic distribution of intraspecific venom proteome variability, may provide hints to delve into the natural history that shaped the current phylogeographical distribution of the species lineages. We also use these data to make inferences about the biogeographic processes that have shaped the current distribution of this species [55,] although we are aware that variable and unpredictable rate of venom toxin sequence diversification conceptually precludes their use for molecular phylogenetic purposes.

2. Materials and methods

2.1. Snake venoms

Venom samples from 36 adult and 9 juvenile (ju) *B. asper* specimens

were collected in different localities of eleven Ecuadorian provinces: Esmeraldas (E, $n = 9$ individuals), Manabí (M, $n = 9$), Pichincha (P, $n = 3$), Cotopaxi (C, $n = 2$), Bolívar (B, $n = 1$), Los Ríos (LR, $n = 3$), Guayas (G, $n = 4$), Santa Elena (SE, $n = 1$), El Oro (EO, $n = 2$), Loja (L, $n = 6$), and Azuay (A, $n = 5$) (Fig. 1, Table S1). The Esmeraldas province samples were from individuals of the CHOCO lineage; snake venoms from southern Esmeraldas to Santa Elena were collected from specimens of the PEC lineage; and the samples collected in the Loja and Azuay provinces were from individuals of the HEC2 lineage. The PECju venom pool included 7 samples from *B. asper* groups Norte P1 (BANP1) ($n = 1$), Norte P2 (BANP2) ($n = 4$), and Norte-Centro (BANC) ($n = 2$). The HECju venom group was pooled from 2 specimens. Venoms samples from ten adult snakes from the Department of Cauca in south-western Colombia were also collected: two samples of *B. asper* sensu stricto (CHOCO lineage) from the municipalities of Playa Rica and Huisitó (El Tambo in the Pacific coast, Choco Province (CH)), four samples of the *B. rhombeatus* lineage (BRH) from Cauca river (CR) valley (Popayán and Cajibío municipalities), and four samples from snakes of the *B. ayerbei* lineage (BAY) from the Alto Patía river (PR) valley (El Tambo, San Joaquín, Pomorroso, Cauca) (Fig. 1, Table S1). In addition, the Grupo de Ofidismo y Escorpionismo (University of Antioquia, Colombia) and the Costa Rican Clodomiro Picado Institute kindly donated, respectively, *B. asper* venom samples from i) Antioquia (Colombia) (BAANT), and ii) from the Caribbean and Pacific sides of Costa Rica, and México, CICA, PICA, and BAMEX. Venoms were collected by snakes biting on glass beakers or glass conical funnel covered with Parafilm®, after which samples were immediately dried in a vacuum container containing anhydrous calcium sulfate (Drierite desiccant) or directly lyophilized, and stored at −20 °C until analysis.

2.2. Venomics

2.2.1. Reverse-phase HPLC profiling and preparation of venom pools

A total of 150–300 μg of venom individually collected from 46 adult and 9 juvenile specimens belonging to *B. asper* lineages from Ecuador and south-western Colombia were individually fractionated by reverse-phase HPLC. Briefly, the venom samples were dissolved in 100 μL of MilliQ® water containing 0.05% trifluoroacetic acid (TFA) and 5%

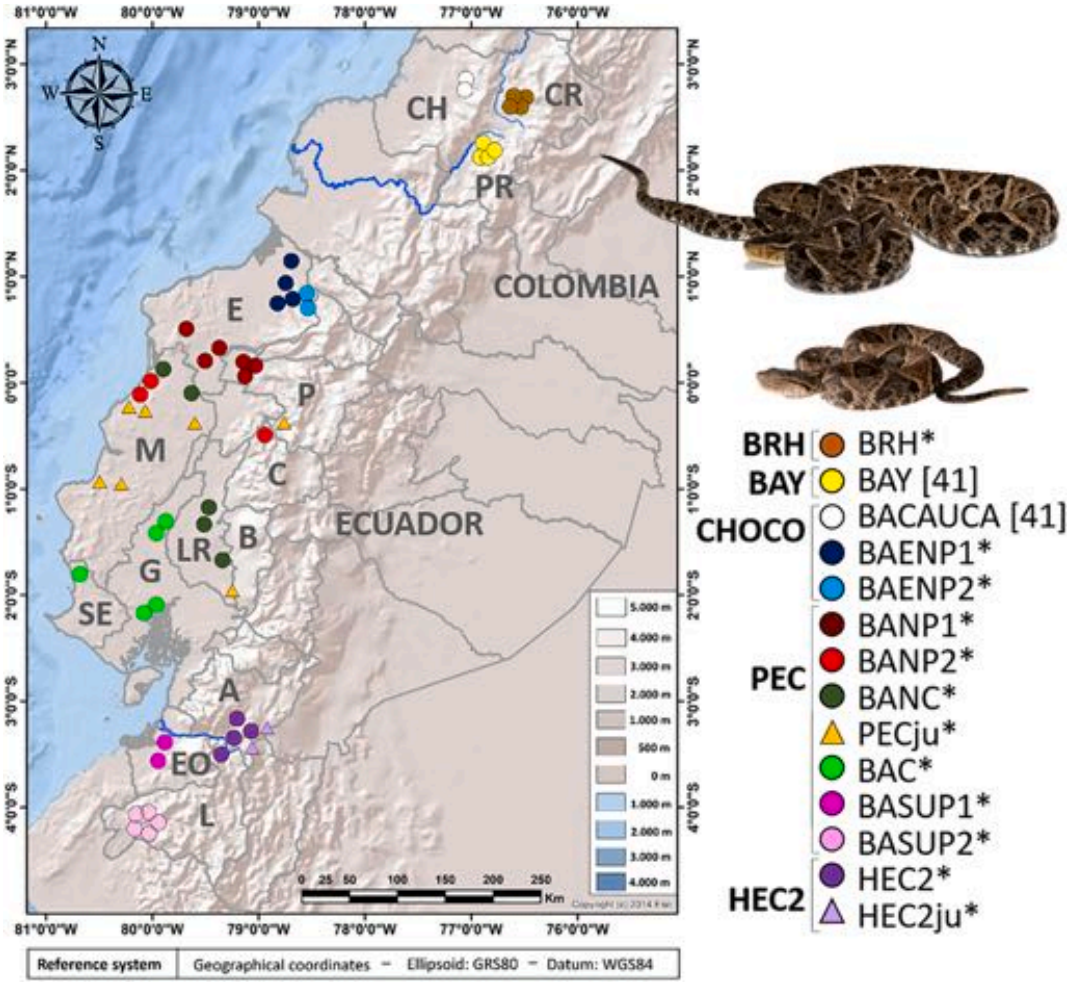


Fig. 1. Geographic distribution of *B. asper* in south-western Colombia and Ecuador. Physical map showing the sampling localities of *B. asper* venoms investigated in this work: El Tambo (Playa Rica and Huisitó, white circles), El Tambo (San Joaquín, yellow circles), Popayán and Cajibío (La Yunga and La Venta, brown circles) municipalities from Department of Cauca, south-western Colombia, and the Ecuadorian Provinces of Esmeraldas (E), Los Ríos (LR), Manabí (M), Cotopaxi (C), Pichincha (P), El Oro (EO), Guayas (G), Santa Elena (SE), Loja (L), and Azuay (A). The acronyms shown at the right refer to the *B. asper* venom groups described in Materials and Methods, Supplementary Table S1, and Fig. 2. Asterisks highlight venom groups characterized in this work. The venom proteomes of *B. ayerbei* from Valle Alto del Río Patía, Department of Cauca, Colombia (BAY) and *B. asper* from El Tambo in the Pacific coast of the Department of Cauca (BACAUCA) have been reported [41]. Circles represent adult snake specimens; triangles, juvenile snakes. BRH, BAY, CHOCO, PEC, and HEC2, acronyms of the *B. asper* lineages described by Salazar-Valenzuela et al. [21]. Inter-Andean valleys are highlighted: Cauca (CR) and Patía (PR) (Colombia) and Jubones (Ecuador) rivers valleys. Map prepared by María José Sevilla-Sánchez. Inset upper: Photos of adult and juvenile specimens of *B. asper* from Ecuador (CC BY-NC-ND 4.0 License, Omar Torres-Carvajal, https://bioweb.bio). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acetonitrile, and centrifuged at 13000 ×*g* for 10 min at room temperature to remove insoluble material. Venom proteins and peptides were separated by RP-HPLC on a C_{18} (250 × 4 mm, 5 μm particle size, 300 Å pore size, Teknokroma Europa) column using Agilent LC 1100 and 1260 High-Pressure Gradient Systems. Elution was performed at 1 mL/min by applying a gradient from 0.1% TFA in MilliQ® water (solution A) towards 0.1% TFA in acetonitrile (solution B), isocratically (5% B) for 5 min, followed by 5–25% B for 10 min, 25–45% B for 60 min, and 45–70% for 10 min. Protein/peptide detection was carried out at 215 nm with a reference wavelength of 400 nm.

The chromatographic profiles of the 55 *B. asper* venoms were classified into 14 groups (Table S1) according to the similarity of their binomial reverse-phase HPLC profile and geographic proximity. The nine groups of adult venoms from Ecuador were named *Bothrops asper* Extremo Norte P1 (BAENP1), *B. asper* Extremo Norte P2 (BAENP2), *B. asper* Norte P1 (BANP1), *B. asper* Norte P2 (BANP2), *B. asper* Norte-Centro (BANC), *B. asper* Centro (BAC), *B. asper* Sur P1 (BASUP1), *B. asper* Sur P2 (BASUP2), and *B. asper* Valle interandino (HEC2). The two groups of juvenile venoms from Ecuador were designated *B. asper* Norte P1, P2, Norte-Centro (PECju), and *B. asper* Valle interandino (HECju). *B. asper* Cauca (BACAUCA), *B. rhombeatus* (BRH), and *B. ayerbei* (BAY) represented the three groups of adult venoms from south-western Colombia (Table S1).

2.2.2. Decomplexation and proteomics analysis of the B. asper venom pools

Equal quantities of each venom classified in the same group were pooled and the peptidome and proteome of 1 mg of each venom pool were fractionated applying our bottom-up 'snake venomics' strategy [57] with minor modifications [58]. The key distinctive feature of our approach to protocols applied in other laboratories lies in taking advantage of the venom decomplexation steps (RP-HPLC followed by SDS-PAGE analysis of the isolated chromatographic fractions) for simultaneously quantify the relative abundance of the venom components. The conceptual and operational principles underlying our protocol, as well as the pros and cons regarding other venomics strategies (e.g. gel-only based or shotgun LC-MS/MS with label-free quantification workflows) have been the subject of a number of reviews [58–63]. Supplementary Fig. S1 (panel 1) displays a scheme of the venomics platform applied here to analyze venom variation across *B. asper* lineages from north-western South America.

The initial step of the protocol, venom decomplexation by RP-HPLC, was achieved as described above for individual venoms profiling and preparation of venom pools. Chromatographic fractions were collected manually, dried in a vacuum centrifuge (SPD SpeedVac®, ThermoSavant), redissolved in MilliQ® water and separated by SDS-PAGE under non-reducing and reducing (5% 2-mercaptoethanol at 100 °C for 5 min) conditions in 14% polyacrylamide gels and finally stained with Coomassie Brilliant Blue G-250. Protein bands were excised and subjected to in-gel reduction (10 mM dithiothreitol, 30 min at 65 °C), alkylation (50 mM iodoacetamide 2 h in the dark at room temperature), and overnight digestion with porcine pancreas trypsin (66 ng/μL in 25 mM ammonium bicarbonate containing 10% ACN, 0.25 μg/sample) in a ProGest™ digestor (Genomic Solutions), following manufacturer's instructions. Tryptic digests were dried in a vacuum centrifuge (SPD SpeedVac®, ThermoSavant), dissolved in 5% ACN containing 0.1% formic acid, and separated by nano-Acquity Ultra Performance LC® (UPLC®) using BEH130 C_{18} (100 μm × 100 mm, 1.7 μm particle size) column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 μL/min with a linear gradient of 0.1% formic acid in MilliQ® water (solution A) and 0.1% formic acid in ACN (solution B) with the following conditions: isocratically 1% B for 1 min, followed by 1–12% B for 1 min, 12–40% B for 15 min, 40–85% B for 2 min. Doubly- and triply-charged ions were selected for CID-MS/MS. The proteins were identified by interpretation of fragmentation spectra: a) manually (*de novo* sequencing), b) searched against the NCBIprot/SwissProt non-redundant databases using the on-line form of the MASCOT Server (version 2.6) at http://www.matrixscience.com (Release 234 of October 15th, 2019) or in an automated way, and c) processed in Waters Corporation's ProteinLynx Global SERVER 2013 version 2.5.2. (with Expression version 2.0) The search parameters were: Taxonomy: bony vertebrates; enzyme: trypsin (two-missed cleavage allowed); MS/MS mass tolerance was set to ± 0.6 Da; carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively. All matched MS/MS data were manually checked. Peptide sequences assigned by *de novo* MS/MS were matched to homologous proteins available in the NCBI non-redundant protein sequences database using the default parameters of the BLASTP program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [64].

Early eluting venom fractions containing low molecular mass peptides (e.g. bradykinin-potentiating-like peptides - BPP, snake venom Zn^{2+}-metalloproteinase inhibitors - SVMPi) were redissolved in aqueous 0.1% formic acid prior to nano-Acquity Ultra Performance LC® (UPLC®) fractionation and downstream de novo MS/MS peptide sequencing. For fragmentation, the electrospray ionization source was operated in positive ion mode and both singly- and multiply-charged ions were selected for CID-MS/MS at sample cone voltage of 28 V and source temperature of 100 °C. The LC–MS eluate was continuously scanned from 300 to 1990 *m/z* in 1 s and peptide ion MS/MS analysis was performed over the range m/z 50–2000 with scan time of 0.6 s. The tripeptide inhibitors of SVMPs, ZNW (pyroGlu/Gln-Asn-Trp) and ZBW (pyroGlu/Gln-Lys/Gln-Trp) are proteolytically released into PIII-SVMP-rich *Bothrops* venoms from larger precursors, e.g., *Bothrops jararaca* [Q6LEM5], *Bothrops jararacussu* [Q7T1M3], *Bothrops insularis* [P68515], which also encode bradykinin-potentiating peptides (BPPs). SVMPi are easily recognized by MS/MS owing to their characteristic $M + H^+$ at m/z 444.1 (ZBW) and 430.1 (ZNW) and their daugther ions at m/z 112.2 (b1 = Z), 205.2 (y1 = W), and 159.2 (immonium ion of W). Sequence-specific y1 and y2 ions at m/z 116.1 (P) and 213.1 (PP), commonly accompanied by y3 IPP at m/z 326.2, or VPP at m/z 311.2, were taken as a signature to tentatively identify parent BPP ions for CID-MS/MS sequencing [65,66]. Fragmentation spectra were interpreted manually to derive de novo amino acid sequences as described [66,67]. To illustrate this point, Supplementary Fig. S2 displays annotated examples of de novo sequence interpretation of representative CID-MS/MS fragmentation spectra of intact naturally occurring peptides in *Bothrops asper* lineage venoms.

2.2.3. Relative quantification of the venom proteomes

The 3-step hierarchical venom proteome quantification protocol developed in our laboratory [58,60–62], and illustrated in panel 2 of the Supplementary Fig. S1, was applied here to compile the relative composition of toxin families in the *B. asper* lineage venom proteomes. In the first step, the relative abundances of the reverse-phase chromatographic peaks obtained by reverse-phase HPLC fractionation of the whole venom were calculated as "% of total peptide bond concentration in the peak" by dividing the peak area by the total area of the chromatogram using ChemStation B.01.01.069 of the Agilent LC 1100 and 1260 High-Pressure Gradient Systems [58,66]. Recording the eluate at the absorbance wavelength of the peptide bond [190–230 nm], and applying the Lambert-Beer law ($A = \varepsilon cl$, where A = absorbance; ε is the molar absorption [extinction] coefficient, [M^{-1} cm^{-1}]; c = concentration [M]; and l = light path length [cm], these percentages correspond to the "% of total peptide bond concentration in the peak". For chromatographic peaks containing single components (as judged by SDS-PAGE and/or MS1 profiling), this figure is a good estimate of the % by weight (g/100 g) of the pure venom component [68]. When more than one venom protein was present in a reverse-phase fraction, their proportions (% of total protein bands area) were estimated in the second quantification step by densitometry of Coomassie-stained SDS polyacrylamide gels using Image Studio Lite®, version 5.2 (LI-COR Biosciences) software. Conversely, the relative abundances of toxins

from different protein classes comigrated in the same SDS-PAGE band were estimated in the third level of quantification based on the relative ion intensities of the three (or an equal number) most abundant peptide ions associated with each protein by MS/MS analysis. The relative abundances of the protein families present in the venom were calculated as the ratio of the sum of the percentages of the individual proteins from the same family to the total area of venom protein peaks in the reverse phase chromatogram [58].

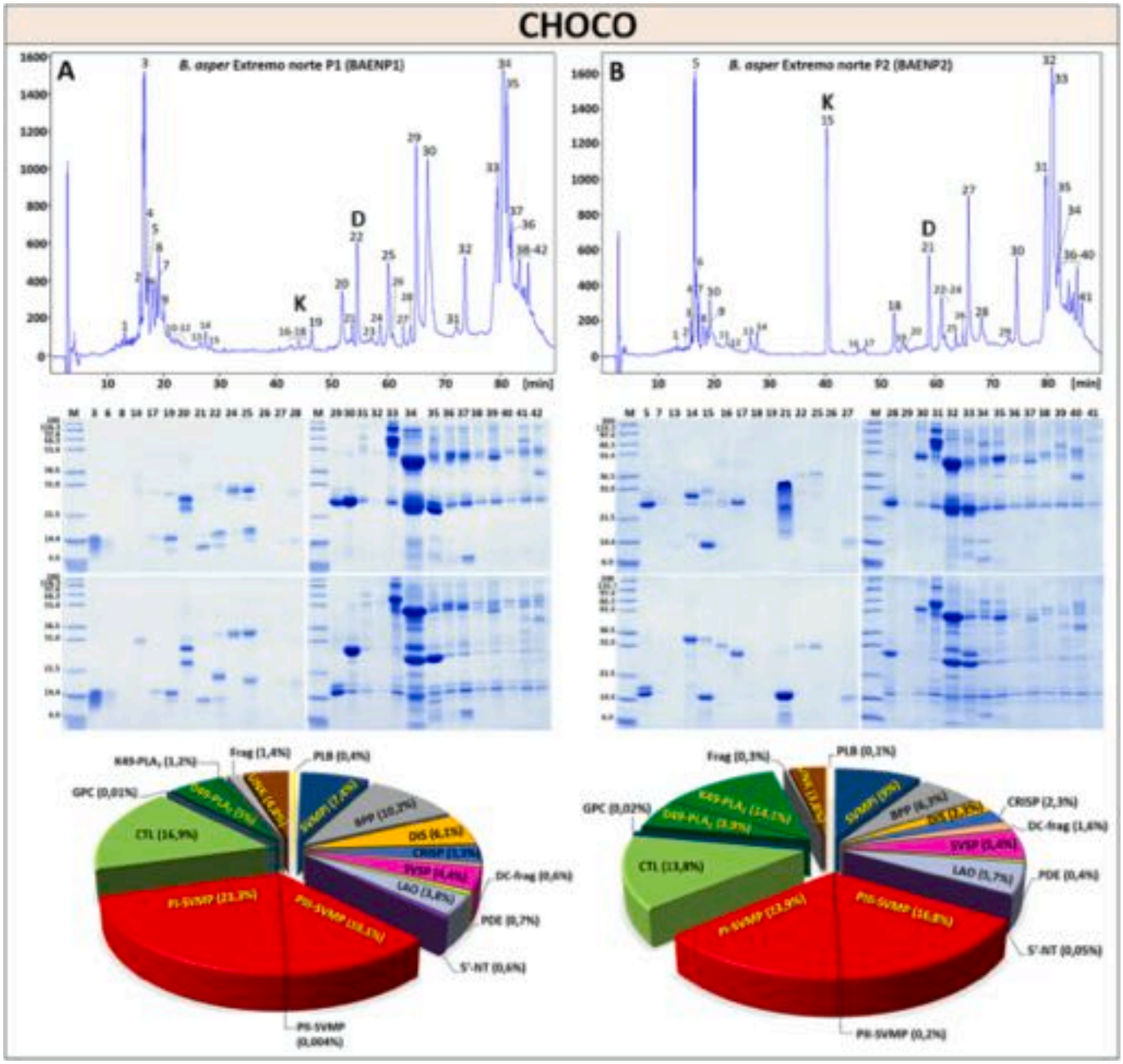


Fig. 2. Venomics analyses of the toxin arsenal of *B. asper* groups from Ecuador and south-western Colombia. Panels A-L illustrate the decomplexation, by reverse-phase chromatography (upper graphic) and SDS-PAGE gels (run under non-reduced (upper gels) and reduced (lower gels) conditions) of the RP-HPLC peaks (middle display), of the following Ecuadorian *B. asper* venoms groups from adult or juvenile (ju) specimens collected in the localities listed in Supplementary Table S1 and highlighted in Fig. 1: (A) Ecuador Extremo Norte P1 (BAENP1); (B) Extremo Norte P2 (BAENP2); (C) Norte P1 (BANP1); (D) Norte P2 (BANP2); (E) Norte-Centro (BANC); (F) Norte P1, P2, Norte-Centro (PECju); (G) Centro (BAC); (H) Sur P1 (BASUP1); (I) Sur P2 (BASUP2); (J) Valle interandino (HEC2); and (K) Valle interandino (HECju). (L) venom group from *B. rhombeatus* lineage (BRH) from the Cauca river valley, Department of Cauca, south-western Colombia. These venom groups correspond to the *B. asper* lineages described by Salazar-Valenzuela et al. [21]: panels A and B, CHOCO; panels C through I, PEC; panels J and K, HEC2; panel L, BRH. Chromatographic peaks labeled K and D contained, respectively, K49- and D49-PLA_2s whose ESI-MS masses are listed in Table 2. The lower graphics of each panel display pie charts showing the relative abundance (in percentage of the total venom proteins) of the different toxin classes found in the venom's groups. Acronyms, SVMP, snake venom Zn^{2+}-dependent metalloproteinase of PIII, PII or PI class; CTL, C-type lectin-like; SVSP, snake venom serine proteinase; K49- and D49-PLA_2, phospholipases A_2 of K49 and D49 subfamilies; BPP, bradykinin-potentiating-like peptides; SVMPi, endogenous tripeptide inhibitors of SVMPs; DIS, disintegrin; LAO, L-amino acid oxidase; CRISP, cysteine-rich secretory protein; 5'-NT, 5′-nucleotidase; PDE, phosphodiesterase; PLB, phospholipase B; DC-frag, Disintegrin/Cysteine-rich fragment of PIII-SVMP; GPC, glutaminyl-peptide cyclotransferase; HyA, hyaluronidase; NGF, nerve growth factor; DIPEP2, dipeptidase-2 like; VEGF, vascular endothelial grow factor; Frag, protein fragments; SA, serum albumin; UNK, unknown.

2.2.4. Determination of the intact molecular mass of RP-HPLC-isolated venom proteins

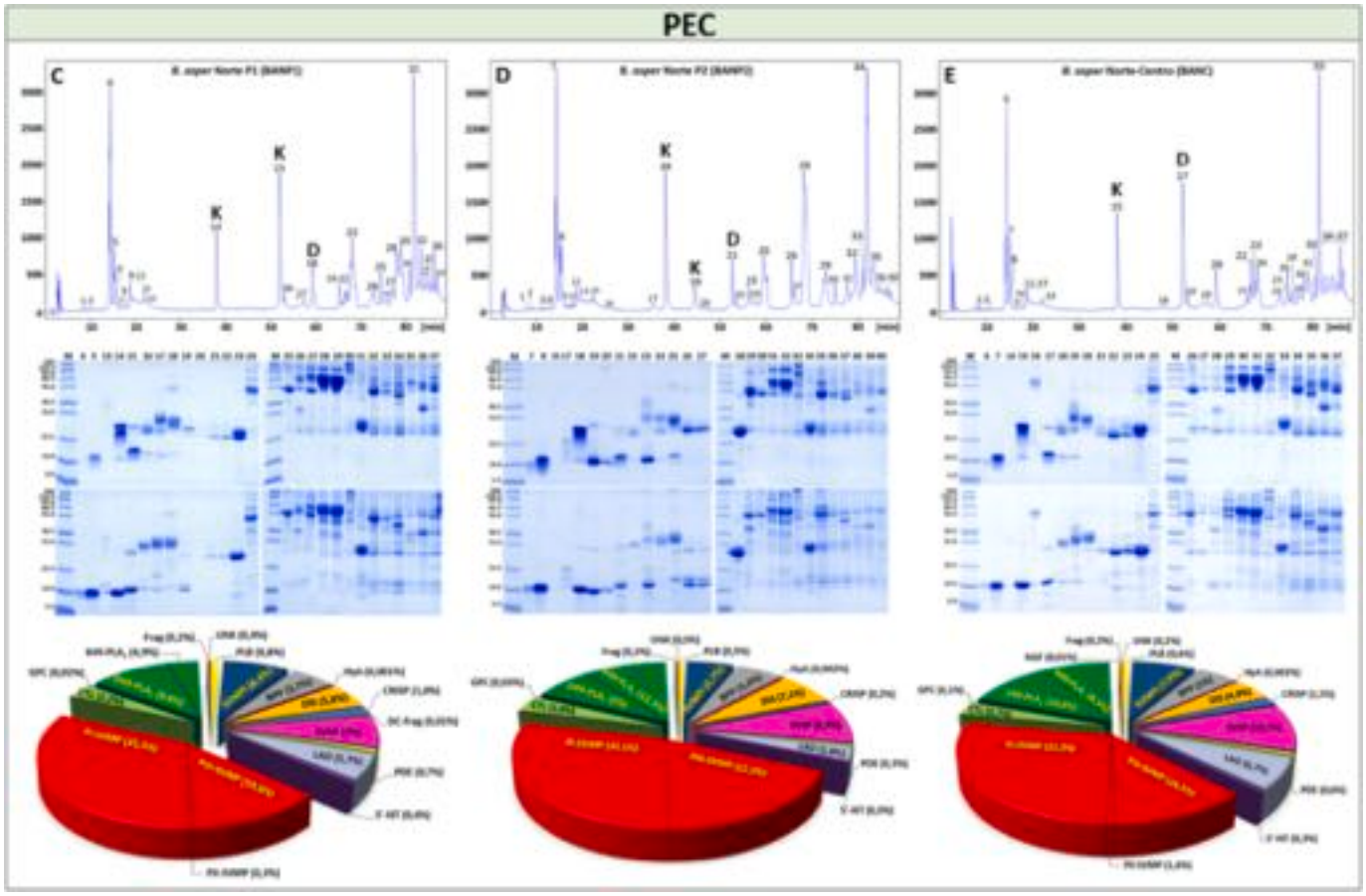
Molecular masses of venoms proteins were estimated by non-reduced and reduced SDS-PAGE (on 14% polyacrylamide gels) or determined by electrospray ionization (ESI) mass spectrometry. For SDS-PAGE analysis sample aliquots were mixed with ¼ volume of 4× sample buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 30% glycerol, 0.02% bromophenol blue, with or without 10% 2-mercaptoethanol) and heated at 100 °C for 5 min. Gels were stained with Coomassie Brilliant Blue G-250. For ESI-MS mass profiling, the proteins eluted in the different RP-HPLC fractions were separated by nano-Acquity UltraPerformance LC® (UPLC®) using BEH130 C_{18} (100 μm × 100 mm, 1.7 μm particle size) column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 μL/min and the column was developed with a linear gradient of 0.1% formic acid in water (solution A) and 0.1% formic acid in ACN (solution B), isocratically 1% B for 1 min, followed by 1–12% B for 1 min, 12–40% B for 15 min, 40–85% B for 2 min. Monoisotopic and isotope-averaged molecular masses were calculated by manually deconvolution of the isotope-resolved multiply-charged MS1 mass spectra [58,69].

2.2.5. Principal component analysis

Principal component analyses (PCA) were conducted using the relative abundances of individual components identified using the "venomics strategy" described above to identify correlated combinations of venom components that successively maximize between venom variance. In addition, the contribution of the variables to the variation among *B. asper* groups was shown using bar charts. These analyses were performed with Programming Language R (Version 1.2.1335 © 2009–2019 RStudio, Inc.) with the extension Graphic Package rgl (version 0.100.30) available from https://www.R-Project.org.

3. Results and discussion

To identify patterns of phenotypic variability across Ecuadorian and south-western Colombian *B. asper* toxin arsenals, single samples of each of the 12 venom groups highlighted in Fig. 1 were decomplexed by RP-HPLC/SDS-PAGE (Fig. 2) and all the 150–210 Coomassie blue-stained protein bands, excised mainly from the reduced SDS-PAGE gels of each venom sample, were processed and submitted to bottom-up venomics characterization. Samples included venoms from 36 adults and 9 juveniles collected in different localities of eleven Ecuadorian provinces, and four venoms of *B. rhombeatus* lineage from the south-western Colombian Cauca river (CR) valley (Table S1). These venom groups represent 3 (CHOCO, PEC, and HEC2) *B. asper* lineages (Fig. 1) and the close phylogenetically related *B. rhombeatus* lineage (BRH) recently identified by Salazar-Valenzuela et al. [21] in inter-Andean valleys of south-western Colombia and Ecuador. The success identification (ID) rate was > 95% of which the vast majority (90%) corresponded to peptide spectrum matches (PSM) of tryptic peptides and the remaining 10% were de novo interpreted sequences of intact naturally occurring peptides found in the early eluting fractions from RP-HPLC decomplexation of the venoms, e.g., SVMPi, BPPs and a few toxin degradation fragments (Fig. S2 and Supplementary Tables S2–S13). PSM IDs were based on at least 2, but often much larger number of non-overlapping peptides matching the same or a taxonomically homolog protein class. However, single unambiguous manually validated peptide sequences uniquely hitting an entry in the "serpentes" taxonomically restricted NCBI database were also regarded as toxin-family-level confident identifications. The proteomic data gathered in this study (summarized in Fig. 2 and presented in detail in the Supplementary Tables S2–S13) are discussed below in the context of functional and structural venomics data reported for other phylogeographic lineages of *B. asper* in northern



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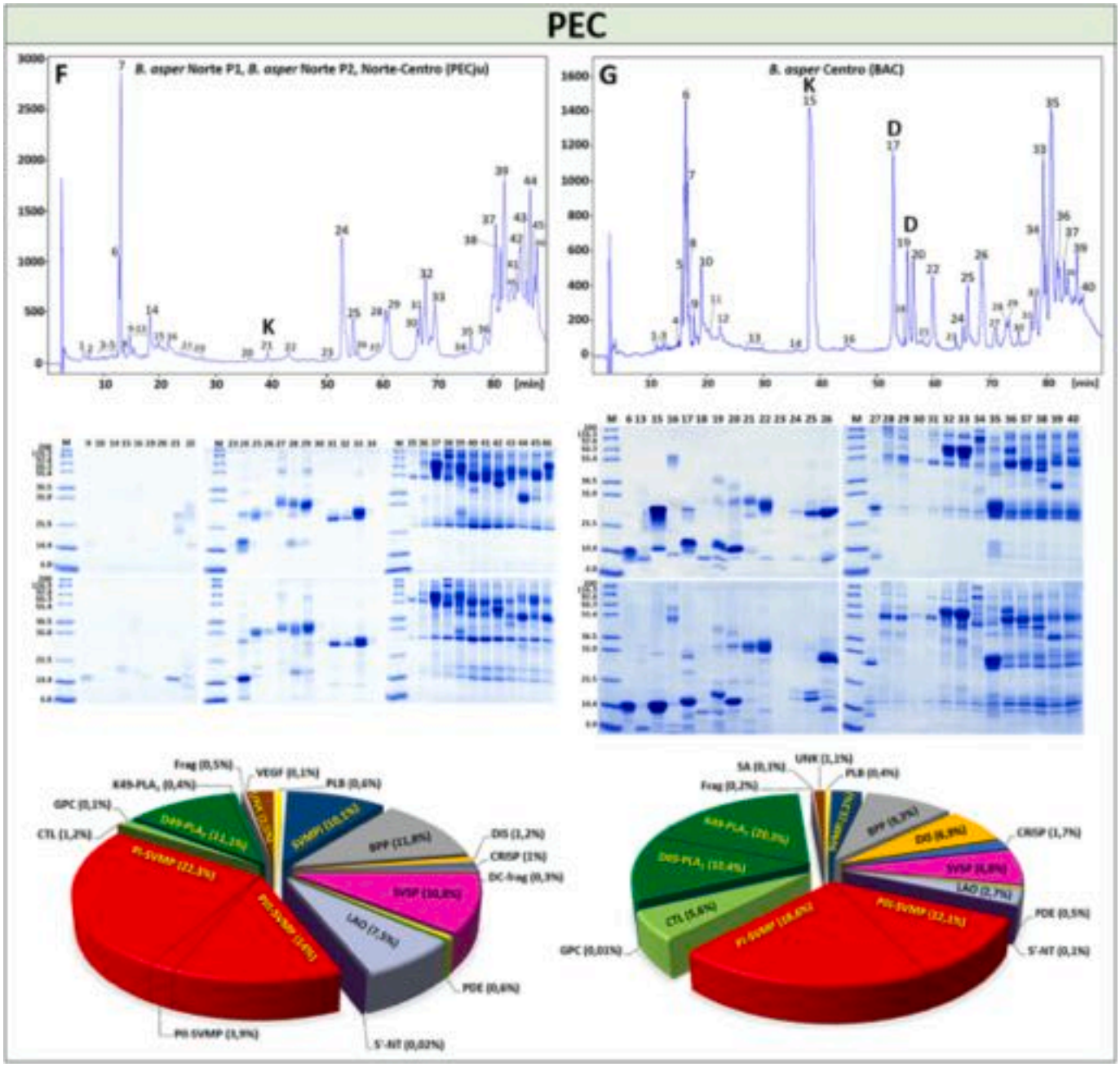

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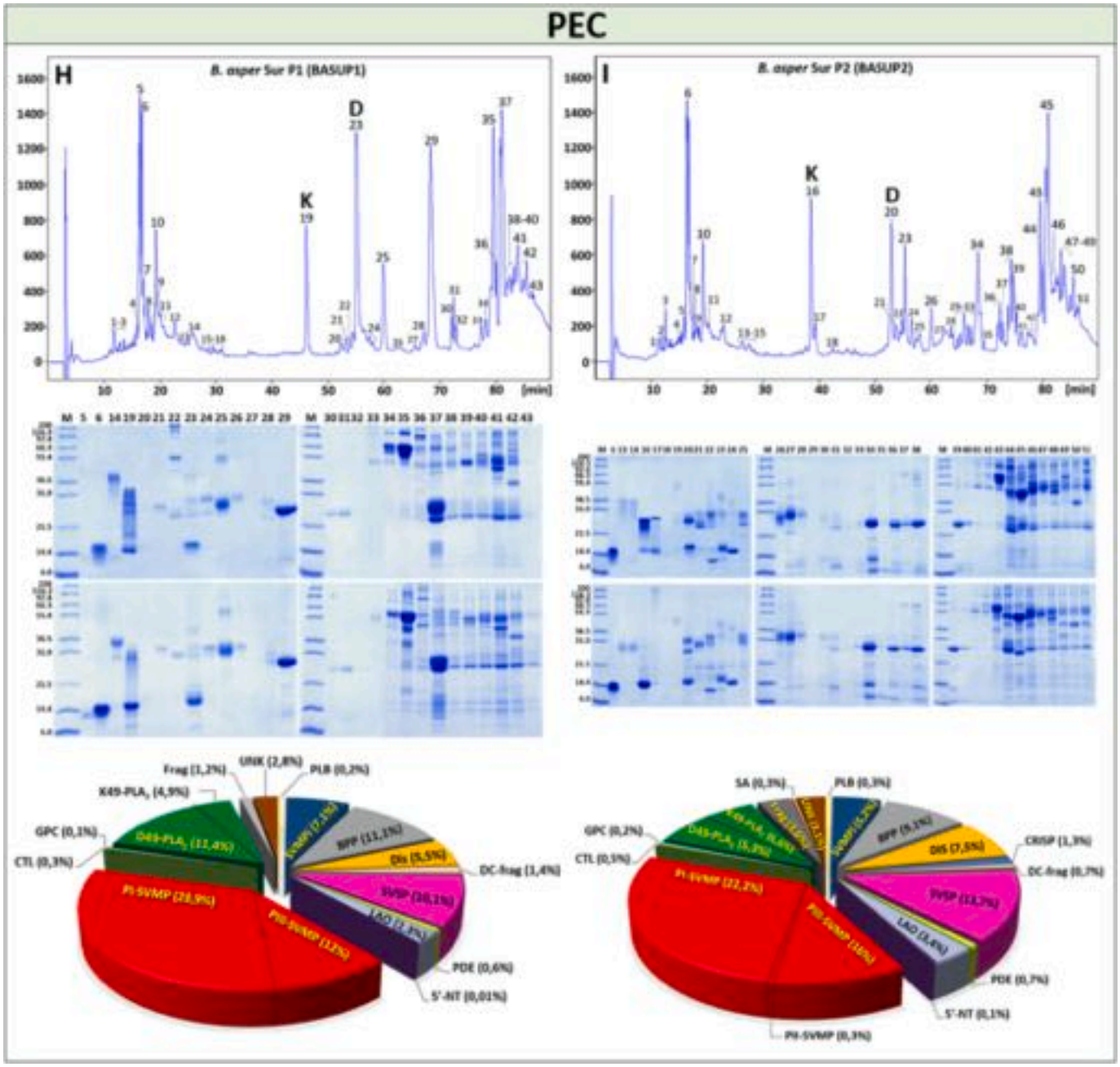
South America and Mesoamerica.

3.1. The divergent venom proteomes of Colombian B. asper and B. rhombeatus, and Ecuadorian lineages

Comparison of the RP-HPLC and SDS-PAGE decomplexation profiles of the venoms of the different *B. asper* age-groups between themselves (Fig. 2, panels A-E and G-J, adult snakes; F and K, juvenile specimens) showed similar levels of complexity in the number, identity, and overall relative abundance of the toxin families present in the venoms (Table 1). However, there were also notable differences particularly among the adult snake venoms (Table 1). More specifically, the proteins eluted in the 37–51 manually collected chromatographic fractions from the 11 venom groups (Fig. 2, panels A-K) of the three *B. asper* lineages (Fig. 1) were assigned to 13–18 protein classes of 15 toxin gene families. These included the following toxin proteins: snake venom metalloprotease (SVMP) and their proteolytic products (Disintegrin/Cysteine-rich fragments, DC-frag; and disintegrin, DIS); C-type Lectin-like (CTL); snake venom serine proteinase (SVSP); phospholipase A_2 (PLA_2); bradykinin-potentiating peptide-like (BPPs) and tripeptide inhibitors of SVMPs (SVMPi) derived from the same precursor; L-amino acid oxidase (LAO); cysteine-rich secretory protein (CRISP); 5′-nucleotidase (5'-NT); phosphodiesterase (PDE); phospholipase B (PDB); glutaminyl-peptide cyclotransferase (GPC); hyaluronidase (HyA); snake venom nerve growth factor (NGF); dipeptidase-2 like (DIPEP2); and snake venom vascular endothelial growth factor (VEGF) (Table 1). With the exception of CRISP which is absent in the BASUP1 venom group (Fig. 2, panel I; Supplementary Table S9), all the toxin families with relative abundance ≥1% of the total venom proteome were present in all the other venom groups, although the relative abundance of protein families and of toxin classes within the same family varies among the venom groups (Fig. 2; Table 1; Supplementary Tables 2–12). Finally, a



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small number of minor protein families were only detected in certain venoms, i.e. HyA (BANP1, BANP2 and BANC) and VEGF (PECju) (Table 1).

PI-SVMPs (20–35%) represent the most abundant toxin class in the majority of Ecuadorian samples with the exception being that BAENP2 and BAC, were PIII-SVMPs (16.8%) and K49-PLA_2 (20.3%) rich (Table 1). Different combinations of 6 classes of toxins, each accounting for 9–17% of the venom proteome, are the second most represented toxin families: [BAENP1: CTL, 16.9%, PIII-SVMP and BPP, 10.1–10.2%]; [BAENP2: CTL, K49-PLA_2, PI-SVMP, 13.8–14.1%]; [BANP1: PIII-SVMP, 19.8%, and D49-PLA_2, 9.4%]; [BANP2: K49-PLA_2, PIII-SVMP, and SVSP, 8.9–12.3%]; [BANC: PIII-SVMP, SVSP, D49-PLA_2 and K49-PLA_2, BPP, 8.3–16.5%]; [BAC: PI-SVMP, PIII-SVMP, D49-PLA_2, and BPP, 9.3–18.6%]; [BASUP1 and PECju: PIII-SVMP, SVSP, D49-PLA_2, and BPP, 10.1–14%]; [BASUP2, BPP, 9.1%; SVSP and PIII-SVMP, 13.7–16%); [HEC2: PIII-SVMP, SVSP, D49-PLA_2, and BPP, 8.2–15.7%] and [HECju: PIII-SVMP, SVSP, and BPP, 10.6–12.9%] (Table 1). With the exception of DIS, which was not found in the HECju proteome, this toxin class and LAO exhibit wide medium abundance concentration ranges, i.e. 1.1–7.5%, among all the Ecuadorian *B. asper* venoms groups (Table 1). On the other hand, CRISP was identified in 11 of the 12 venom groups in average relative quantities of 1.7 ± 1.5% of the total venom arsenals, and the minor 5'-NT, PDE, and PLB venom components exhibited the largest variation among the Ecuadorian *B. asper* venom groups (Table 1).

The venom proteome of the *B. rhombeatus* lineage (Fig. 2, panel L) from the Colombian Cauca river (CR) valley (Fig. 1), showed high abundances (10.1–25.5%) of PI-SVMP, PIII-SVMP, D49- and K49-PLA_2 toxin proteins (Table 1)], which matches the overall toxin-class distribution of the Ecuadorian Norte-Centro (BANC) venom group (Fig. 2, panel E) from the *B. asper* Pacific Ecuador (PEC) lineage [21,24] (Fig. 1). Strikingly, the venom of *B. ayerbei* (BAY lineage), which

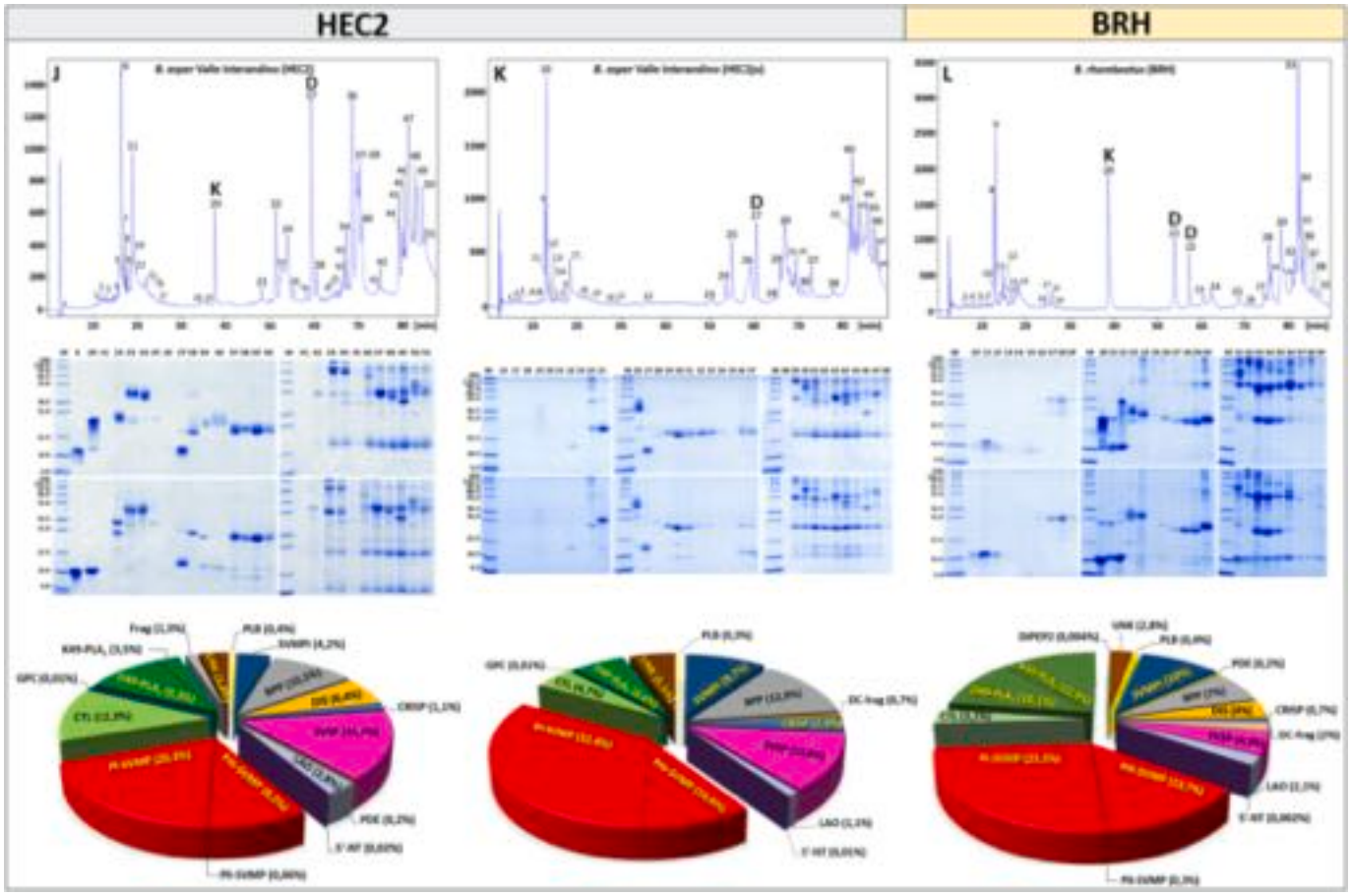

Fig. 2. (*continued*)

together with *B. rhombeatus* lineage forms a sister clade of the Darien Panamá and Choco (CHOCO) *B. asper* lineage [21,24], is characterized by the toxin combination [PI-SVMP (26.6%) > PIII-SVMP (20%), CTL (10.1%) and SVSP (9.3%)] [34], which strongly departs from the PLA_2-rich venom phenotypes of Ecuadorian *B. asper* lineages (Table 1) and Colombian *B. asper* from the Pacific coast of the Department of Cauca [41]. Fig. 3 highlights RP-HPLC profiles and the corresponding compositional pie charts of the venom of *B. rhombeatus* lineage from the Cauca River Valley in the Popayán municipality (middle chromatogram, Fig. 3). These patterns contrast with the composition of *B. asper* (sensu stricto) from the Pacific slopes of the mountain range in the El Tambo municipality (labeled BACAUCA in Fig. 1) (upper chromatogram), and *B. ayerbei* (labeled BAY in Fig. 1) from the Patía River Valley in the Rosas municipality [41].

Intraspecific geographic and ontogenetic variation in the relative abundances of PI- versus PIII-SVMP, D49- versus K49-PLA_2, and SVSP within and between the venoms of *B. asper* populations from the Caribbean and Pacific regions of Costa Rica has also been previously reported [40,42]. Likewise, chromatographic comparison of *B. asper* venoms from southern México versus venoms from conspecific specimens collected in the Pacific and Atlantic sides of Costa Rican show variability in their PLA_2s and SVMPs subproteomes which may reflect their different toxicities: Mexican venoms show higher lethal and defibrinogenating activities while Costa Rican venoms show higher hemorrhagic and in vitro coagulant activities [46]. On the other hand, unlike the venoms of Costa Rican snakes, *B. asper* venoms from four regions across Panamá showed only slight variation in their chromatographic and electrophoretic profiles [47]. These patterns indicate that diversification of *B. asper* venom is regionalized and must have resulted from spatial variation in mechanism such as local adaptation, geographic isolation, genetic drift, and purifying selection. The Caribbean and Pacific populations of Costa Rican *B. asper* belong to two divergent lineages that originated 3.20 [CI95%: 2.32–4.31] million years ago (Mya) [23] coinciding with the uplift of the Cordillera de Talamanca, a mountain range that extends 400 km along the Central American Continental Divide, dated at approximately 3–5.4 Mya [70]. This major geologic event imposed a strong barrier to gene exchange between Pacific and Caribbean populations of terrestrial taxa [71,72] and allowed the allopatric accumulation of phenotypic differentiation since the mid-Pliocene underlying the cladistic structure and venom variation between and within the Pacific and Caribbean populations *B. asper* [23].

3.2. Ontogenic variation in the venom of *B. asper* from Ecuadorian lineages

The availability of two groups of juveniles (PECju [BANP1, BANP2, and BANC] and HEC2ju) and adult specimens from the same locations (Fig. 1) provided us with the opportunity to investigate the possible occurrence of ontogenetic changes in the venom composition of these *B. asper* lineages. Both venom pools are characterized by a drastically reduced (PECju, Fig. 2F) or complete lack (HECju, Fig. 2K) of K49-PLA_2 molecules. In addition, the HECju venom pool showed higher relative abundance of PI-SVMPs (32.4%) and lower content of CTLs (4.7%) and lack of disintegrins than the venom of adult snakes (20.3% PI-SVMP, 12.3% CTL, and 6.4% DIS), while the relative abundances of PIII-SVMPs are similar (10.6 vs 8.2%) in the venom pooled from juvenile and adult snakes (Table 1).

In agreement with our results, a previous study on the expression pattern of myotoxin (K49-PLA_2) isoforms in the venoms of newborn (less than 10 days of age) and adult specimens from the Atlantic and Pacific sides of Costa Rica showed individual differences of isoform expression in adults and the completely lacked myotoxin bands in the

Table 1

Venom protein and peptides	Ecuador											Colombia			Costa Rica[2]		
	CHOCO[1]					PEC[1]				HEC2[3]							
	BAENP1*	BAENP2*	BANP1*	BANP2*	BANC*	PECju*	BAC*	BASUP1*	BASUP2*	HEC2*	HECju*	BRH*	BAY*	PICA	PICAne	CICA	CICAne
SVMP	33.4	30.9	45.6	47.4	40.4	40.2	30.7	40.9	38.5	28.5	42.9	39.7	46.6	44.0	65.5	41.0	63.0
PIII-SVMP	10.1	**16.8**	19.8	12.3	16.5	14	12.1	12.0	16	8.2	10.6	13.7	20.0	13.3	58.2	8.8	61.0
PII-SVMP	0.004	0.2	0.3	-	1.6	3.9	-	-	0.3	0.06	-	0.3	-	-	-	-	-
PI-SVMP	**23.3**	13.9	**25.5**	**35.1**	**22.3**	**22.3**	18.6	**28.9**	**22.2**	**20.3**	**32.4**	**25.5**	**26.6**	30.5	7.1	32.2	2.9
CTL	16.9	13.8	3.2	3.4	2.7	1.2	5.6	0.3	0.5	12.3	4.7	3.1	10.1	0.5	0.2	0.5	<0.1
SVSP	4.4	5.4	7.0	8.9	10.1	10.8	6.8	10.1	13.7	15.7	12.6	4.9	9.3	4.4	2.6	18.2	6.7
PLA_2	6.2	18.0	14.3	17.3	18.9	11.5	30.7	16.2	11.9	12.8	6.6	23.0	0.7	45.1	27.7	28.8	23.7
D49-PLA_2	5.0	3.9	9.4	5.0	10.6	11.1	10.4	11.4	5.3	9.3	6.6	10.1	-	9.1	23.5	10.0	21.6
K49-PLA_2	1.2	14.1	4.9	12.3	8.3	0.4	**20.3**	4.9	6.6	3.5	-	12.9	-	36.0	4.2	18.8	2.1
BPP	10.2	6.3	5.7	5.6	5.0	11.8	9.3	11.1	9.1	10.5	12.9	7.0	8.3	-	-	-	-
SVMPI	7.4	9.0	8.4	5.7	7.9	10.1	3.2	7.1	5.2	4.2	9.7	10.0	8.7	-	-	-	-
DIS	6.1	2.3	5.8	7.1	4.9	1.2	6.9	5.5	7.5	6.4	-	4.0	2.3	1.4	0.6	2.1	1.6
LAO	3.8	5.7	5.7	2.8	6.7	7.5	2.7	2.3	3.4	2.8	1.1	2.1	3.3	4.6	3.4	9.2	2.5
CRISP	3.3	2.3	1.8	0.2	1.5	1.0	1.7	-	1.3	1.1	2.9	0.7	1.1	0.1	<0.1	0.1	2.5
5'-NT	0.6	0.05	0.4	0.3	0.3	0.02	0.1	0.007	0.1	0.02	0.01	0.002	-	-	-	-	-
PDE	0.7	0.4	0.7	0.3	0.6	0.6	0.5	0.6	0.7	0.2	-	0.2	0.7	-	-	-	-
PLB	0.4	0.1	0.8	0.5	0.6	0.6	0.4	0.2	0.3	0.4	0.3	0.4	-	-	-	-	-
DC-frag	0.6	1.6	0.007	-	-	0.3	-	1.4	0.7	-	0.7	2.0	-	<0.1		<0.1	
GPC	0.01	0.02	0.02	0.03	0.1	0.1	0.01	0.1	0.2	0.01	0.01	-	-	-	-	-	-
HyA	-	-	0.001	0.002	0.002	-	-	-	-	-	-	-	-	-	-	-	-
NGF	-	-	-	-	0.01	-	-	-	-	-	-		0.1	-	-	-	-
DIPEP2	-	-	-	-	-	-	-	-	-	-	-	0.004	-	-	-	-	-
VEGF	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-

Table 1. Relative abundance (in percentage of the total venom proteome) of the components identified in the *B. asper* venoms from different lineages of Ecuador, Colombia and Costa Rica.The major venom component in each venom is in bold face; the second most abundant toxin classes are highlighted in gray background.
[1]Acronyms of the *B. asper* lineages identified by Salazar-Valenzuela et al. [21]. *Acronyms of the *B. asper* groups investigated in this work. [2]Data from Alape et al. [40]: [3]Data from Mora-Obando et al. [41]. ju, and ne, venom from juvenile and neonate specimens, respectively.

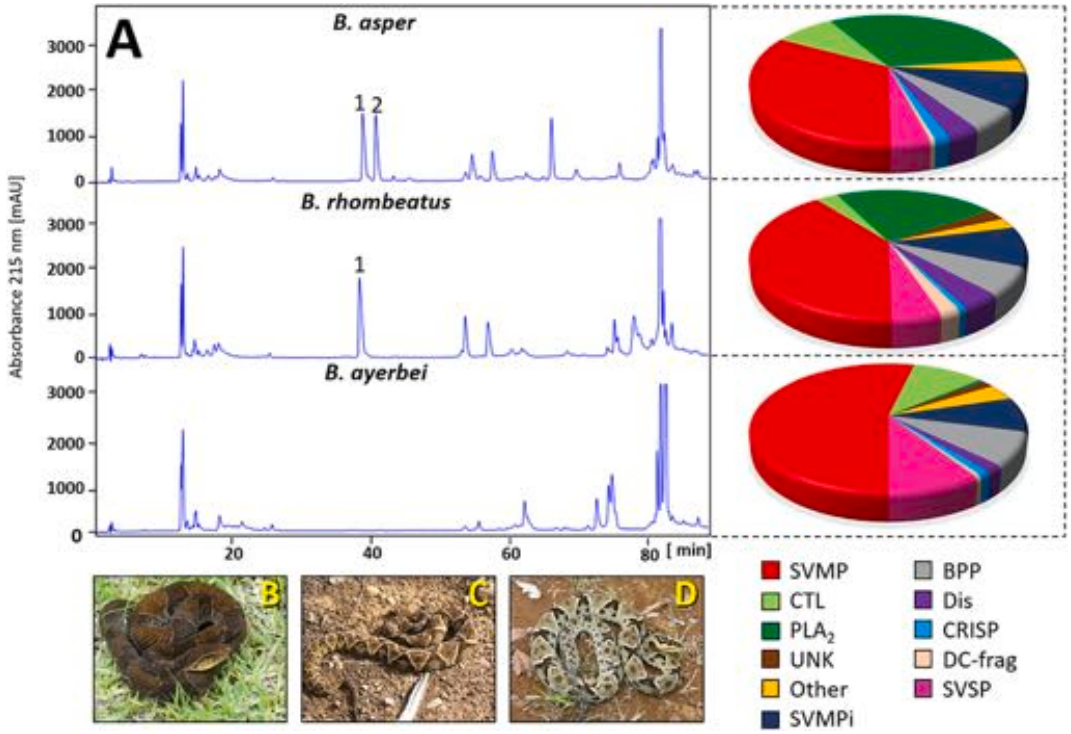

Fig. 3. Comparison of the chromatographic venom profiles of *B. asper* lineages from south-western Colombia, Department of Cauca. Panel A compares the RP-HPLC profiles and the corresponding compositional pie charts of the venom of *B. rhombeatus* lineage from the Cauca River Valley in the Popayán municipality (middle chromatogram) [this work], with those of *B. asper* (sensu stricto) from the Pacific slope of the occidental mountain range in the El Tambo municipality (upper chromatogram), and *B. ayerbei* captured in the Patía River Valley in the Rosas municipality, reported by Mora-Obando et al. [41]. The molecular masses of the PLA_2 molecules recovered from the chromatographic fractionations of the *B. asper* and *B. rhombeatus* lineage venom proteomes (numbered) were measured by ESI-MS1 and their deconvoluted values are reported in Table 2. Photos of *B. asper* (B), *B. rhombeatus* (C), and *B. ayerbei* (D), Santiago Ayerbe-González©, Centro de Investigaciones Biomédicas, Universidad del Cauca, Popayán, Colombia.

newborns [73]. These results clearly indicate the occurrence of an ontogenetic regulation in the expression of K49-PLA_2s in *B. asper*. Shifts from a PIII-SVMP-rich to a PI-SVMP-rich venom and relative abundance of K49-PLA_2 > D49-PLA_2 in venom from adults vs. neonates, have been described in the venoms of Costa Rican *B. asper* specimens from both the Caribbean and the Pacific lineages [40,74]. The finding that, compared to adults, newborn specimens from both sides of Costa Rica have more proteolytic, hemorrhagic, edema-forming and lethal venoms, whereas venom from adult specimens are more hemolytic and myotoxic [75,76], supports the view that the age-dependent transition in the composition of the venom may be functionally relevant. In accordance with this evidence, optimal foraging theory predicts that predators should either maximize energy gains or minimize time spent obtaining a fixed amount of energy [77,78], and ontogenetic shift in diet from ectothermic prey (arthropods, lizards, and amphibians) to endothermic prey (mammals) has been associated with a shift in snake venom toxicity, with juvenile venom being more toxic to ectotherms and adult venom more toxic towards mammals [4,79–81].

3.3. Principal component analysis

Principal component analysis (PCA), using the percentages of the venom components as a variable, was applied to explain determinants of compositional variation among the *B. asper* venom groups displayed in (Table 1). PC1, PC2 and, PC3 loadings explain, respectively, 48.56%, 12.13% and 11.75% of the variability among the venoms of Ecuadorian CHOCO, PEC and HEC2 *B. asper* lineages (Fig. 4A). PC1 mainly differentiated these venoms groups by their relative content of PI- and PIII-SVMP. PC2 further discriminated the venom groups by their relative abundance of CTL. PC3 loadings separated venoms per their relative abundance of K49-PLA_2. The same multivariate analysis but including the venom composition of the Colombian *B. ayerbei* (BAY) and *B. rhombeatus* (BRH) lineages, segregated BAY from all the other venom groups and placed BRH within the Ecuadorian Pacific lineage (PEC) (Fig. 4B). The relative abundances of SVMPs, PLA_2s, and CTL proteins represent the major contributors to the PC1, PC2, and PC3 scores that summarize venom variability. When the venom proteomes of *B. asper* from the Caribbean (CICA) and Pacific (PICA) sides of Costa Rica were included in the same multivariate analysis, the Costa Rican venoms, which belong to the México and Nuclear Central America (MNCA) clade [23], were clearly differentiated from all other venoms (Fig. 4C). The toxin classes contributing to the loadings were SVMP (PC1), PLA_2 (PC2) and SVSP (PC3). PCA of the ontogenetic trends in *B. asper* PEC and HEC2 lineages showed that the major contributors of the age-dependent venom compositional shift is essentially driven by the relative abundance of PI-SVMP (PC1 explaining 53.67% of the variability), K49-PLA_2 (PC2, 14.88%) and CTL, PIII-SVMP and SVSP (PC3, 10.87%) (Fig. 4D). A similar conceptual scenario has been described in the Mojave rattlesnake (*Crotalus scutulatus*) (82). In this species the transcriptomic expression of members of four toxin families, PLA_2, SVMP, CTL, and myotoxin (MYO), appeared to be primarily responsible for the diversity within and between the neurotoxic (Type A) and hemorrhagic (Type B) venom phenotypes [82]. These examples conform to Barua and Mikheyev's "many options/few solutions" pattern of venom evolution [83] that, although there is no evidence of phylogenetic constraints on the number of possible venom combinations, a small number of molecular strategies centered on a few major toxin families have evolved via ecological filtering. An extreme situation is the occurrence of toxin family dichotomies in venom composition, such as 3FTx/PLA_2 in *Micrurus*, 3FTx/DenTx in *Dendroaspis* [34,84–86]. Intrageneric and intraspecific venom dichotomies may be maintained by the action of balancing selection, an evolutionary scenario where local adaptation

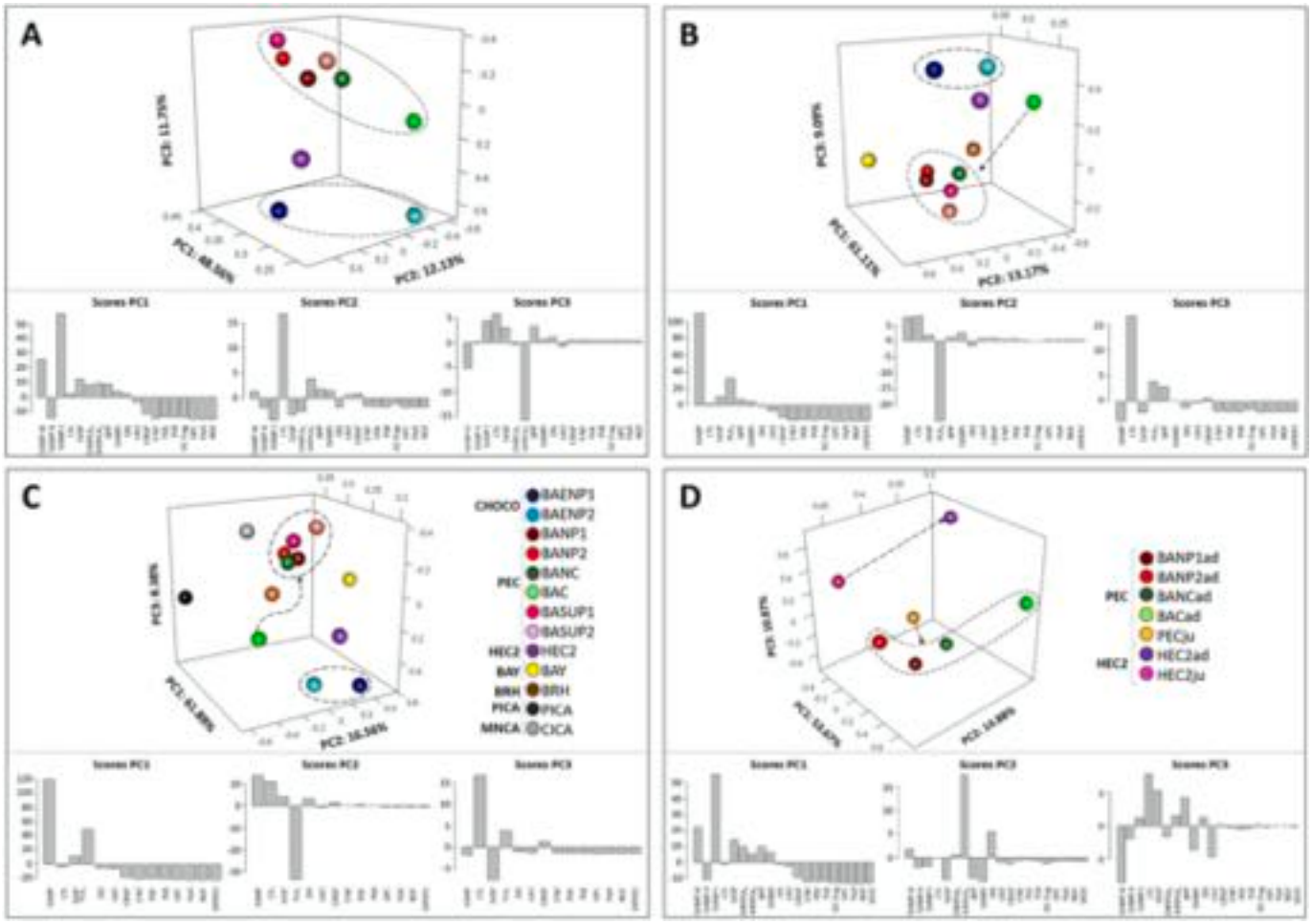

Fig. 4. Principal component analysis (PCA). Panel A, multivariate analysis of the variability among the venom groups of adult specimens of the Ecuadorian CHOCO, PEC and HEC2 *B. asper* lineages [24], showing the percentages of the variability explained by PC1, PC2, PC3 scores and the contributions of the different venom components to each score. Panel B, PCA of the same set of venoms analyzed in panel A plus the venoms from south-western Colombian *B. rhombeatus* lineage (BRH) and *B. ayerbei* (BAY) lineages. Panel C, multivariate analysis of the variability among all the venom groups of adult specimens listed in Table 1, including those included in the analyses displayed in panels A and B and the Costa Rican Caribbean (CICA) and Pacific (PICA) Isthmian Central America *B. asper* lineages from the México and Nuclear Central America (MNCA) clade [23]. In panels B and C, the BAC group is connected to its respective lineage (PEC) by means of an arrow. Panel D, PCA of from pooled age-group (ad, adult; ju, juvenile) venoms from the *B. asper* lineages HEC2, and PEC. The PEC pooled comprise venoms from *B. asper* lineages BANP1, BANP2, and BANC. Venom groups are represented by color-coded circles and identified by their acronyms described in Figs. 1 and 2.

depends on the frequencies of the two traits, with the hetero-phenotype having an advantage over the homo-phenotypes [87,88]. *Bothrops asper* venom tetrachotomy characterized by combinations of the relative abundance of toxins belonging to four protein families, SVMPs, PLA_2, CTL and SVSP (Fig. 4), could represent a more complex version of the same evolutionary mechanism.

3.4. Comparison of the chromatographic profiles and ESI-MS PLA_2 mass profiling of Mesoamerican and northern South American B. asper venoms

According to Bayesian estimations of divergence time the origin of the *B. asper-B. atrox* group occurred approximately 3.91 Mya (CI95% = 2.53–5.26 Mya) when this clade diverged from the Antillean lanceheads (*B. caribbaeus* and *B. lanceolatus*) [23]. The ancestor of *B. asper* split from the *B. atrox-B. asper* species complex during the mid-Pliocene, 3.34 ($CI_{95\%}$ = 2.44–4.48) Mya, and soon after (3.20 Mya (95% CI = 2.32–4.31 Mya) dispersal events northward through the Pacific coast of Isthmian Central America into Caribbean Mesoamerica and southward along the foothills of the Pacific side of northern South America into northwestern Perú molded the present-day biogeographic distribution of *B. asper* lineages [23–25]. These events occurred coincidentally with the rapid final uplift of the Eastern Cordillera of the Andes, at rates of 0.6–3 mm/year [89] and the closure of Isthmian Central America [90] during the mid-Pliocene, 3.34 (95% CI = 2.44–4.48) Mya. The allopatric distribution of *B. atrox* and *B. asper* lineages on each side of the Eastern Andes, and of the Costa Rican Pacific and Caribbean *B. asper* populations at both versants of the Cordillera Central, a volcanic mountain range in central Costa Rica, suggests that orogenic events along the Continental Divide of the Americas played a significant role in driving the diversification of *B. asper* lineages. In this context, and to understand the presumably adaptive venom divergences among the South American *B. asper* lineages described above, we compared the chromatographic profiles and the phylogeographic distribution of PLA_2 markers between South American and Mesoamerican *B. asper* venoms.

Fig. 5 displays reverse-phase chromatographic profiles of *B. asper* venoms from México (BAMEX) [46], Costa Rica (CICA and PICA) [40], Panamá (BAPAN1, BAPAN2, BAPAN3, BAPAN4) [47] and from the Department of Antioquia, Colombia (BAANT), run under the same conditions as the venoms from Ecuador and south-western Colombia (Fig. 2). These Mesoamerican and Colombian venoms belong to the conspecific haplogroups MY (México-Yucatan, BAMEX), CICA (Caribbean Isthmian Central America, CICA), PICA (Pacific Isthmian Central America, PICA, BAPAN1, BAPAN2, BAPAN3), and CHOCO1 (Darien-

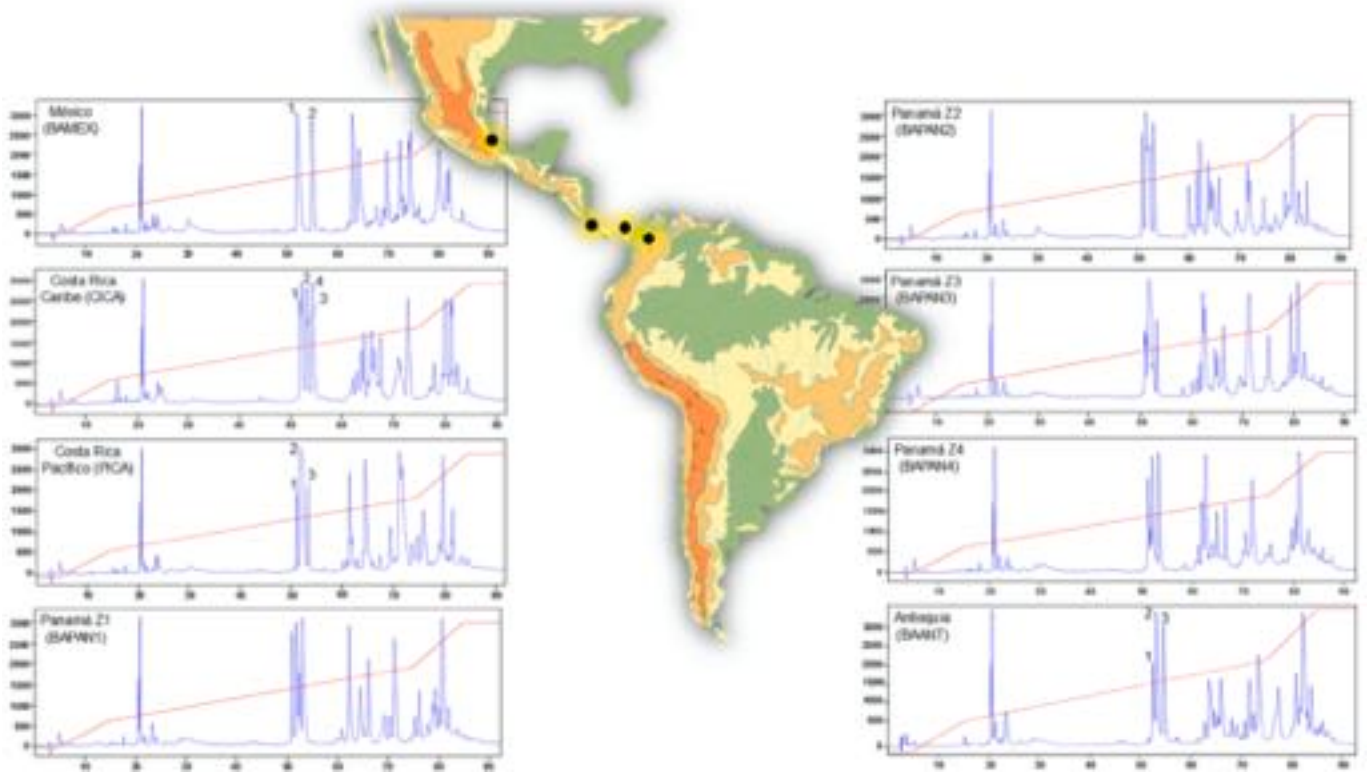

Fig. 5. Chromatographic profiles of *B. asper* venoms from Mesoamerica and northwestern South America. The geographic origin of the venoms is specified in each chromatogram and represented with a black-filled circle in the map. The molecular masses of the PLA_2 molecules recovered from the chromatographic fractionations of the BAMEX, CICA, PICA y BAANT venom proteomes (numbered) were measured by ESI-MS1 and their deconvoluted values are reported in Table 2.Chromatographic profiles of venoms from different Panamanian regions (BAPAN1–4) are from Vélez et al. [47] and are shown with permission of the publisher, Elsevier.

Colombian Chocó from central and eastern Panamá, BAPAN4, and Pacific coast of Colombia, BAANT) described by Saldarriaga-Córdoba et al. [23]. They exhibit remarkable similar chromatographic peak distributions between themselves, which matches their conserved qualitative toxicity profile, as all of them induce murine lethality, hemorrhagic, edema-forming, in vitro coagulant, defibrinogenating, and myotoxic activities ([47] and references therein) (Table S14). However, only the venoms of Costa Rican CICA and PICA *B. asper* have been proteomically characterized [40]. Comparison of the chromatographic profiles of the Ecuadorian and south-western Colombian *B. asper* venoms displayed in Fig. 2 and those of *B. asper* from Mesoamerica and northern South America (Fig. 5) evidences notable differences between their PLA_2 subproteomes.

To explore possible insights into the biogeographic history of these snakes, we focused on this toxin class because this venom subproteome has been used as population marker for tracing dispersal paths of *B. atrox* in its Amazonian range [91,92]. Table 2 lists the molecular masses of the PLA_2 molecules recorded in the venoms of the different *B. asper* lineages sampled and in *B. atrox* venoms from the Colombian Magdalena Medio Valley and from Puerto Ayacucho and El Paují in the Venezuelan States of Amazonas and Orinoquía, respectively. Table 2 highlights molecular masses shared between venoms and Fig. 6 shows their mapping onto the *B. asper* range across Meso and South America. The pattern of distribution of identical molecules provides a venomics perspective that complement phylogenetic analysis using genetic markers for reconstructing the dispersal routes that account for the current biogeographic distribution of the described species' phylogroups [21,23–25,93]. The finding of PLA_2 molecules shared between the venom of *B. atrox* from El Paují (Bolivar, Venezuela), Puerto Ayacucho (Amazonas, Venezuela), a sample from Magdalena Medio Valley (Colombia), originally assigned to *B. atrox* [92] although from its geographical origin could correspond to *B. asper*, and all the South American and Mesoamerican *B. asper* lineages (Table 2, Fig. 6) is consistent with a model of initial dispersal of an ancestor *B. asper* species complex, originated approximately 3.3 Mya in north-western South America [21]. This occurred along a northeast to northwest path, presumably through corridors between the Venezuelan Cordillera de Mérida and the Colombian Cordillera Oriental and the Sierra de Perijá and Sierra Nevada de Santa Marta, reaching the Pacific coast of Isthmian Central America. The pattern of PLA_2 conservation among venoms of Ecuadorian, Colombian, Costa Rican, and Mexican *B. asper* lineages can be explained by at least two dispersal events, a Y-shaped path with one branch of the fork running southwards along the narrow strip of lowlands of the Pacific slopes of the Colombian and Ecuadorian Andes ("West Andes route"), with the other branch entering the Isthmus of Panamá towards Costa Rica and México ("Panamá-México route"), and another radiation from southern Ecuador northward into Mesoamerica ("Colombia-Costa Rica route"). The forked route carried the genes coding for PLA_2s of 13,775 ± 1 Da, 13,935 ± 1 Da (MM), 13,724.8 Da and 13,822.8 (BAANT) in both directions, and genes encoding PLA_2s of Mw 13,966 ± 1 Da and 14,193 ± 1 Da (MM) southward through the West Andes route. Along this radiation, a PLA_2 of 13,966.6 Da was exclusively found in the BRH lineage, whereas the 14,196 ± 1 Da appeared to be widely distributed from southwestern Colombia (BANP2) through southern Ecuador (BASUP2) (Fig. 6). The Colombia-Costa Rica route carried the gene encoding PLA_2 of 14,033.8 Da from BACAUCA (El Tambo, Department of Cauca, southwestern Colombia) to the Pacific versant of Costa Rica, respectively (Fig. 7, Table 2).

This hypothesized venomics-informed dispersal scenario is compatible with the two-step dispersal-pulse hypothesis put forth by Savage (2002) to explain the unequal distribution of amphibians and reptiles of

Table 2

Table 2. Isotope-averaged molecular masses (in Da) of PLA_2 molecules determined by electrospray ionization mass spectrometry (ESI-MS) recorded from the venoms of *B. asper* lineages from Ecuador (EC), Colombia (COL) (Fig. 1), México (MX), and Costa Rica (CR) (Fig. 5). ESI-MS masses for PLA_2s from *B. atrox* from Venezuelan locales (El Paují and Puerto Ayacucho) and from a *B. atrox/asper* specimen from Colombian Magdalena Medio (MM) valley of uncertain taxonomy are from Calvete et al. [91]. Masses shared among South American venoms and between South American and Mesoamerican venoms are highlighted in gray and ocher backgrounds.

	B. asper		K49-PLA_2			D49-PLA_2		
EC	BAENP1	17	13727.5	13745.8	13711.9			
		19	13647.7	13691.1	13164.3			
		21				13586.6	12985.7	13403.6
		22				13772.9	13789.1	13804.8
		25				14194.0	14210.6	
	BAENP2	15	13934.3	14048.3	13951.4			
		16	14162.8					
		21				13702.6	13718.5	13734.8
		22				14192.6	14208.6	14224.5
	BANP1	14	13724.6	13838.6				
		15				14179.1	14195.8	14211.1
		16	14162.3					
		17	13952.6			13926.4	13968.5	
		18				13804.8	13820.8	13837.8
	BANP2	18	13724.6	13838.6	13787.0			
		19	13532.5	13576.4	13687.5			
		20	13775.4	13748.5				
		21				14179.1	14195.1	14210.1
		25				13804.8	14194.9	13820.8
	BANC	15	13724.6	13836.5	13787.6			
		17				14179.1	14195.1	14211.1
		19				13926.4	13723.6	
	PECja	21	13724.6	13838.6	13952.7			
		22				14196.1	14211.1	
		24				14179.2	14196.2	14237.2
	BAC	15	13725.0	13823.0	13839.0			
		16	13741.9					
		17				14179.5	14195.5	14236.5
		19				14208.5	14224.4	
		20				13926.9	13941.9	14040.9
	BASUP1	19	13817.7	13933.7	13833.7			
		23				13772.8	13788.9	13754.9
	BASUP2	16	13724.6	13740.6	13838.7			
		17	13633.4	13666.3				
		20				14179.1	14195.1	
		23				14208.1	14224.1	13773.0
		24				13926.4	13942.5	14040.4
	HEC2	20	13724.5	13740.6				
		27				14194.9	14210.8	
	HEC2ja	27				14194.1	14210.6	14252.1
COL	BRH	20	13724.7	13838.6	13952.7			
		21				14180.2	13724.6	14196.1
		22				13966.6	14080.5	13982.6
MX	BAMEX	1	13724.7	13757.8	13935.6			
		2	13873.7	13889.7	13971.7			
CR	CICA	1	13717.9	13774.9	13733.8			
		2	13725.0	13822.9				
		3	13758.7	13856.6				
		4	13935.6	13859.6				
	PICA	1	13774.7	13717.6	13687.6			
		2	13758.6	13856.6				
		3	13935.6	13859.7	14033.3			
COL	BAANT	1	13868.1	13774.8	13832.8			
		2	13724.8	13822.8				
		3	13935.8	13951.8				
	BACALCA	1	13724.8	13801.8	13822.5			
		2	14033.8	13935.8				
	MM		13775	13935		14193	13966	
	B. atrox							
VE	El Paují					13865	13966	
	El Paují ja		13801					
	P. Ayacucho		13831			13966	14198	

South American origin inhabiting Middle America [93]. Savage (2002) suggested that the first episode took place some 3.4 Mya when the sea level fell and South American taxa were able to invade Mesoamerica before the closure of the Panamanian land bridge [94]. Our data suggest that basal PLA_2s [13,935 Da, 13,775 Da, 13,724 Da and 13,822 Da] became fixed in the gene pool of Caribbean and Pacific Costa Rican *B. asper* before the separation of these phylogroups due to the uplift of the Central Mountain Chain during the late Pliocene/early Pleistocene (~ 2.8–2.4 Mya), a geological event that has been determined to occur at an average lifting speed of 1–2 mm/year [95]. In accordance with our conclusion, Saldarriaga-Cordoba et al. (2017), have estimated the separation between *B. asper* clades A (CICA) and B (PICA) at mid-Pliocene, approximately 3.20 Mya (CI95% = 2.32–4.31 Mya) [23]. Savage's second dispersal episode presumably occurred a million years later,

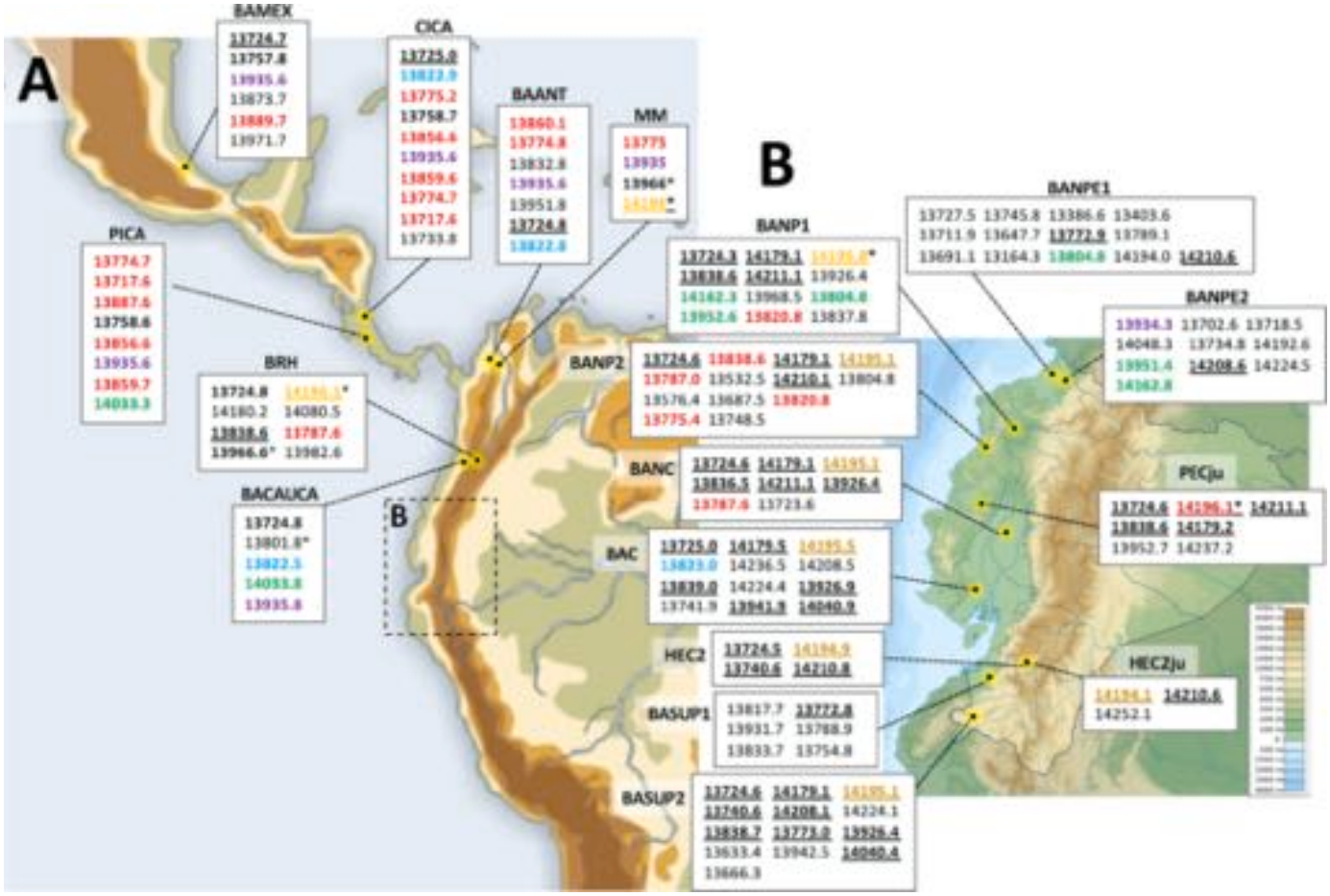

Fig. 6. Distribution of PLA_2 molecules across the *B. asper* range in Meso and South America. Panel A, ESI-MS molecular masses (in Da) of K49- and D49-PLA_2 molecules present in the venoms of South American *B. asper* and *B. rhombeatus* lineages investigated in this work (Fig. 2; Table 2; Supplementary Tables 2–12), in venom from *B. asper* from Antioquia (BAANT, Colombia), and venoms from *B. asper* lineages from México and Costa Rica (Fig. 5), listed in Tables 2, are mapped over the geographic distribution of *B. asper* lineages. The area framed in the box "B" with a discontinuous outline is shown in panel B. Molecular masses recorded in the venom of the specimen of controversial taxonomy (*B. asper*/*B. atrox*) from the Colombian Magdalena Medio (MM) valley are also shown. Identical molecular masses are highlighted in bold and underlined or in the same color. Asterisks denote PLA_2 masses shared between venoms from Venezuelan (El Paují, Bolivar and Puerto Ayacucho, Amazonas) *B. atrox* [92] and *B. asper* venoms sampled in this work.

after the final closure of the Isthmian bridge and after the isolation of the Caribbean and Pacific Costa Rican *B. asper* phylogroups [93]. Our finding that the second incursion of *B. asper* in Mesoamerica was characterized by the introgression of PLA_2s [14,033 Da] in the Pacific population but not in the *B. asper* stock of the Atlantic side of Costa Rica (Fig. 7), also implies that this dispersion event must have occurred after the separation of the CICA and PICA lineages.

4. Concluding remarks – clinical and ecological correlates of the geographic variation in *B. asper* venom

Despite remarkable differences in morphological and natural history traits exhibited among *B. asper* lineages [11,18,19], the overall toxin-family compositional trends observed among their venoms (Fig. 4A) appears to be mirrored by the qualitatively similar profile of clinical manifestations of envenomations throughout its wide distribution from lowland southern Mexico and Central America in Middle America and northwestern South America [47]. However, an unusual case of envenomation by *B. asper*, i.e. with the development of hemothorax as a hemorrhagic complication, has been reported [96]. A detailed comparison of the LD_{50} values and the minimum doses of hemorrhagic, coagulant and defibrinogenating activities of the venoms of geographic variants of adult specimens of *B. asper* revealed differences in their biological effects [41,46,48–51,76,97,98] (Table S14). Different bothropic antivenoms (Birmex, México; ICP, Costa Rica) effectively neutralized, albeit with different potencies, the various toxic effects of venoms from the Caribbean and Pacific Costa Rican populations of *B. asper* [46,48,50,97,99–101].

From an evolutionary ecology perspective, most human snake envenomations result from defensive bites inflicted when snakes and humans have chance encounters in nature. Snake venom evolved under selective pressure to subjugate prey, deter competitors, or defense themselves from predators through an escalating arms race characterized by asymmetrical selection between the predator's toxic arsenal and the prey's evolved counter adaptive resistance mechanisms. Subtle differences in abundance within and between venom toxin family members, which in this study essentially involve PI- and PIII-SVMPs, K49- and D49-PLA_2s, CTLs, and SVSPs (Fig. 4), may not have relevant clinical consequences but may have been instrumental over ones to select the evolutionary changes that maximized the organism's fitness in local environments, e.g., the snake foraging success on preferred prey.

Bothrops asper is considered a generalist species and its diet likely reflects opportunistic encounters with prey as the species exhibits great variation in diet composition [4,11], a circumstance that has been shown to influence spatial ecology, habitat selection, and foraging behavior of this ambush-hunting snake [102]. However, even for a generalist snake capable of subduing different prey items, non-consumptive habitat use, such as dietary decisions, define its trophic niche.

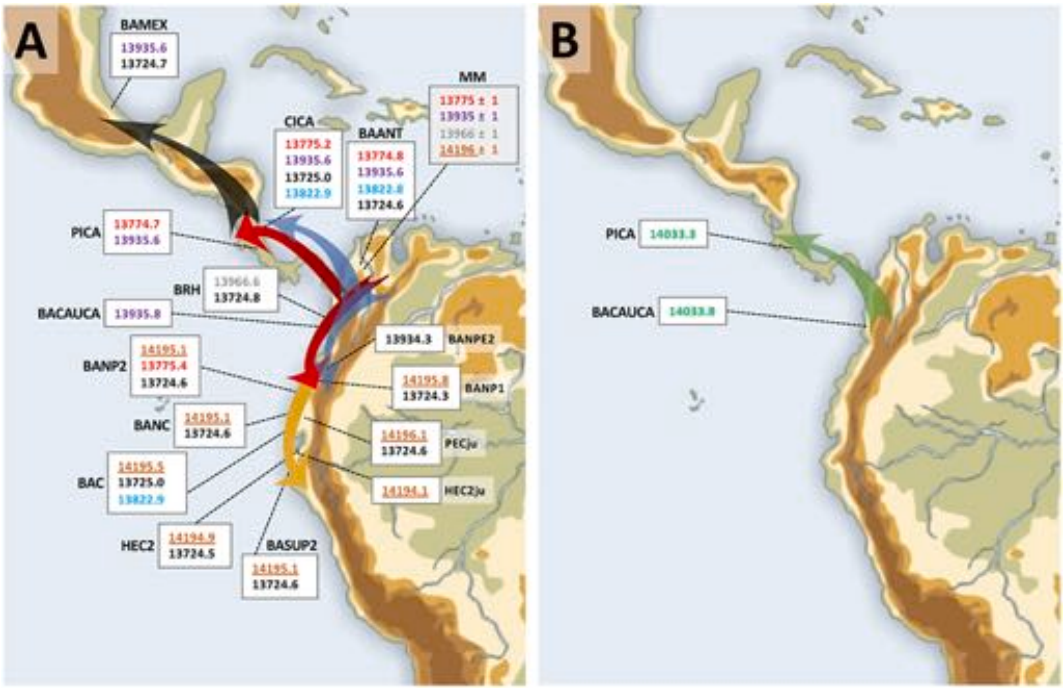

Fig. 7. Proposed dispersal routes of *B. asper*. Panel A displays the distribution of conserved PLA_2 molecules between *B. atrox* and *B. asper* lineages used as molecular markers for tracing the initial dispersal events of ancestral *B. asper* from northwestern Colombia southwards along the Pacific slopes of the Colombian and Ecuadorian Andes ("West Andes route") and northwestwards entering the Isthmus of Panamá towards Costa Rica and México ("Panamá-México route"). Panel B highlights a proposed more recent "Colombia-Costa Rica" dispersal route from the south-western Colombian population (BACAUCA) to the Pacific versant of Costa Rica (PICA).

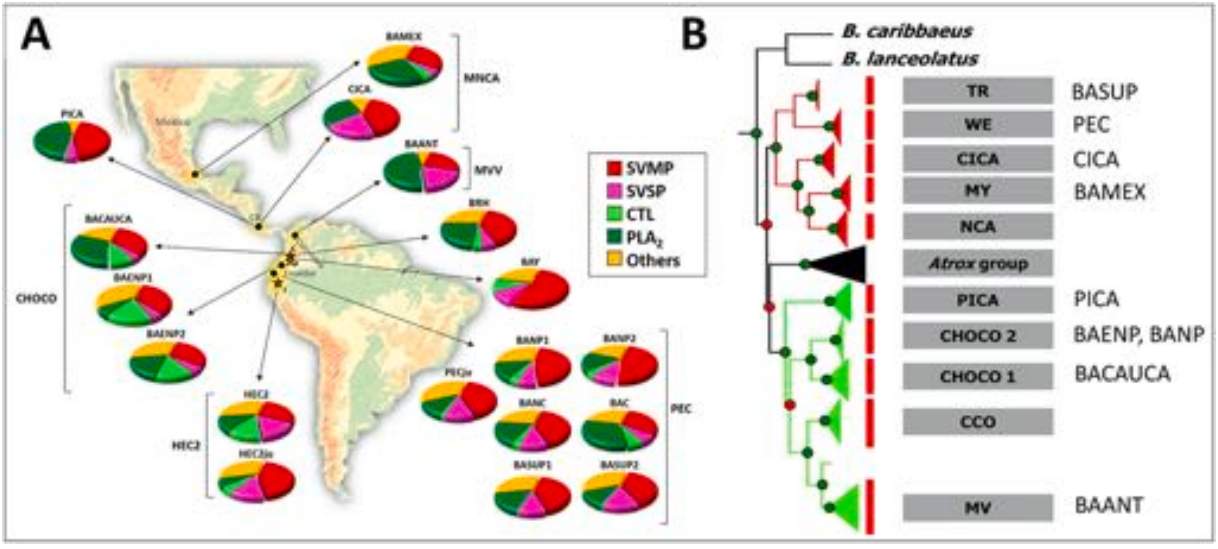

Fig. 8. Phylogeographic and phylogenetic affinities of *B. asper* proteomes across its distribution from southern México to southwestern Ecuador. Panel A, Mapping of the overall compositions of *Bothrops* (*asper* and *ayerbei*) species and *B. rhombeatus* lineage venoms in northern South America and Mesoamerica. Pie charts display the relative abundances (as percentage of total venom proteins) of the major venom toxin families that explain > 80% of the PCA compositional variability between *B. asper* groups. Pie chart BAMEX (México) was compiled through integration of the chromatographic profile shown in Fig. 5, and protein families were assigned by comparison with *B. asper* venom from Costa Rica [40]. Proteomic data of *B. asper* from Costa Rica (PICA and CICA) were retrieved from Alape-Girón et al. [40]. Composition data of the venom from Colombian Magdalena Medio (MM) valley of dispute *B. atrox*/*B. asper* taxonomy are from Calvete et al. [92]. The venom proteomes of *B. asper* (BACAUCA) and *B. ayerbei* from (BAY) are from Mora-Obando et al. [41]. The venom proteomes of adult and juvenile (ju) *B. asper* specimens from Ecuador (BAENP (1 and 2), BANP (1 and 2), BANC, PEC, BAC, BASUP (1 and 2) and HEC2) and that of *B. rhombeatus* (BRH) are displayed in Fig. 2 and presented in detail in the Supplementary Tables S2–S13. Panel B, The grouping of venom pools in the phylogenetic tree of *B. asper* lineages proposed by Saldarriaga-Córdoba et al. [23] and Salazar-Valenzuela et al. [21] is highlighted. South American clade B and clade A, which includes *B. asper* specimens from the Caribbean coast of Middle America and southwestern Ecuador, are depicted in green and red, respectively. The geographic origin of the venoms is represented by dots in the map, and the venoms from Colombian Cauca (C), and Patía (P), and Ecuadorian Jubones (J) river valleys are highlighted by red stars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Environmental conditions modulate the metabolic state of both the predator and the prey, and thus their interactions [103–105]. Venoms of snakes that feed predominantly upon homeothermic endotherms (e.g. mammals) may be under different pressures than those of snakes specialize in poikilothermic ectotherms (e.g. reptiles, amphibians), whose physiological state fluctuates widely according to time of the day, level of activity, and seasonally [106]. While venoms with a high percentage of enzymatic toxins, which are rate-limited by temperature, may be an option for both mammalian predatory snakes and snakes that hunts lizards during the day (when the lizards are active and may have been basking or otherwise maintaining an elevated body temperature and metabolic rate), for a snake that feeds on the same species of lizard at night, when it is sheltering and its metabolic rate is depressed, another strategy, perhaps non-enzymatic neurotoxins may be preferable [35,107].

As a complement to molecular genetic studies [21,23,24], our multivariate analyses of the venom composition among *B. asper* lineages (Fig. 4) favors a model of local adaptations for the diversification of *B. asper* venoms driven by the evolvability of only four toxin families, likely selected by the ecological opportunities created by the complicated orogenic and climatic fluctuation events during the Pliocene-Pleistocene along the Central and South American Continental Divide, and the species' own ability to spread into new niches [21,23,24]. The tectonic growth of the Andes and the regional climate have evolved simultaneously and have influenced each other [108]. Inter-Andean valleys, such as the Patía, Cauca, Jubones and Magdalena river valleys home to the BAY, BRH, HEC2, and MVV lineages, respectively, include distinct ecosystems with humidity patterns oscillating from dry bottoms to wet summits, and semi-arid pockets in the foothills, creating differentiated patterns of ecoregional altitudinal niches endowed with endemic flora and fauna. [33]. The highest species richness in this general area has been identified in the Pacific and eastern sides of the Andes, and the lowest in the Cauca and Magdalena valley bottoms [33]. A strong climatic gradient across the Costa Rican Cordillera Central results in greater weathering and erosion including fluvial canyons, waterfalls, and more frequent landslides on the humid Caribbean slope [109].

The lack of perfect match between the phylogeography (Fig. 8A) of venoms and the *B. asper* phylogroups recovered by phylogenetic analyses of concatenated genes using genomic data and Bayesian clustering analyses (Fig. 8B), particularly the placement of PICA in a clade otherwise populated by the Colombian CHOCO and Magdalena river valley (MV) lineages may indicate the occurrence of genetic exchange between specimens from the BAENP, BANP, BACAUCA and/or BAANT and PICA populations, presumably during the second wave of *B. asper* migration into Mesoamerica along the Colombia-Costa Rica route. The presence of PLA_2 [14,033 Da] in the populations of BACAUCA and PICA (Fig. 7B) may represent evidence of a putative hybridization event. A future broader sampling carried out with more resolutive techniques, e.g. top-down venomics with lineage-specific transcriptomic or genomic database matching will deepen our understanding of the genetic affinities and radiation patterns of the present-day *B. asper* lineages in its vast range of distribution.

Declaration of Competing Interest

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

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Author credit statement

DM-O, DS-V and JJC were responsible for the conception of the work; JJC supervised the experimental work. DM-O and DP performed the proteomic analyses. BL, JAG-V, and SA provided materials; DM-O, DS-V, DP, BL, JAG-V, SA, HLG and JJC analyzed and participated in the discussion of the results. DM-O and JJC drafted the manuscript. All the authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103945.

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