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Identification and molecular characterization of five putative toxins from the venom gland of the snake *Philodryas chamissonis* (Serpentes: Dipsadidae)

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ABSTRACT

Philodryas chamissonis is a rear-fanged snake endemic to Chile. Its bite produces mild to moderate symptoms with proteolytic and anti-coagulant effects. Presently, the composition of the venom, as well as, the biochemical and structural characteristics of its toxins, remains unknown. In this study, we cloned and reported the first full-length sequences of five toxin-encoding genes from the venom gland of this species: Type III snake venom metalloprotease (SVMP), snake venom serine protease (SVSP), Cysteine-rich secretory protein (CRISP), α and β subunits of C-type lectin-like protein (CLP) and C-type natriuretic peptide (NP). These genes are highly expressed in the venom gland and their sequences exhibited a putative signal peptide, suggesting that these are components of the venom. These putative toxins had different evolutionary relationships with those reported for some front-fanged snakes, being SVMP, SVSP and CRISP of *P. chamissonis* closely related to the toxins present in Elapidae species, while NP was more related to those of Viperidae species. In addition, analyses suggest that the α and β subunits of CLP of *P. chamissonis* might have a α-subunit scaffold in common with Viperidae species, whose highly variable C-terminal region might have allowed the diversification in α and β subunits. Our results provide the first molecular description of the toxins possibly implicated in the envenomation of prey and humans by the bite of *P. chamissonis*.

1. Introduction

Snake venoms vary in complexity and composition, which is related to the species efficiency to capture and digest their prey (Gibbs and Mackessy, 2009). Venoms are a mix of several organic molecules, containing mainly toxins that produce diverse biological effects on prey (Ramos and Selistre-de-Araujo, 2006). These toxins are enzymes, such as snake venom metalloproteases (SVMP), serine proteases (SVSP), L-amino oxidases and phospholipases (Kang et al., 2011) and proteins without enzymatic activities, such as cysteine-rich secretory proteins (CRISP), natriuretic peptides (NP), C-type lectins (CTL) and C-type lectin-like proteins (CLP), which act as ligands to specific receptors (Morita, 2005; Yamazaki and Morita, 2004). At the present, there is an important accumulation of information concerning the composition, biological effects, and evolutionary implications of the front-fanged snakes' venom, particularly of those produced by snakes with medical importance (i.e. Viperidae and Elapidae families), which deeply contrasts with the very scarce information on the rear-fanged snakes' venom (Fry et al., 2008, 2012).

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Philodryas is a genus of rear-fanged snakes widely distributed in South America, which comprises 21 species (Grazziotin et al., 2012; Zaher et al., 2014). These species are generally considered harmless for humans, although there are reports of accidents with medical significance caused by *Philodryas baroni* (Kuch and Jesberger, 1993), *Philodryas chamissonis* (Neira et al., 2007), *Philodryas olfersii* (Ribeiro et al., 1999), *Philodryas patagoniensis* (de Medeiros et al., 2010) and *Philodryas viridissima* (Means, 2010). Moreover, the symptoms produced by the bites of some *Philodryas* species resemble to the local symptoms of envenomation produced by the lethal front-fanged *Bothrops* species (Rocha et al., 2006). In the last years, the components of venom secretion of two *Philodryas* species have been studied using transcriptomic and biochemical approaches, reporting in *P. olfersii* the presence of at least five classes of toxins, SVMP, SVSP, CRISP, CLP and NP (Assakura et al., 1994; Ching et al., 2006; Fry et al., 2006), whereas in *P. patagoniensis* studies describe SVMP and CRISP toxins (Peichoto et al., 2009, 2010, 2011). These toxins may determine the main effects observed after a bite of *Philodryas* snakes in different prey and humans (Peichoto et al., 2007).

The available information about the toxicology of the trans-Andean *Philodryas* species *P. chamissonis*, *Philodryas amaru*, *Philodryas simonsii* and *Philodryas tachymenoides* (Thomas, 1976, 1977; Zaher et al., 2014) is very scarce (Neira et al., 2007). From these species, *P. chamissonis* (Fig. 1A) is endemic to Chile and inhabits Mediterranean areas close to human populations (Sallaberry-Pincheira et al., 2011). Its bite produces in humans, edema, intense pain, bleeding for few minutes, ecchymosis and in some cases, lymphadenopathy (Arzola and Schenone, 1994; Neira et al., 2007), effects that can last between one to seven days without produce death (Arzola and Schenone, 1994; Donoso Barros, 1966; Neira et al., 2007). In small rats and lizards, the injection of lyophilized venom gland of *P. chamissonis* causes degenerative and hemorrhagic actions and *in vitro*, venom produces a strong proteolytic activity (Donoso-Barros and Cárdenas, 1959). All these effects are similar to those produced by the venom of other *Philodryas* species (e.g. Acosta de Perez et al., 2003; Peichoto et al., 2004, 2005), suggesting that the venom of *P. chamissonis* may have a similar composition as the venom of *P. olfersii* (Ching et al., 2006) and *P. patagoniensis* (Peichoto et al., 2004, 2009, 2007). Further than these indirect evidence, however, the composition of the venom secretion and the biochemical and structural characteristics of *P. chamissonis* toxins remains unknown. Here, we aimed to identify the venom components of this species, evaluating the presence of five putative toxins (SVMP, SVSP, CRISP, CLP and NP) described in other *Philodryas* species, which will allow providing the molecular bases to understand the effects of the bite produced by *P. chamissonis*. Finally, we explore the evolutionary relationships of these toxins, to shed some light on the evolution of *P. chamissonis* venom.

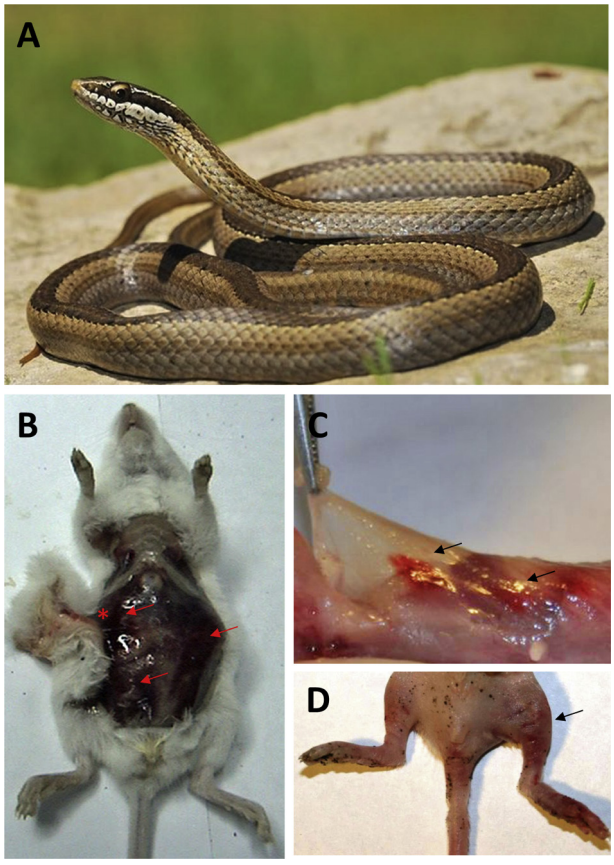

Fig. 1. Venom secretion of *P. chamissonis* produces hemorrhage in mice. (A) Specimen of *P. chamissonis*. (B) Hemorrhagic zones (arrows) observed after 1.5 h of an intraperitoneal injection of *P. chamissonis* venom (0.5% in saline). The asterisk indicates the injection site of the venom. (C) Hemorrhagic zones in the peritoneum (arrows) and (D) edema in a limb (arrow) produced after 15 min of *P. chamissonis* bites.

2. Materials and methods

2.1. Animals

Three individuals of *P. chamissonis* were collected in Central Chile. They were transported and maintained at the Laboratorio de Neuroetología, Universidad de Chile, following a previously tested protocol (Troncoso-Palacios and Labra, 2012). Capture, maintenance and experimentation with snakes was approved by Servicio Agricola y Ganadero (Resolution No. 7466), and the local ethic committee of the Facultad de Medicina (Protocol CBA#0286 FMUCH).

2.2. Collection of venom secretion and effects on mice

Venom secretions were collected in a plastic petri dish covered with parafilm. Individuals were induced to bite the parafilm with the opistoglyphous fangs and secretions were collected from the plate in saline solution (0.5%) and immediately injected intraperitoneally in a mouse. One hour after the injection, the mouse was sacrificed by cervical dislocation to analyze the venom effects on the peritoneum and internal organs. In addition, during a feeding session of the snakes, a mouse that was bitten several times by a snake, we took it before it was constrained and swallowed. This mouse was sacrificed by cervical dislocation 15 min after being bitten, to explore directly in a prey the effects of a real bite on the peritoneum and internal organs.

2.3. Tissue dissection

Two individuals of *P. chamissonis* were returned to their collecting sites, while the third one was sacrificed three days after extract the venom, as venom glands reach their maximal production of mRNA and protein synthesis between two to four days after the venom extraction (Casewell et al., 2009; Paine et al., 1992; Rotenberg et al., 1971). The sex of the individual, a male, was determined during the dissection. We removed the venom glands, liver and testes, and transferred them immediately to RNA*later* solution (Thermo Scientific, USA) and maintained at −80 °C overnight. Liver and testes were used as controls to evaluate the differential gene expression of these toxins.

2.4. RNA extraction and cDNA synthesis

Tissues were homogenized with the electronic homogenizer Tissue Master 125, OMNI (International) and the total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) following the manufacturer recommendations. The purity of the total RNA was assessed by photometric analysis (260/280 ratio). RNA was treated with DNAse I (Ambion, Austin, USA), and RNA integrity was evaluated by denaturant gel electrophoresis. One μg of total RNA was used as template for the reverse transcription reactions to synthesize a single strand cDNA using MMLV-RT reverse transcriptase (Promega, USA) and oligodT primers (Invitrogen, USA), according to standard procedures.

2.5. Primers design and standard PCR

Using the software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), we designed a set of primers for the amplification of the five genes of interest (SVMP, SVSP, CRISP, CLP and NP; Supplementary Table 1). These were designed based on the available sequence data of homologous genes from different snake species, including front-fanged (Viperidae, Elapidae) and rear-fanged snakes. Primers were designed to match the conserved regions of each gene. Products obtained from PCR were sequenced to confirm the presence of toxin-encoded genes. We used the following thermal cycle profile for standard PCR: 94 °C for 120 s, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min followed by 4 °C for 15 min. Products were fractionated by electrophoresis in 1.5% agarose gel, and the bands containing the main PCR products were cut from the gel, purified with Wizard SV Gel and PCR Clean-Up System (Promega, USA). Eluted DNA fragments were sequenced with the dideoxy chain termination technique in both directions by standard-DNA sequencing facility in Macrogen Inc. (Rockvill, MD, USA).

2.6. Quantitative real-time PCR

Quantitative Real-Time PCR (qPCR) amplifications and fluorescence detection were performed using the LightCycler 1.5 Instrument (Roche, Switzerland) and Light Cycler Fast Start DNA Master SYBR Green I (Roche). Supplementary Table 2 shows the primer sequences used for qPCR. The following thermal cycle profile was used for qPCR: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, primer annealing at 60–65 °C for 15 s, and 72 °C for 15 s. The specificity of primers was evaluated by melting curve analyses and the expected sizes of the qPCR products were confirmed in a 3% agarose gel. The stability of candidates housekeeping genes, *gapdh*, *hsp* and *actin* reported in literature (Currier et al., 2012) was evaluated using the web-tool Redfinder (http://www.leonxie.com/referencegene.php), and the best candidate, *gapdh*, was used as the internal reference gene. We determined the relative expression levels of the genes, using the method described by Pfaffl (2001). The transcript levels of the studied genes are shown as transcript abundance relative to the reference gene.

2.7. RACE-PCR and cloning

To obtain the full-sequences of the five putative toxins encoded in the venom gland of *P. chamissonis*, cDNA was used as template to perform 3′- and 5′-RACE-PCR, cloning and then sequencing them. Gene Racer kit (Invitrogen, USA) was used for RACE-PCR of 5′and 3′extrems according to the manufacturer's instructions. For these reactions, we used *Pfu* DNA polymerase high fidelity (Promega, USA) and the primer pairs used are shown in Supplementary Table 3. Amplified fragments were separated on 1.5% agarose gel electrophoresis and bands of interest were cut, purified and cloned into pCR 2.1 TOPO vector (TOPO-TA cloning, Invitrogen). *Escherichia coli* Top-10 competent cells were transformed with plasmids that contained the cDNA fragments of interest and selected with ampicillin. Colonies for each reaction were chosen to isolate plasmid DNA by miniprep, checking the presence of the inserts by PCR with the appropriate primers. Between three to five confirmed colonies were analyzed by DNA sequencing with universal primers.

2.8. Sequence analysis, molecular phylogeny and homology modeling

The toxin sequences were obtained through NCBI BLAST searching (http://www.ncbi.nlm.nih.gov/) and aligned with the ClustalW software (http://www.ebi.ac.uk/clustalw; Thompson et al., 1994). Amino acid sequences from nucleotide sequences were predicted using the GeneMark server (http://opal.biology.gatech.edu/). These sequences were used for the phylogenetic analyses, which were performed with MEGA software, and using the Maximum Likelihood Method based on the JTT matrix-based model (Tamura et al., 2011); the confidential intervals were estimated at each node by bootstrap tests (5000 replicates). Signal peptides were predicted with Signal P 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and the protein domains were predicted with INTERPRO (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The sequences reported in this article were deposited in GenBank with the following accession numbers: KM527180 (SVMP-Pch), KM527181 (SVSP-Pch), KM527182 (CRISP-Pch), KM527183 (α-CLP-Pch), KM527184 (β-CLP-Pch) and KM527185 (NP-Pch). Toxin models were generated using at least one template protein 3D-structure, identified from Protein Database (PDB, http://www.rcsb.org/pdb/home/home.do). Homology modeling were performed through I-TASSER software (Roy et al., 2010; Zhang, 2008), and information about the templates and models are shown in Supplementary Table 4. The modeling of the toxins were displayed with VMD v1.9.2 software (http://www.ks.uiuc.edu/Research/vmd/).

2.9. Statistics

Data on gene expression are shown as mean ± standard deviation, based on five replicates. The expressions of the different genes among the tissues were compared using an one-way ANOVA followed by Tukey tests. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. Venom secretion of P. chamissonis produces local hemorrhage and mild edema in mice

After 1.5 h of the intraperitoneally injection of *P. chamissonis* venom, it was observed an extensive hemorrhagic zones in the peritoneum (Fig. 1B, red arrows), distant from the injection point (Fig. 1B, red asterisk); however, there was no visible damage in the internal organs (heart, liver, stomach, and intestine). The dissection of a mouse 15 min after been bitten by a snake, revealed only damage in the peritoneum with incipient hemorrhagic zones (Fig. 1C, black arrows). Bites produced wide hemorrhagic zones in the limb muscle (not shown) with significant edema (Fig. 1D), and small coagulopathies in the subcutaneous tissue. These observations indicate that venom of *P. chamissonis* mainly induce local hemorrhage and mild edema.

3.2. Five toxin-encoding genes are expressed in venom gland of P. chamissonis

BLAST searches revealed that the nucleotide sequences, previously amplified and sequenced, corresponded to the five groups of snake venom toxins (SVMP, SVSP, CRISP, CLP and NP), showing between 93 and 98% of identity to the sequences described in *P. olfersii* (Ching et al., 2006; Fry et al., 2006). The qPCR results showed that the venom gland exhibits high levels of transcripts of these toxins, suggesting that their putative proteins would be relevant in the venom composition (Fig. 2). Liver and testes had high expression of SVSP and CRISP, respectively, in accordance with the descriptions of high mRNA expression of serine proteases in the liver of several species (Degen and Sun, 1998; Neth et al., 2001) and CRISPs in the male reproductive system of mammals (Song et al., 2011; Vadnais et al., 2008). On the other hand, because CLP toxins are composed of heterodimers, and C-type lectins present in venom of other snakes are composed of homodimers (Morita, 2005), we examined in *P. chamissonis* venom the abundance of putative CLP subunits. Data show that the α and β subunits had similar relative expression, suggesting that CLP of *P. chamissonis* is a heterodimer (Fig. 2E). Altogether, these results indicate that the venom gland of *P. chamissonis* exhibits high levels of transcript abundance of the five toxin-encoding genes.

3.3. Structural characteristics of the putative toxins with catalytic domains: SVMP and SVSP

Fig. 3A shows a representation of the three types of SVMPs (grouped in PI, PII, PIII) and disintegrin present in the venom of several snake species (Takeda et al., 2012). We found a full-length cDNA sequence of SVMP type-III in *P. chamissonis* (SVMP-Pch), which encodes a polypeptide of 615 amino acids that contains a putative signal peptide sequence (amino acids 1 to 18) and a pro-domain composed of 173 amino acid residues (Supplementary Fig. 1). The metalloproteinase domain (M-domain) of the SVMP-Pch has an extension of 196 amino acids, containing a highly conserved catalytic Zn^{2+}-binding motif (HELGHNLGINHD) and a Met-turn motif (CXM) with methionine in position 373 (Fig. 3B, boxes *a* and *b* and Fig. 3C). The latter is a structural characteristic of all metzincin metalloendopeptidases required to form a hydrophobic pillow for the catalytic Zn^{2+} (Igarashi et al., 2006; Takeda et al., 2006, 2012). The disintegrin-like domain (D-domain) exhibits a DCD motif (Supplementary Fig. 1, box *c*) and the cysteine-rich domain (C-domain) has a variable and a hypervariable region between 356–566 residues and 578–615 residues, respectively (Supplementary Fig. 1, boxes *d* and *e*). SVMPs type-III (Fig. 3A) have been defined according to their structural characteristics in three classes: III a/b, c and d (Igarashi et al., 2006; Takeda et al., 2006, 2012). SVMP-Pch can be classified as a non-dimeric class a/b, considering that it does not exhibit the consensus sequence QDH(S/N)K presents in the dimeric SVMPs (Igarashi et al., 2007) and it lacks the Cys374 residue required for an inter-chain disulfide bond (Takeda et al., 2006; Supplementary Fig. 2). The homology modeling suggests that SVMP-Pch, as other SVMPs type-III, exhibits a C-shaped configuration, where C-domain is close to the catalytic site in M-domain (Fig. 3D). Three conserved Ca^{2+}-binding sites are contained in the M-domain, opposite to the catalytic site (Site I: E215, D299, N407 residues) and D-domain (Site II: N422, E426, E429, D432 residues and Site III: D483, E486, D498 residues), suggesting that calcium ions are required for the folding of SVMP-Pch.

The identified full-length cDNA sequence of SVSP from *P. chamissonis* (SVSP-Pch) encodes an open reading frame (ORF) of

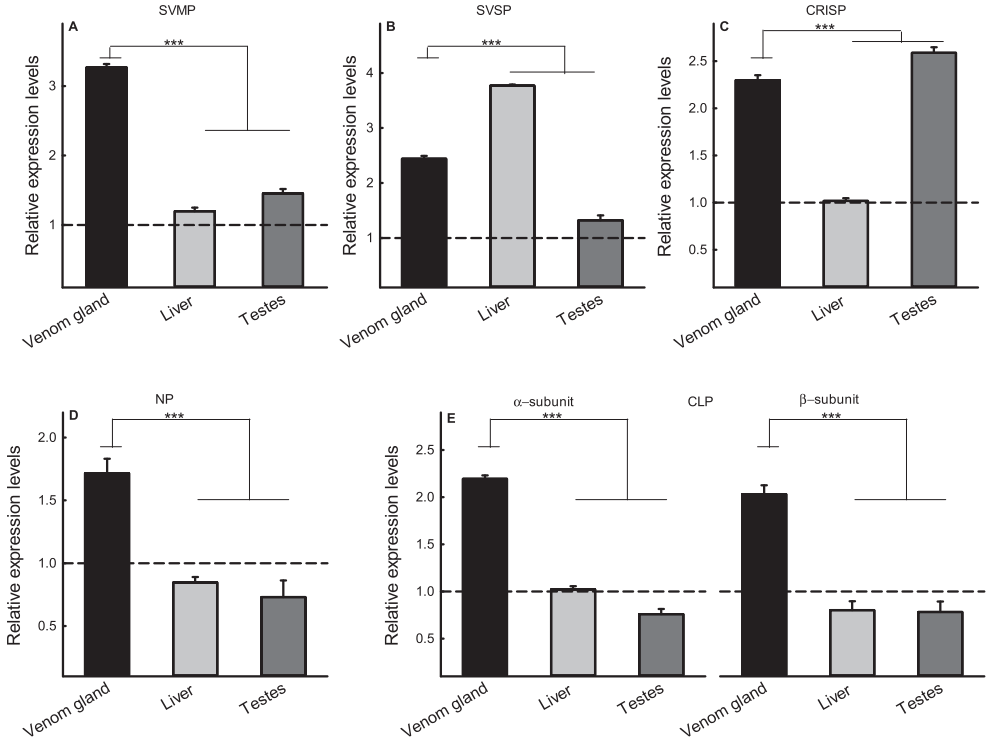

Fig. 2. Expression of five toxin-encoding genes in the venom gland and non-toxic tissues (liver and testes) of *P. chamissonis*. (A) Snake venom metalloprotease (SVMP), (B) snake venom serine protease (SVSP), (C) cysteine-rich secretory peptide (CRISP), (D) natriuretic peptide (NP), (E) C-type lectin-like peptide (CLP), α- and β-subunits. Data are shown as transcript abundance relative to gene reference (*gadph*), and the broken line indicates equal abundance with this gene. Values are expressed as mean ± Standard deviation (N = 5). Connecting lines show the differential expression of the different toxins between venom gland and non-toxic tissues, *** = $p < 0.001$.

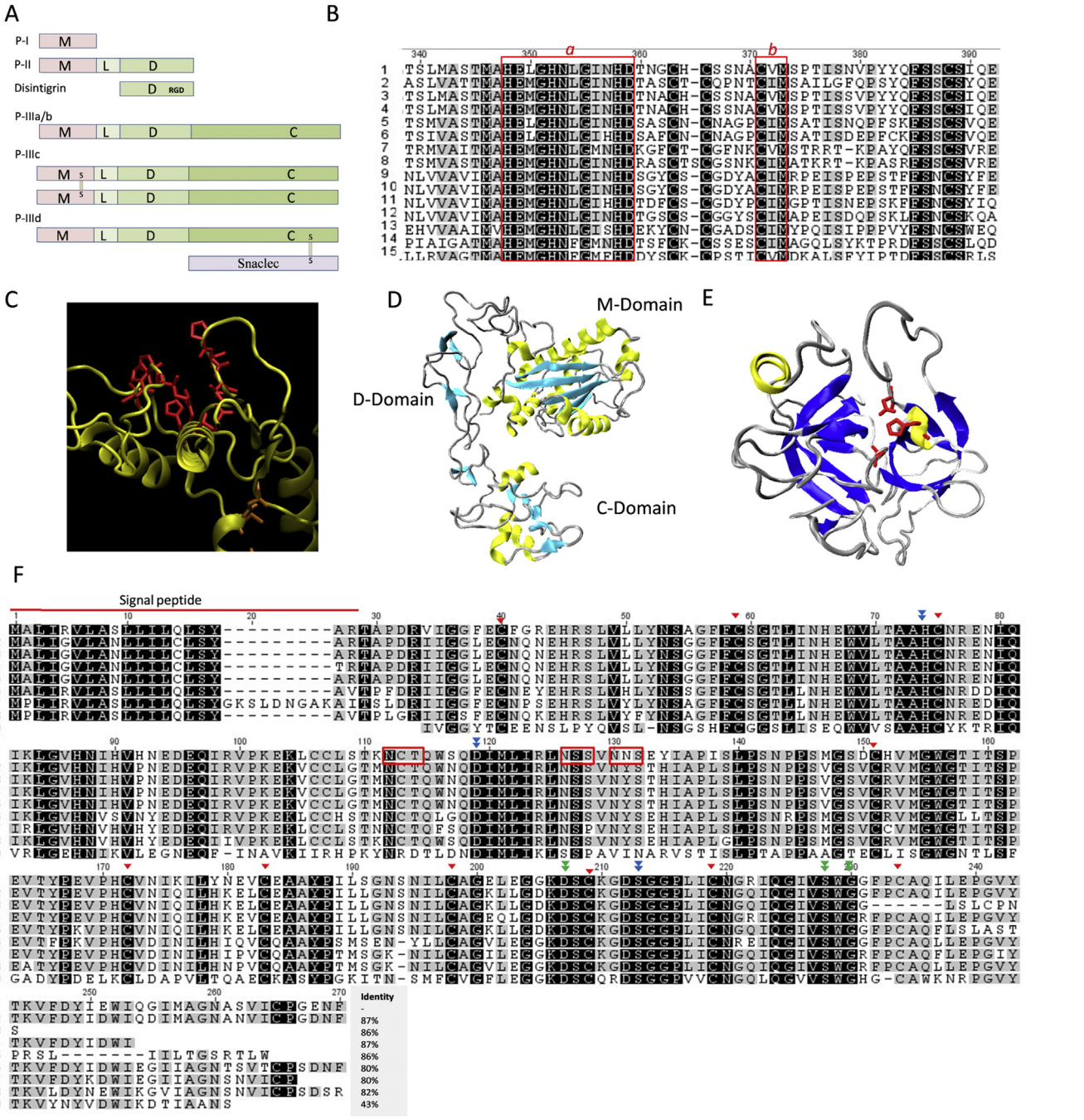

Fig. 3. Snake venom metalloprotease (SVMP) Type-IIIa/b and snake venom serine protease (SVSP) of *P. chamissonis*. (A) Diagram of the domain structure of the three types of SVMPs (grouped in PI, PII, PIII) and disintegrin that are present in the venom of several snake species. Each domain is represented by a different color. M, Metalloprotease domain; L, linker; D, disintegrin-like domain and C, cysteine-rich domain; Snaclec, snake venom C-type lectin-like domain (Takeda et al., 2012). (B) Sequence alignment of M-domain of SVMP from 1: *P. chamissonis* (KM527180), 2–4: *P. olfersii* (ACS74986.1; ACS74987.1; ACS74988.1), 5: *Drysdalia coronoides* (AEH95531.1), 6: *Austrelaps superbus* (ABH10621.1), 7: *Naja naja* (AAF00693.1), 8: *Bungarus multicinctus* (ABN72537.1), 9: *Crotalus atrox* (ACV83931.1), 10: *Crotalus viridis viridis* (ACV83933.1), 11: *Bothrops insularis* (AAM09693.1), 12: *Bothrops jararaca* (AAG48931.5), 13: *Echis ocellatus* (CAJ01680.1), 14: *Anolis carolinensis* (XP_003226913.1), 15: *Homo sapiens* (NP_055080.2). Letters *a* and *b* indicate Zn^{2+}-binding site and CXM motif, respectively. (C) Close up view of M-Domain with the Zn^{2+}-binding site in red. (D) Ribbon representation of the predicted 3D-structure of *P. chamissonis* SVMP Type-IIIa/b. (E) Homology model of SVSP of *P. chamissonis*. Conserved residues H74, D119 and S213 that compose the catalytic site are shown in red. (F) Sequence alignment of SVSP from 1: *P. chamissonis* (KM527181), 2–5: *P. olfersii* (Q09GK1.1; AAZ75626.1; AAZ75628.1; AAZ75629.1, respectively), 6: *Ophiophagus hannah* (ABN72544.1), 7: *Hydrophis hardwickii* (AAV98367.1), 8: *Micropechis ikaheka* (AHZ08801.1), 9: *Homo sapiens* (PDB:1H4W_A). Conserved cysteine residues (red arrowheads), conserved residues within the catalytic site (blue double arrowheads), and the substrate-binding sites (green double arrowheads) are indicated. Putative N-glycosylation sites are highlighted in red open boxes. GenBank or UniProt/Swiss-Prot accession numbers of sequences used are shown in parenthesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

261 amino acid residues, including a putative signal peptide of 18 residues, an activation peptide of six residues and a native serine protease of 226 amino acids (Fig. 3F). The activation peptide of SVSP-Pch exhibits the same motif RTAPDR as the SVSP of *P. olfersii* (Ching et al., 2006; Fry et al., 2006), which is different from the canonical motif QKSSEL described for other snake species (Serrano

and Maroun, 2005). SVSP-Pch is a monomeric enzyme that has a catalytic site composed by a conserved triad (H74, D119 and S213), three substrate recognition sites (D207, S228 and G230) and three putative N-glycosylation sites (NXS/T) at ^{112}NCT^{114}, ^{126}NSS^{128} and ^{130}NNS^{132} (Fig. 3F). The three-dimensional conformation shows that SVSP-Pch exhibits two domains with antiparallel β-sheets and little α-helix (Fig. 3E).

3.4. Structural characteristics of the putative toxins without catalytic domains: CRISP, CLP and NP

We determined the nucleotide sequence of a full-length cDNA encoding cysteine-rich secretory protein of *P. chamissonis*, CRISP-Pch. This sequence comprises an open reading frame of 720 nucleotides in length, encoding a polypeptide of 239 amino acids, which contains a putative signal peptide of 18 residues (Fig. 4A). Comparative sequence analysis revealed a high identity of the CRISP-Pch with some CRISPs reported from *P. olfersii* and Elapidae species (Fig. 4A). CRISP-Pch has sixteen cysteine residues that form the eight conserved disulfide bridge in CRISP toxins (Yamazaki and Morita, 2004), exhibiting the two characteristic domains of this family (Fig. 5A): a pathogenesis-related domain (PR-1 domain; residues 20–182) and the cysteine-rich domain (CRD; residues 202–240), which have a linker sequence from G183 to P201 (Fig. 4A). CRISP toxins can interact with several ion channels, such as voltage-gated Ca^{2+} channels, voltage-gated K^+ channels and ryanodine receptors (Yamazaki and Morita, 2004; Zhou et al., 2008). As is shown in Fig. 4A, CRISP-Pch, however, lacks of all the reported motifs involved in the inhibition of L-type Ca^{2+} channel (^{206}DVF^{208} and ^{205}ENEF^{208}; Yamazaki et al., 2003), voltage-gated K^+ channel Kv1.3 (Q217, S219; Q221, D223 and R236; Wang et al., 2006) and the cyclic nucleotide-gated (CNG) ion channels (K196, K203 and R204; Yamazaki et al., 2002a), containing only the highly conserved bivalent cation-binding residues (H79 and H134), which may bind Zn^{2+} ions (Shikamoto et al., 2005). Homology modeling of CRISP-Pch (Fig. 5A) suggests that the PR-1 domain has an α-β-α sandwich organization and CRD exhibits three α-helixes with several tails, similar to others CRISP toxins (Wang et al., 2006).

We also identified two full-length cDNA sequences of C-type lectin-like proteins, CLP-Pch, corresponding to α and β subunits of a heterodimer. cDNA of α and β subunits encode an open reading frame of 160 and 156 amino acid residues, respectively, both having a putative signal peptide of 23 amino acids. The α and β subunits of CLP-Pch exhibit 96% and 97% of identity with the same subunits reported for *P. olfersii* (GenBank access number: Q09GJ8.1 and Q09GK0.1, respectively; Ching et al., 2006), and both subunits have a 40% of identity with C-type lectin reported for *P. olfersii* (Fry et al., 2006, Fig. 4B). Both subunits of CLP-Pch have six cysteine residues

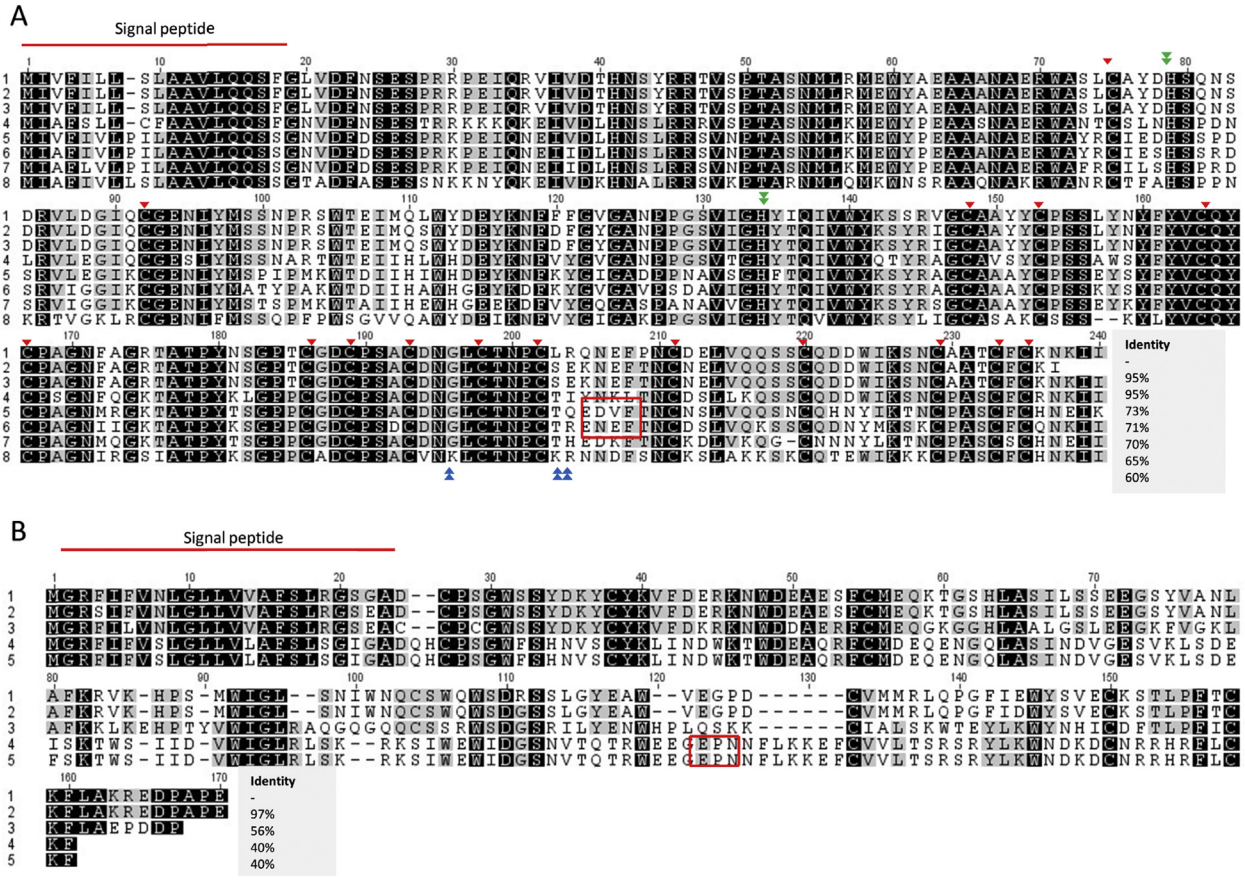

Fig. 4. Full-length sequences of Cysteine-rich secretory protein (CRISP) and β-subunit of C-type lectin-like protein (CLP) of *P. chamissonis*. (A) Sequence alignment for CRISP of 1: *P. chamissonis* (KM527182), 2–3: *P. olfersii* (AAZ75604.1; Q09GJ9.1, respectively), 4: natrin from *Naja atra* (AAP85301.1), 5: ablomin from *Gloydius blomhoffi* (Q8JI40.1), 6: triflin from *Protobothrops flavoviridis* (AAM45665.1), 7: *Vipera berus* (CAP74089.1), 8: pseudechetoxin from *Pseudechis australis* (AAL65291.1). Conserved cysteine residues (red arrowheads), conserved bivalent-cation binding residues (green double arrowheads), putative motif for inhibition of CNG-channels (blue double arrowheads) and L-type Ca^{2+} channels (red open box) are indicated. (B) Multiple alignment for β-subunit of C-type lectin-like proteins and C-type lectins reported for *Philodryas* species. Red open box indicates the carbohydrate-binding site of C-type lectin. Numbers indicate 1: *P. chamissonis* (KM527184), 2–5: *P. olfersii* (ABI74695.1, ABI74697.1, ABU68500.1, ABU68501.1, respectively). GenBank or UniProt/Swiss-Prot accession numbers of sequences used are shown in parenthesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

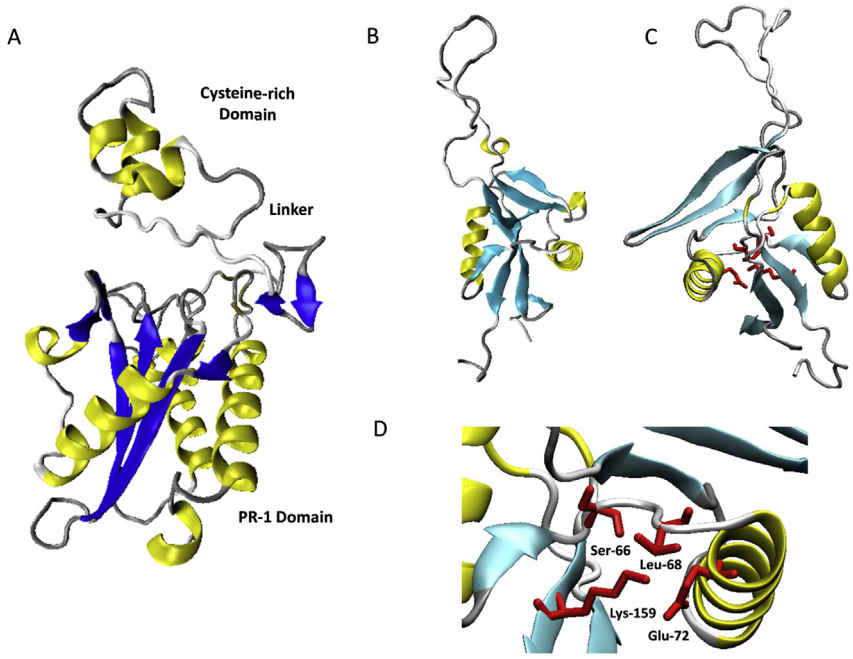

Fig. 5. Homology models of Cysteine-rich secretory protein (CRISP) and α and β-subunits of C-type lectin-like protein (CLP) of *P. chamissonis*. (A) Homology model of CRISP-Pch. Pathogenesis-related domain (PR-1 domain), linker and Cysteine-rich domain (CRD) are indicated. (B) Homology model of α-subunit and (C) β-subunit CLP. (D) Detail of amino acidic residues of the β-subunit (Ser66, Leu68, Glu72 and Lys159), which does not form the Ca^{2+}-binding site.

(C27, C38, C55, C133, C150 and C158) and five tryptophan residues (W31, W48, W92, W108 and W145), which are conserved in CTL of other snakes (Braud et al., 2002; Jebali et al., 2009). On the other hand, CLP-Pch sequences do not have a carbohydrate-binding site (EPN or QPD motif; Fig. 4A red open box for *P. olfersii*), suggesting a putative biological activity on coagulation system by binding to the coagulation factor IX/X, a mechanism that can be Ca^{+2}-dependent or -independent, requiring for the former case, Ca^{+2}-binding sites (S66, E/Q68, E72 and E159; Atoda et al., 2002). CLP-Pch does not exhibit these Ca^{+2}-binding sites, containing S66, G68, E72, K159 residues in α-subunit and S66, L68, E72, K159 residues in β-subunit (Fig. 5D). Homology modeling of α and β subunits CLP-Pch shows a similar configuration as others CLP (Fig. 5B and C), suggesting that the central portion of the putative protein is projected outward, a structure known as swapping domain, which is the putative site of association between the α and β subunits and the ligand-binding site (Ogawa et al., 2005).

The full sequence of the natriuretic peptide identified from *P. chamissonis* (NP-Pch) contains an ORF that encodes a precursor protein of 165 residues with a signal peptide of 20 amino acids. NP-Pch exhibits the common ring of the vasoactive natriuretic peptides constituted by 17 amino acids ($CFGX_{12}GC$ motif), bridged by an intra-molecular disulfide bond (Fig. 6B and C). NP-Pch can be classified as a C-type NP (Fig. 6A, *a*), because it lacks the N- and C-terminal extensions of the 17 amino acids loop, which are characteristics of A-type and B-type NPs (Sudoh et al., 1990). In addition, NP-Pch does not exhibit the bradykinin-potentiating peptide domains (BPP, Fig. 6A, *b*) nor the C-terminal extension (Fig. 6A, *c*), which are present in C-type NP from Viperidae and Elapidae snakes, respectively. Finally, NP-Pch has a high identity (99% for ABI74693; 74% for BAI59771) to C-type NP of the rear-fanged snakes, *P. olfersii* and *Rhabdophis tigrinus*, respectively (Fig. 6C).

3.5. Putative toxins of P. chamissonis have different evolutionary relationships with those toxins of Elapidae and Viperid species

The toxin sequences identified from the venom gland of *P. chamissonis* are highly related to those of *P. olfersii*, constituting a well-supported group for all the toxins studied (Figs. 7–9). The phylogenetic analyses indicate that the two toxins with putative enzymatic activities (SVMP and SVSP) from *P. chamissonis* are closely related to those of Elapidae species (Fig. 7A and B, respectively). In addition, CRISP from *P. chamissonis* constitutes a well-supported group with CRISP sequences from other species of Dipsadidae family (*P. olfersii* and *Liophis poecilogyrus*) and this group is close to the CRISP sequences of Elapidae and Colubridae species (Fig. 7C). In contrast, the natriuretic peptide sequence from *P. chamissonis* is closely related to bradykinin-potentiating and C-type natriuretic peptides (BPP–CNP) of Viperidae species (Fig. 8). On the other hand, C-type lectin-like proteins have been only described from Viperidae species, in contrast to the C-type lectins, which have been reported in Viperidae and Elapidae species (Morita, 2005; Ogawa et al., 2005). The molecular phylogeny of C-type lectin-like proteins of Viperidae and *Philodryas* species (Fig. 9), shows that α and β subunits of CLP-Pch are closely related to the α-subunits CLP of Viperidae species. Therefore, these results show that the putative toxins of *P. chamissonis* exhibit different evolutionary relationships with the toxins of snakes from the Elapidae and Viperidae families.

4. Discussion

In a seminal study, Donoso-Barros and Cárdenas (1959) showed that the injection of venom gland extracts of *P. chamissonis* in mice and lizards produced degenerative and hemorrhagic actions, and *in vitro* conditions, these extracts had a strong proteolytic activity. Our results confirm that its venom induces in mice local hemorrhage and edema in the bitten zone and that its venom gland has genes that encode for the five toxin classes (SVMP, SVSP, CRISP, CLP and NP) described in several snakes (Ching et al., 2006; Fry et al., 2008). This suggests the presence of toxins that act upon the coagulation system and possibly, they may affect the blood pressure and induce myotoxicity.

SVMPs have been identified as toxins responsible for the

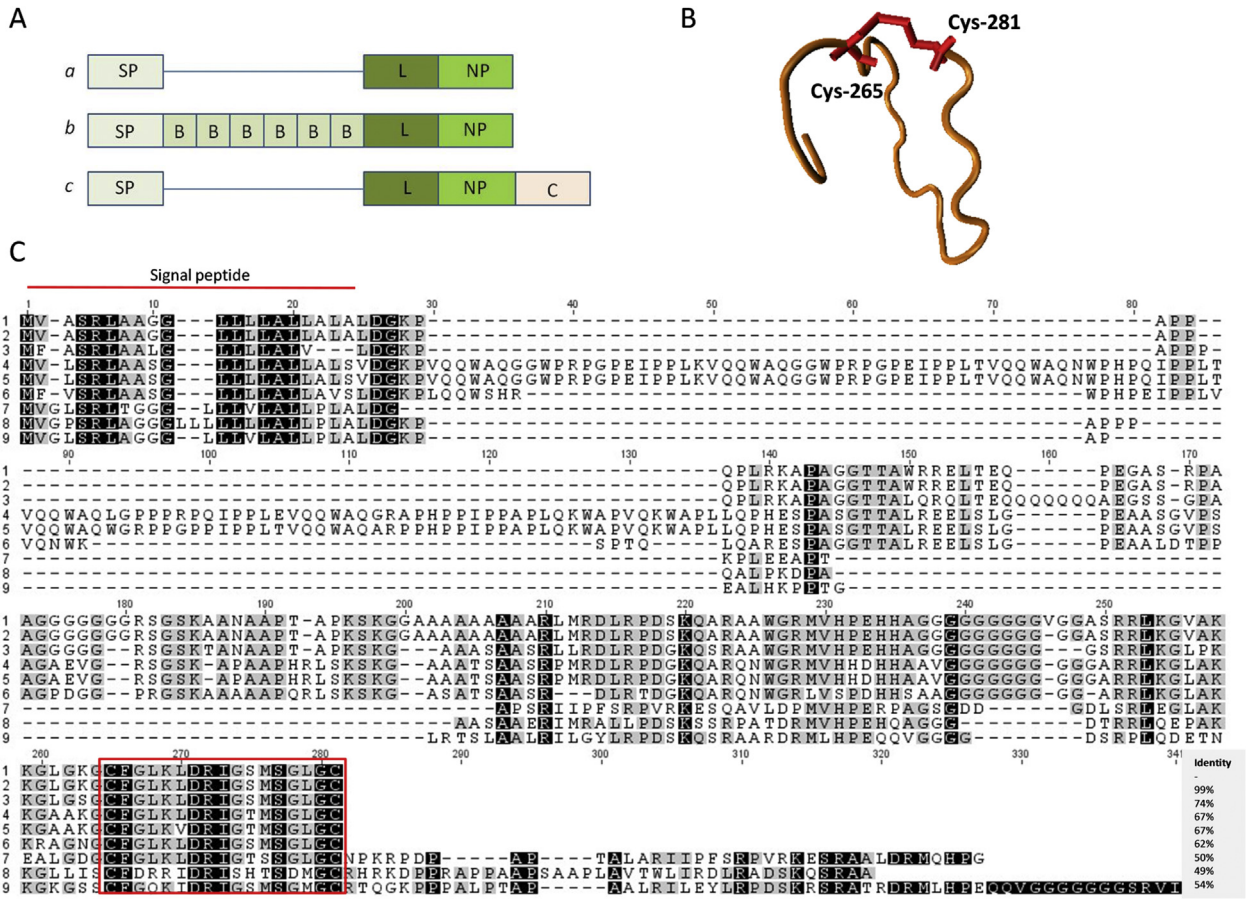

Fig. 6. Full-length sequence of natriuretic peptide (NP) from *P. chamissonis*. (A) Diagram of the domain structure of C-type natriuretic peptides. Each domain is represented by a different color. SP, signal peptide; B, Bradykinin-potentiating peptide domain; L, linker; NP, natriuretic peptide domain and C, C-terminal extension. (B) Homology model of NP-Pch, the conserved intramolecular disulfide bond (Cys265—Cys281) is shown in red. (C) Multiple alignment for C-type natriuretic peptides reported for *Philodryas* species and other snake species. Red open box indicates the 17-amino acid residues ($CFX_{12}GC$) that constitute a characteristic ring of this peptide family. Numbers are: 1. *P. chamissonis* (KM527185), 2: *P. olfersii* (ABI74693.1), 3: *Rhabdophis tigrinus* (BAI59771.1), 4: *Bothrops insularis* (P68515.2), 5: *Bothrops jararaca* (Q9PW56.2), 6: *Crotalus durissus terrificus* (AAL09427.1), 7: *Micrurus fulvius* (AAZ39879.1), 8: *Bungarus flaviceps* (D5J9S0.1), 9: *Naja atra* (D9IX97.1). GenBank or UniProt/Swiss-Prot accession numbers of sequences used are shown in parenthesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hemorrhagic and pro-inflammatory effects produced by Viperidae snakes (Gutiérrez et al., 2005; Lopes et al., 2009; Teixeira et al., 2009), which are closely related to the mammalian ADAM (a disintegrin and metalloproteinase) and ADAMTS (ADAM with thrombospondin type-1 motif) family of proteins (Takeda et al., 2012). P-III class of SVMPs exhibits a modular architecture with two non-catalytic domains (disintegrin-like and cysteine-rich domains, Fig. 3A) and has high hemorrhagic activity associated with other effects, such as a degradation of the components of the basement membrane as well as an interaction with endothelial cell integrins, interfering with their adhesion to the extracellular matrix (Gutiérrez et al., 2005). SVMP Type-IIIa/b of *P. chamissonis* (SVMP-Pch) was predicted as a single chain protein with M- and D-domains similar to the sequences reported for *P. olfersii* (Ching et al., 2006). This suggests that the putative protein of SVMP-Pch may exhibit biological effects related to the metalloprotease activity as the one described for the metalloproteases isolated from the venom of other *Philodryas* species (Assakura et al., 1994; Peichoto et al., 2007). For example, patagonfibrase, a single chain SVMP from *P. patagoniensis*, and four metalloproteases (PofibC1, C2, C3 and H) isolated from *P. olfersii* degrade exclusively the α-chain of fibringen without effects on prothrombin, having a α-fibrinogenolytic activity. Additionally, hemorrhagic and pro-inflammatory effects have been identified as other proteolytic activity-dependent actions for both PofibH (Assakura et al., 1994) and patagonfibrase (Peichoto et al., 2007, 2011). These effects are inhibited by adding chelanting agents such as 1,10-phenanthroline and Na_2EDTA *in vivo* and *in vitro*, suggesting that the protease activity is dependent of divalent cations as has been demonstrated for *P. patagoniensis* and *P. baroni* (Peichoto et al., 2007, 2011; Sánchez et al., 2014). This dependence of divalent cations is consistent with the three conserved Ca^{2+}-binding sites found in the SVMP-Pch sequence, which can be required for its folding and consequently its enzymatic activity. Therefore, the putative protein SVMP-Pch may exhibit α-fibrinogenolytic activity, hemorrhagic and pro-inflammatory effects.

The putative serine protease obtained from *P. chamissonis* (SVSP-Pch) has the sites required for protease activity: three substrate-binding sites and a catalytic triad. Usually, snake serine proteases contain 12 conserved cysteine residues (He et al., 2007), 10 of them are required to form disulfide bonds (Itoh et al., 1987), similar to trypsin in *Homo sapiens* (Fig. 3E). However, SVSP-Pch has two additional cysteine residues, one of which is part of an extension of the C-terminal region, a typical motif of SVSP (Jin et al., 2007). This additional cysteine residue and the three putative N-glycosylation sites identified in SVSP-Pch may contribute to the enzyme stability (He et al., 2007) and of its macromolecular selectivity, similar as other SVSPs (Siigur et al., 2011; Zhu et al., 2003). SVSPs with

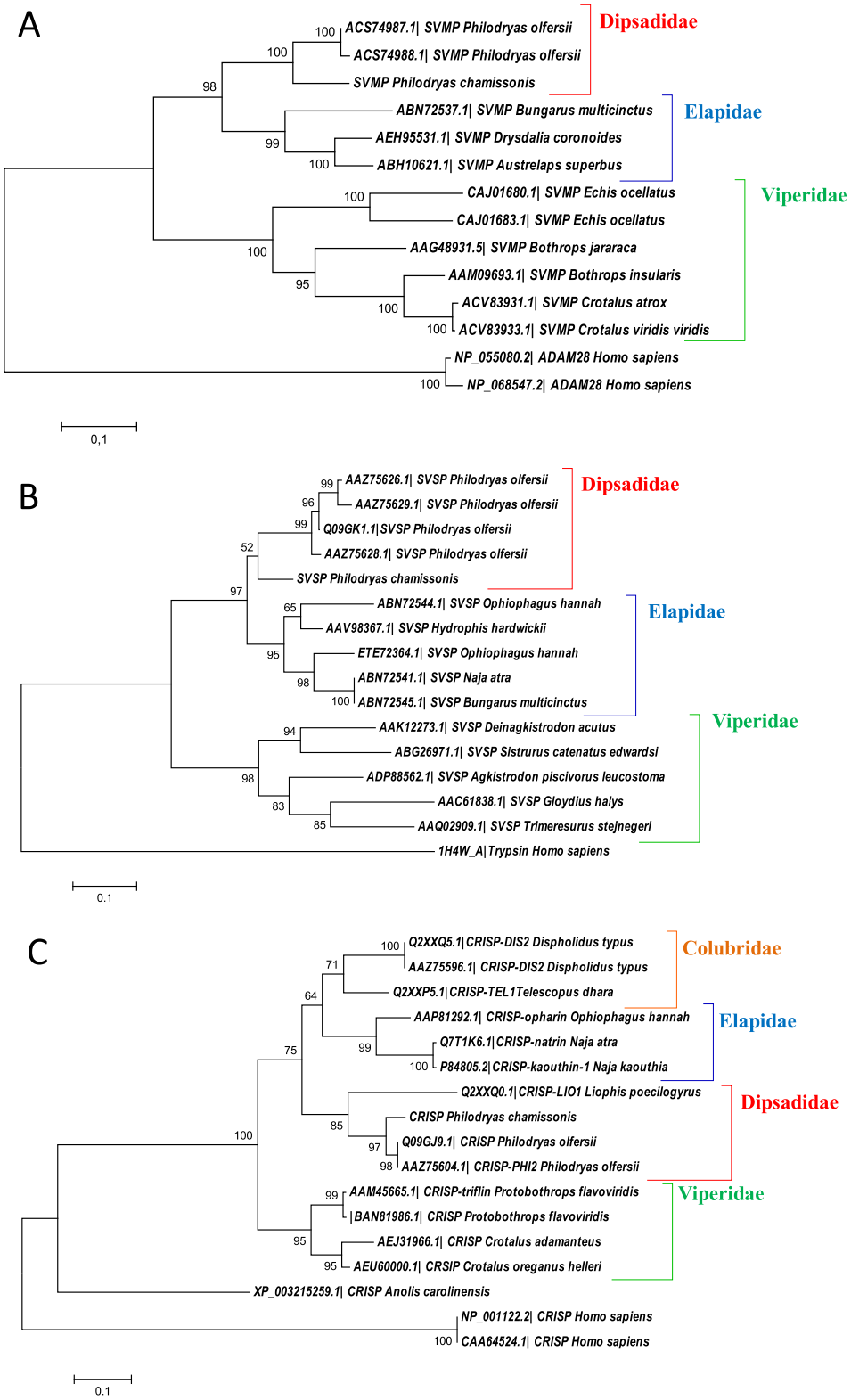

Fig. 7. Molecular phylogeny of three toxins identified in *P. chamissonis* and other snake species: SVMP (A), SVSP (B) and CRISP (C). Phylogenetic analyses were performed on the amino acid sequences, using the Maximum Likelihood Method based on the JTT matrix-based model. Confidential intervals at each node were estimated by bootstrap test (5000 replicates). GenBank or UniProt/Swiss-Prot accession numbers of sequences used are shown.

fibrinogenolytic activities has been described from the venom of *P. olfersii* (Assakura et al., 1994) and *P. patagoniensis* (Peichoto et al., 2005), degrading the Aalpha-chain and affecting the blood coagulation in mice (Peichoto et al., 2005). Considering the similarities between the SVSP-Pch and the SVSP sequences reported for *P. olfersii* (Ching et al., 2006), they probably have similar biological

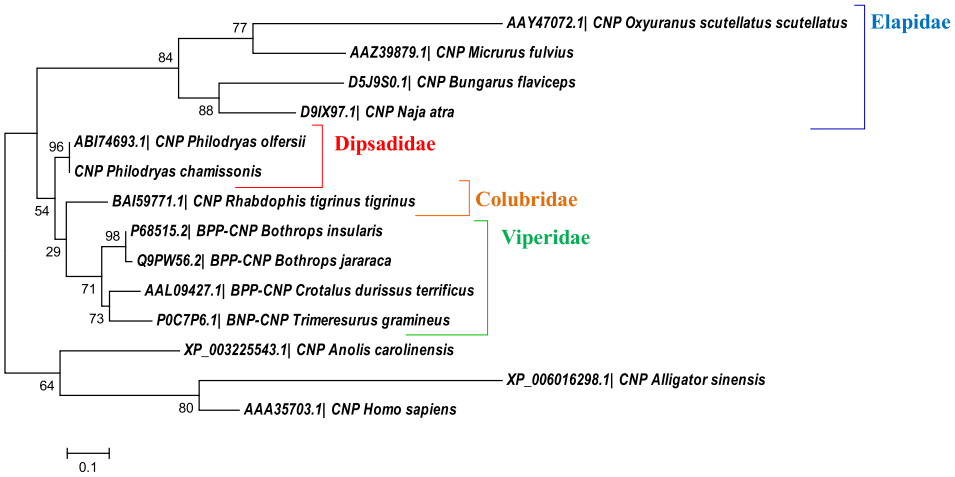

Fig. 8. Molecular phylogeny of C-type natriuretic peptides identified for *P. chamissonis* and other snakes. Phylogenetic analyses were performed on the amino acid sequences, using the Maximum Likelihood method based on the JTT matrix-based model. Confidential intervals at each node were estimated by bootstrap test (5000 replicates). GenBank or UniProt/Swiss-Prot accession numbers of the sequences used are shown. Tree only includes C-type natriuretic peptides (NP).

effects. In summary, SVMP and SVSP from *P. chamissonis* may have a relevant participation in the envenomation, being the main responsible for the observed effects after the snake bite; local hemorrhage and edema both in humans (Neira et al., 2007) and mice (this study; Donoso-Barros and Cárdenas, 1959).

Some CRISP toxins from front-fanged snakes inhibit depolarization-induced contraction in the arterial smooth muscle of rat tail, interacting with several ion channels, such as L-type Ca^{2+} channels (Yamazaki et al., 2002b), voltage-gated K^{+} channels and ryanodine receptors (Wang et al., 2006; Yamazaki and Morita, 2004; Zhou et al., 2008). In contrast, the biological activities and putative targets of CRISP toxins from rear-fanged snakes are poorly known. It has been suggested that the cysteine-rich domain of several CRISP toxins interacts with biological targets (Matsunaga et al., 2009). However, CRISP-Pch lacks of all the putative binding sites and domains required for the inhibition of ionic channel mediated-actions on smooth muscle described for other snakes (Ramazanova et al., 2009; Shikamoto et al., 2005; Yamazaki et al., 2003). Interestingly, a native CRISP purified from *P. patagoniensis* snake venom (patagonin) produces muscular damage to murine gastrocnemius muscle without affects the smooth muscle contraction, which is an unusual action for a CRISP (Peichoto et al., 2009). The full-sequence of patagonin is unknown, but fragments of the N-terminal and PR-1 domain sequences match the CRISPs of *P. olfersii* and *P. chamissonis* (Supplementary Fig. 3A). Although, the full-length sequence of patagonin is required for further conclusions, it is possibly that these CRISP toxins exhibit different biological effects (e.g. myotoxicity) than those produced by CRISP from front-fanged snakes.

In front-fanged snakes C-type lectin-like proteins (CLP) usually affect the vertebrate blood coagulation and the platelet aggregation (Atoda et al., 2002; Morita, 2005), while the biological effects of these toxins from rear-fanged snakes are unknown and only the nucleotide sequences are currently available (Ching et al., 2006). C-

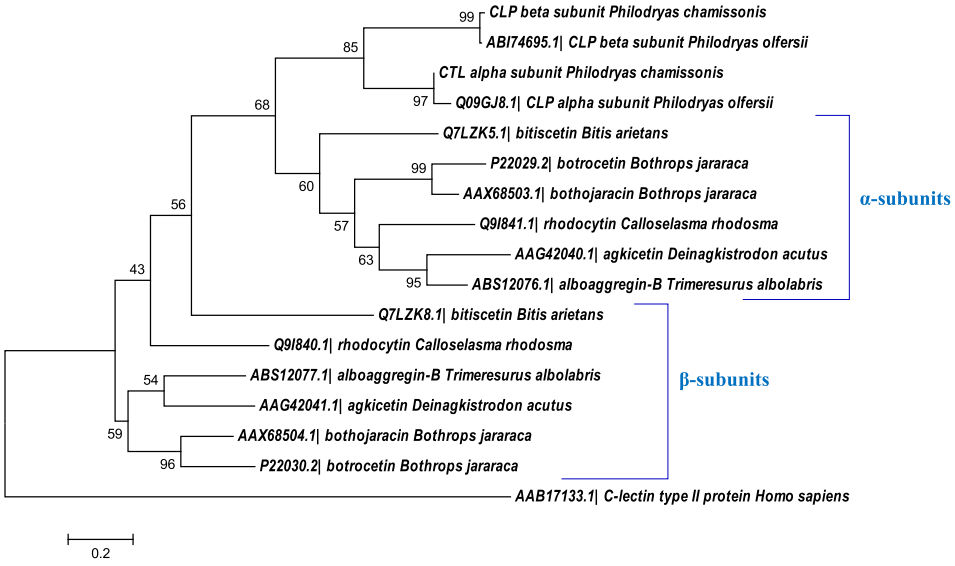

Fig. 9. Molecular phylogeny of C-type lectin-like proteins identified for *P. chamissonis* and other snakes. Phylogenetic analyses were performed on the amino acid sequences, using the Maximum Likelihood method based on the JTT matrix-based model. Confidential intervals at each node were estimated by bootstrap test (5000 replicates). GenBank or UniProt/Swiss-Prot accession numbers of the sequences used are shown. For phylogeny of CLP only sequences of Viperidae species have been reported.

type lectin-like proteins identified from the venom gland of *P. chamissonis* have a putative quaternary organization with α and β subunits, without Ca^{2+}-binding sites. These subunits, similar to the subunits of C-type lectins of Viperidae species (Jebali et al., 2009), exhibit two regions: a more conserved region-A, which extends between residues 24 and 76 and a region-B with notable differences between both subunits in C-terminal sequences, which goes between the residues 77 and 164 (Supplementary Fig. 3B). These characteristics of α and β subunits are consistent with reports of protein-encoding regions highly variable as compared to its non-coding regions (Ogawa et al., 2005) and recombination events between α and β subunits (Jebali et al., 2009) described for CLPs and C-type lectins, respectively. Considering these data and that both α and β subunits of *P. chamissonis* and *P. olfersii* are closely related to α-subunits of Viperidae species, it is possible to propose that the subunits of CLP from *Philodryas* species may have a α-subunit scaffold in common with Viperidae species, whose highly variable C-terminal region might have allowed the diversification in α and β subunits. Further studies are required to demonstrate this point.

Natriuretic peptides isolated from front-fanged snakes produce hypotensive and vasorelaxation effects in rats (Da Silva et al., 2011; Evangelista et al., 2008). Currently, only two sequences of natriuretic peptides from rear-fanged snakes are known (GenBank access number: ABI74693 and BAI59771). The natriuretic peptide sequence from *P. chamissonis* has a very high identity with the NP from *P. olfersii* (Fig. 6). All these NP sequences of rear-fanged snakes are close related to the BPP–CNP from Viperidae species, and contain the highly conserved 17-residues that constitutes the intramolecular disulfide loop required for their biological activities (Nakao et al., 1992). At the present, however, the contribution of NP of rear-fanged snakes in the envenomation remains uncertain.

Several evidence show that the toxins of rear-fanged snakes affect prey differentially, e.g. lizards and mice (Weldon and Mackessy, 2010), only birds (Pawlak et al., 2006), birds and lizards (Pawlak et al., 2009) or only affecting lizards and being harmless to mice (Heyborne and Mackessy, 2013). The effects of the *P. chamissonis* venom on different animals is poorly known, although, it seem to be more harmful to its primarily prey, lizards (Donoso-Barros and Candiani, 1950; Greene and Jaksic, 1992). This suggests a specialization of its venom with taxa- and prey-specific adaptations, which possibly include a venom with low diversity of components, relation already proposed for other rear-fanged snakes (Ching et al., 2006; Mackessy et al., 2006; Weldon and Mackessy, 2010).

In conclusion, we here described the first full-length sequences of five toxins, SVMP, SVSP, CRISP, α and β CPL and NP, from the venom gland of *P. chamissonis*, a rear-fanged snake endemic to Chile with scarce toxicological information. Our results provide the molecular basis to understand the effects of envenomation produced by this species, especially hemorrhage and edema, in prey and humans.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2015.09.032.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2015.09.032.

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