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Some aspects of the venom proteome of the Colubridae snake *Philodryas olfersii* revealed from a Duvernoy's (venom) gland transcriptome

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Abstract We investigated the putative toxins of *Philodryas olfersii* (Colubridae), a representative of a family of snakes neglected in venom studies despite their growing medical importance. Transcriptomic data of the venom gland complemented by proteomic analysis of the gland secretion revealed the presence of major toxin classes from the Viperidae family, including serine proteases, metalloproteases, C-type lectins, Crisps, and a C-type natriuretic peptide (CNP). Interestingly, the phylogenetic analysis of the CNP precursor showed it as a linker between two related precursors found in Viperidae and Elapidae snakes. We suggest that these precursors constitute a monophyletic group derived from the vertebrate CNPs.

Keywords: Snake venom; Transcriptome; Molecular evolution; Natriuretic peptide; 2D electrophoresis; Colubridae

1. Introduction

Despite the fact that most species of snakes currently described are placed in the family Colubridae, studies on their venom secretions have been in general neglected. On the other hand, the complexity, diversity and similarity of Viperidae and Elapidae snakes venom transcriptomes and proteomes have been of tremendous research interest and are well known [1–6]. The Colubridae snakes have not been regarded as clinically important due to their less dangerous venom inoculation apparatus. However, a growing number of accidents caused by snakes of the Colubridae family, especially of the Xenodontinae subfamily, have been reported in epidemiological studies [7]. Various species of this family produce toxic secretions from their Duvenoy's glands, which are homologous to the venom glands of other venomous snakes [8]. But only few Colubridae toxins were recently confirmed by sequence determination, such as three-finger toxins, phospholipases A2 (PLA2s), Crisps and serine proteases [9–11].

Philodryas olfersii (Colubridae, Xenodontinae), an opisthoglyphous (rear-fanged) snake widespread over South America, is involved in many of the reported cases of envenoming in Brazil [7]. The envenoming, showing both local and systemic effects, can be easily misinterpreted with those from *Bothrops* (Viperidae) [12]. Haemorrhagic, oedematogenic, myotoxic and fibrin(ogen)olytic activities, along with shared epitopes with *Bothrops* toxins, have been reported on the venom of *P. olfersii* [12–14].

Besides being important to the management of snake envenoming, the knowledge about the bioactive components from snake venoms has been proved to be useful to understand various mammalian physiological processes, to analyze toxin evolution and to mine models for drug design. In order to explore the putative venom components of *P. olfersii* we carried out a transcriptomic analysis of its Duvernoy's (venom) gland. Together with a complementing analysis of the venom by two dimensional (2D) electrophoresis and mass spectrometry, this expressed sequence tags (ESTs) database will hopefully help to understand the evolutionary relationships of Colubridae toxins with those of the Viperidae and Elapidae families.

2. Materials and methods

2.1. cDNA Library Construction and EST generation

The specimens of *P. olfersii* were kept at the Herpetology Laboratory of Instituto Butantan (São Paulo, Brazil) and the venom was milked periodically after injection of pillocarpin (10 mg/kg in 0.15 M NaCl), pooled and lyophilized. Pairs of venom glands were obtained 5 and 10 days (two female specimens) and 15 days (one male specimen) after milking. Messenger RNA purification was performed on a column of oligo-dT cellulose (Amersham Biosciences) and the cDNAs were synthesized from 5 μg of mRNA using the Superscript Plasmid System for cDNA Synthesis and Cloning (Invitrogen), directionally cloned in the pSPORT-1 plasmid and transformed in *Escherichia coli* DH5 α cells. Plasmid DNA was isolated using alkaline lysis from randomly chosen clones as described [6]. The DNA was sequenced on an ABI 3100 sequencer using BigDye2 kit (Applied Biosystems) with standard 5′ primer.

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Abbreviations: 2D, two dimensional; A/BNP, atrial and brain natriuretic peptide; BPP, bradykinin-potentiating peptide; CNP, C-type natriuretic peptide; CTL, C-type lectin; ESTs, Expressed Sequence Tags; GRC, group of related cluster; NP, natriuretic peptide; PLA2, phospholipase A2; SP, serine protease; SVMP, snake venom

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2.2. Clusters assemble and identification

A homemade pipeline of EST analysis software was developed and used to remove poor quality sequences, vector, adaptors and short ESTs (<150 bp), as described elsewhere [6]. ESTs were then assembled in clusters of contiguous sequences using the CAP3 program [15], set to join only sequences with at least 95% of base identity. A second round of grouping using a lower stringency (66%) was also performed on the cluster sequences. The clusters were filtered by BLASTN against a dataset of ribosomal RNAs, mitochondrial, *E. coli* and vector sequences to mask them from statistical analysis. Each cluster was then searched against GenBank databases using BLASTX and BLASTN algorithms to identify similar products with an *e*-value cutoff $<10^{-5}$. Unidentified sequences and those with unpredicted function were checked for the presence of signal peptide and for orthologous occurrence throughout reptile ESTs (compiled from various sources). A final annotation table in Microsoft Excel format was generated containing all the relevant information about clusters. Methodologies of other analysis are described within the figure legends.

3. Results and discussion

3.1. EST sequencing, clustering and identification

The random sequencing of a *P. olfersii* cDNA library from three specimens of both genders at different times of their venom production gave readable sequences for a total of 2194 clones. They were grouped in 1285 clusters (1113 singlets, and 172 of two or more ESTs) that were further combined in 1029 groups of related clusters (GRCs) (966 singlets, and 63 of two or more clusters) and compared to GenBank sequences, revealing the profiles shown in Fig. 1 and in Supplementary Fig. 1. All EST sequences were submitted to GenBank dbEST under accession numbers EC780214–EC782407. A complete list with putative identifications of all clusters can be provided under request and the relevant properties of the five classes of toxins identified (Fig. 1, right) are described below.

3.2. Metalloproteases are the most abundant transcripts

Previous studies using chelating agents and immunodetection had indicated that snake venom metalloproteases (SVMPs) play an important role in the envenoming by *Philodryas* spp. [12,13,16]. However, there are no reports on complete SVMP sequences from Colubridae venoms.

Confirming the importance of SVMPs, here we show that 15% of all clones of *P. olfersii* cDNA library encode SVMP sequences, which are also highly diversified (44 clusters arranged in 12 GRCs). Since SVMP precursors of the P-III and P-IV classes may be ~2.4 kb in length [17] it was not possible to assemble unique full-length sequences, although fragments of all domains were detected. All clones encoding sequences that matched the catalytic domain showed the conserved zinc binding active site motif HEXXHXXGXXH, and all those matching additional domains showed a disintegrin-like region containing a DCD (or ECD) motif and a cysteine-rich sequence (Fig. 2A), homologous to those of the P-III class precursors proposed by Bjarnason and Fox [17]. No P-I or P-II class precursors of metalloproteases were identified. In fact, all *P. olfersii* SVMP clusters are similar among themselves, showing higher similarity levels to the few known Elapidae and Atractaspidae SVMPs than to the Viperidae ones. Nevertheless, a Northern-blot analysis probed with one of the clones (from GRC05) showed cross hybridization with Viperidae species and a restricted expression of this protease to the venom glands (Fig. 2B).

Various abundant spots visualized in a 2D gel were at the region expected for P-III class SVMPs (50–65 kDa/pI 4.5–7.0) and five of them could be identified by mass spectrometric analysis of trypsin-digested spots as SVMPs (Fig. 3; Supplementary Table 1). The analysis of four of these spots (labeled 1–4 in Fig. 3) showed matching fragments corresponding to internal segments of the catalytic, disintegrin-like and cysteine-rich domains, indicating that they contain full-length P-III class SVMPs. The spot 5 (~32 kDa) showed peptides matching only to the disintegrin-like/cysteine-rich region, suggesting that this protein is the result of the autolysis of a P-III SVMP. The global analysis of our partial and complete ESTs suggests that P-III class SVMPs are the major components of

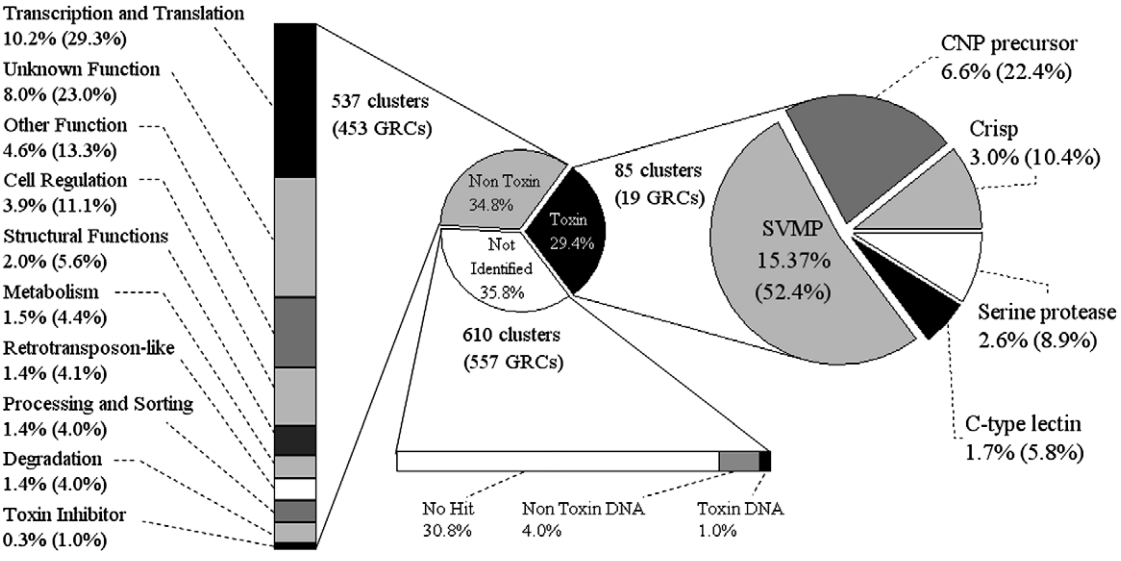

Fig. 1. Relative proportion of each category of product found. Toxin, non-toxin and not identified categories are, respectively, clusters matching GenBank snake toxin sequences, non snake toxin sequences, and no sequence with an *e*-value <10*e*−05 on Blastx search or inconclusive nucleotide matches on Blastn. Non-toxins are categorized by their cellular function, whereas toxins by their structural type. Number of clusters and GRCs, percentages of total ESTs and of ESTs in the category (in brackets) are presented.

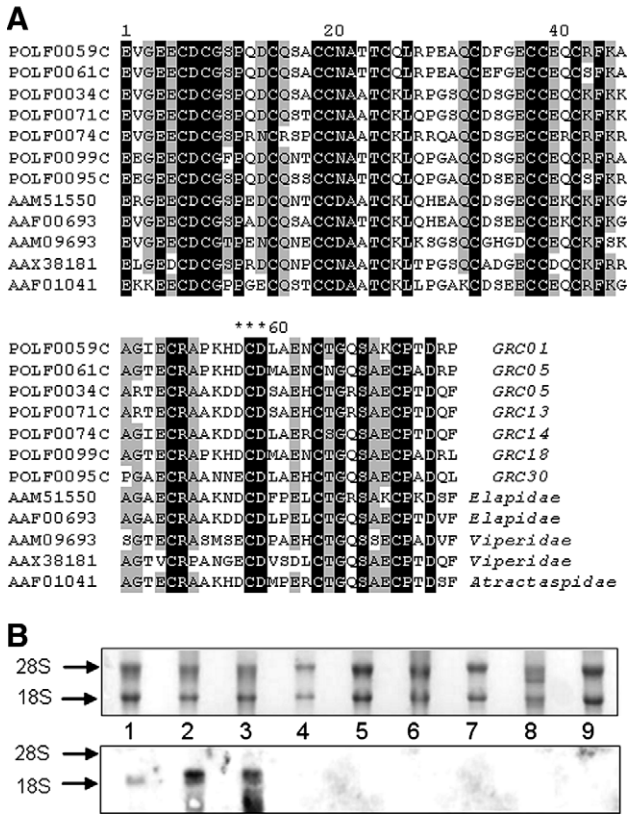

Fig. 2. The SVMPs from *P. olfersii.* (A) Alignment of the disintegrin-like domains of *P. olfersii* clusters coding for SVMPs (POLFs) with representative sequences of P-III SVMP precursors from other snake groups. The sequences are referred to their GenBank accession numbers and *** above the alignment marks the position corresponding to the RGD motif of true disintegrins. (B) Northern Blot analysis. Samples of total RNA (10 μg) from venom glands of: (1) *Crotalus durissus terrificus*, (2) *Bothrops insularis* (3) *P. olfersii* and other *P. olfersii* tissues: (4) brain, (5) lung, (6) kidney, (7) liver, (8) muscle, (9) ovary, were fractionated on a 1% formaldehyde agarose gel and stained with ethidium bromide (upper panel) before transferring to nitrocellulose sheets. A probe was prepared from 200 ng of purified cDNA insert from a clone included in cluster POLF0034C with Alkphos Direct Labelling and Detection System/CDP-star (GE Healthcare), hybridized to the membrane overnight at 55 °C and exposed to Hyperfilm (GE Healthcare) (lower panel).

P. olfersii venom and the main responsible for its most toxic effects.

3.3. Slightly differing serine proteases (SPs)

The other class of proteases commonly found in snake venoms is the SPs, whose precursors occur as tripsin-like enzymes in Viperidae or structured as more complex Factor X-like proteins in terrestrial Elapidae [18]. Until recently, no reports on Colubridae SPs sequences were available, in spite of fibrinogenolytic and amidolytic activities having been demonstrated for a SP isolated from the venom of *P. olfersii* [13]. However, Fry et al. reported five almost full-length sequences of SP isoforms from *P. olfersii* [11]. Here we found 9 clusters of tripsin-like SPs, including two full-length sequences. These are not identical to those previously described, but all of them are very similar to each other and showed typical features of Viperidae SPs (Supplementary Fig. 2A). No factor X-like enzyme was found among the *P. olfersii* ESTs, suggesting that the strategy

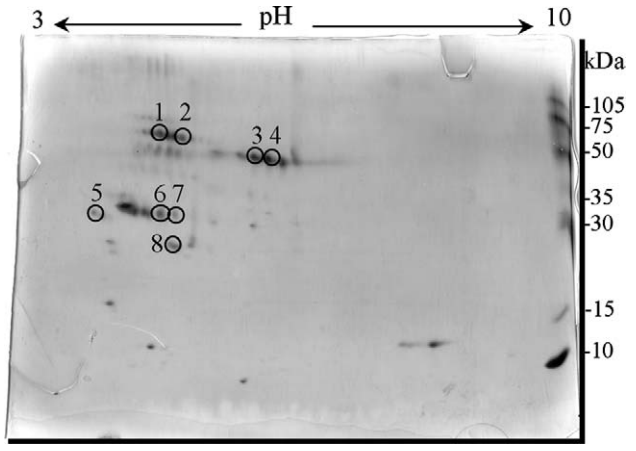

Fig. 3. 2D gel pattern of *P. olfersii* venom. Venom (500 μg) was submitted to first dimension isoelectric focusing (IEF) on a 3–10 IPG strip followed by electrophoresis on an 8–18% polyacrylamide gel. The gel was stained with silver. Spots indicated with numbers were identified by in-gel trypsin digestion and matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) peptide mass fingerprinting. Tryptic peptides were searched against the *P. olfersii* cluster database. Identified spots are: 1–5, SVMPs; 6,7, SPs and 8, Crisp as detailed in Supplementary Table 1.

of its venom to coating enzymes resembles that of the Viperidae snakes.

In the 2D gel, two spots were identified by mass spectrometry as matching SP clusters (Fig. 3, Supplementary Table 1). But contrarily to what happens in Viperidae, where a single specimen may contain a variety of SP sequences [6], all isoforms found here and elsewhere [11] share more than 90% of identity (Supplementary Fig. 2A), suggesting that they would display similar biological activities.

3.4. Non-enzymatic proteins: C-type lectins (CTLs) and Crisps

CTLs are dimeric proteins that contain a carbohydrate recognition domain, present in most snake venoms. The same venom usually contains one or a few forms of "true" lectins, which bind preferably galactose, and several forms of "CTL-like" proteins that have a variety of biological activities mainly upon the coagulation system. We identified six different clusters of CTL, arranged in 4 GRCs. The two most abundant GRCs (GRC03 and GRC11) are 10 times more expressed than the others. This suggests that these proteins might associate to form a heterodimeric CTL. These are the first Colubridae CTL described so far and their full-length sequences are more closely related to Elapidae sequences than to Viperidae ones (Supplementary Fig. 2). None of the clusters seems to encode a galactose binding CTL.

Cysteine rich secretory proteins, named Crisps, are a growing family of ancestral proteins found in the venoms of lizards and various snakes, which are related to proteins present in the mammalian male reproductive tract [19]. We found a full-length Crisp sequence nearly identical to the two almost full-length isoforms from *P. olfersii* reported by Fry et al. [11] (Supplementary Fig. 4). In this sequence, the same Val/Ile polymorphism exists at position 145, although only Glu_{205} but not Lys_{205}, was found in our cluster. The analysis of the spot 8 of the 2D gel by mass spectrometry confirmed the presence of a Crisp in the venom (Fig. 3, Supplementary Table 1).

3.5. *The* P. olfersii *natriuretic peptide (NP) precursor evolutionarily links Elapidae NP and Viperidae BPP/NP precursors*

NPs are hypotensive and vasodilator agents, whose precursors have been reported as organized in two ways among snakes (Fig. 4A). In Elapidae, a 110–140 amino acids ORF contains a signal peptide, a short propeptide and the NP with a C-terminal extension resembling that of vertebrate atrial and brain NPs (A/BNPs) [20]. Differently, in the Viperidae (Crotalinae subfamily), the precursor is a more complex molecule with a 180–270 amino acids ORF containing a signal peptide, a region with a variable number of bradykinin-potentiating peptides (BPPs) (from one to seven in different snake species) followed by a long intervening linker sequence and the C-type NP (CNP), without a C-terminal extension [21]. The BPPs are important inhibitors of the angiotensin converting enzyme that served as basis for the design of the anti-hypertensive drug captopril. However, to the best of our knowledge no BPPs or NPs have been described in Colubridae venoms.

Perhaps the most interesting finding of this report is a very abundant molecule (6.6% of total ESTs), showing an organization similar to that of the Viperidae BPP/CNP precursor, including the signal peptide, the linker and the CNP (without C-terminal extension), but lacking the entire BPPs region (Fig. 4B). Neither any of the typical BPP sequences nor the newly discovered bradykinin inhibitory peptide [22] were found in this Colubridae precursor. So, the only bioactive peptide recognized is the CNP, which is almost identical to the Viperidae CNP-22 consensus sequence, including the Cys_6 and Cys_{22} involved in the disulfide bond that results in the 17 residues ring structure of NPs. Both processing signals for the CNP-22 and for the N-terminally extended CNP-53 are present in the precursor, suggesting that it undergoes the same processing events of the mammalian CNP-53 (Fig. 4B).

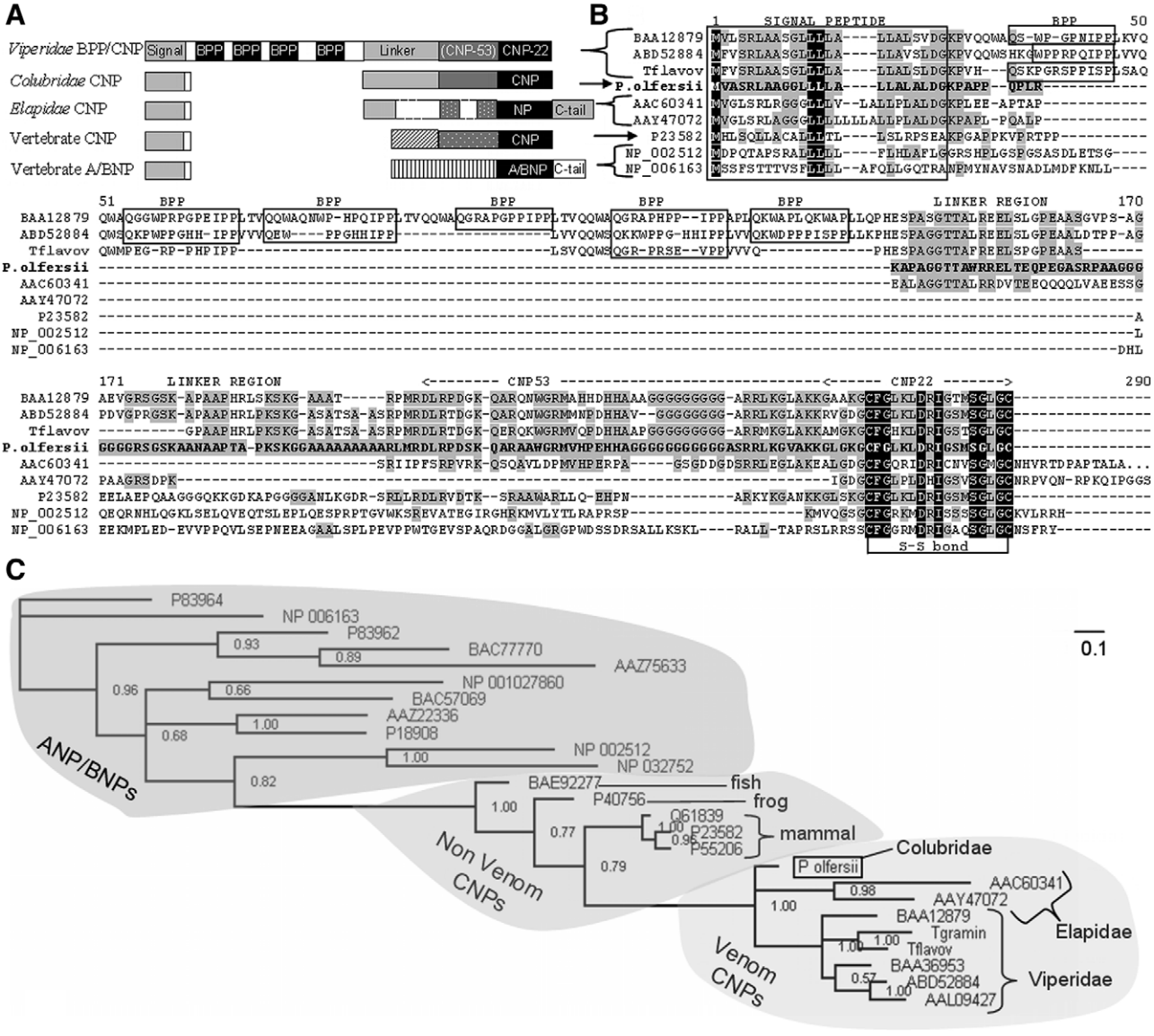

Fig. 4. Primary structure of the *P.olfersii* CNP precursor. (A) Schematic view of NP precursors in the different snake families and other vertebrates. (B) *P. olfersii* precursor (bold) is aligned to some representatives of such precursors, showing high conservation (shaded residues). (C) Bayesian inference of the phylogeny based upon the posterior probability distribution of the trees. MrBayes software version 3.1.1–p1 [27], with 10^6 number of cycles for the Markov chain Monte Carlo algorithm, was used to the analysis of the proteins indicated by their accession numbers on the tree.

It seems that the *P. olfersii* NP precursor possesses features of both kinds of precursors. N-terminally, it resembles the Elapidae ones (without the BPP region) and, C-terminally, the Viperidae CNP precursor. Bayesian phylogenetic analysis of these molecules indicates that, in fact, *P. olfersii* CNP precursor has a clear common origin with NPs from both Elapidae and Viperidae families (Fig. 4C), but it is the one that accumulated less substitutions (evidenced by its short branch length in the tree). Moreover, for the first time, venom and non-venom NPs were grouped in a single monophyletic group. Previous NP phylogenies have suggested a paraphyletic group, with Elapidae NP deriving from vertebrate A/BNPs [23]. This was probably because the previous analysis only took into account the peptide region, due to the poor information on full-length precursor sequences available at that time. The robust grouping of snake venom NP precursors achieved here suggests to us a hypothesis of a single recruitment event of this component to an ancestral snake venom arsenal, followed by a differentiation through segments gaining in each group of snakes. Perhaps in Colubridae, the longer linker was added to a common vertebrate CNP, whereas the Viperidae may have received also the segment containing the BPPs. On the other hand, in the Elapidae, a C-terminal extension was probably acquired de novo from the ancestral venom CNP, conferring their NPs the ability to bind and activate A/BNP specific type of receptors (NPR-A) [24]. In fact, the C-terminal tails of the venom NPs are not conserved, being longer than and different from those of the non-venom A/BNPs. Therefore, a de novo acquisition of the C-terminal extension seems more likely than all the necessary changes for the conversion of an A/BNP precursor into a snake venom NP. The ultimate resolution of venom NPs evolution will certainly depend on obtaining more precursor sequences from other Colubridae and Elapidae species.

3.6. Other transcripts and some candidates to novel toxin sequences

Besides the clusters clearly matching snake toxins, we detected others that would not be classified as toxins at a first glance. Their general profile (Fig. 1, left) is similar to that of other snake transcriptomes, including the presence of retrotransposon-like elements and toxin inhibitors [1,6]. However, some of these products could be pointed out as putative toxins due to some special features such as sequence, function and abundance (Supplementary Table 2).

Whereas most of the "no match" GRCs are at low redundance (~1 clone/cluster), GRC08 grouped 64 ESTs in 6 clusters, forming a 1.6 kb sequence with hundreds of stop codons in all frames. Although it is a non-coding sequence, it could be a very long 3'UTR at an abundance level equivalent to that of the most expressed toxins found here. We investigate its expression profile through a Northern-blot analysis (Fig. 5). It showed messages ubiquitously expressed in all *P. olfersii* tissues tested, but none in the other snake samples, suggesting that it is not a toxin. A major band far above the 28S ribosomal RNA (4800 pb) and a wide range of minor bands were detected in these samples, except for the venom gland, where the major band is not present. Interestingly, it was recently reported another abundant non-coding sequence in the transcriptome of *Lachesis muta* [6], but both sequences do not align at all. This kind of transcript seems to be important for snakes and should be further investigated.

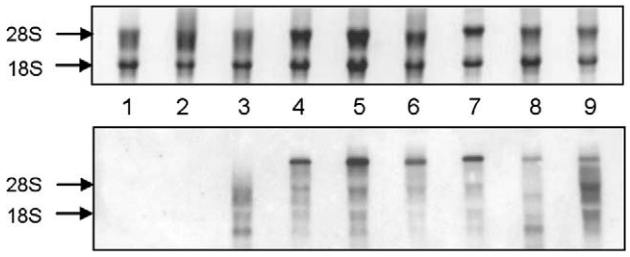

Fig. 5. Expression profile of GRC08. The samples of total RNA were prepared as described in Fig. 2B, except that the probe was prepared from a clone included in cluster POLF0042C (GRC08). The agarose gel (upper panel) and the revealed X-ray film (lower panel) are presented.

3.7. Conclusions on the repertoire of toxins of the Colubridae P. olfersii

Direct sequence comparisons revealed five toxin classes in this Colubridae (Fig. 2, right). All these classes are commonly observed in Viperidae, what could explain the molecular basis of the efficacy of anti-bothropic antiserum to neutralize almost all the *P. olfersii* venom toxicity [12]. Sequences of three of these classes (SVMP, CNP and CTL) are reported for the first time in a Colubridae venom. The high abundance of toxin transcripts in *P. olfersii* (~30%) is comparable to that previously reported from snake venom gland transcriptomes; however, the diversity of toxin classes is lower[1,3,5,6]. In *L. muta* (Viperidae) transcriptome, for example, 11 different types of toxins were found, but this number varies between 7 and 10 in the other catalogs. We did not observe sequences coding for one of the most ubiquitous component of snake venoms, the PLA2 enzymes. Although it has been observed in other Colubridae [10], our previous studies showed that the PLA2 activity of *P. olfersii* venom was minimal compared to that of other Viperidae venoms [12]. Moreover, no transcript coding for three-finger toxins was detected among our sequences, in spite of the fact that this is by far the major scaffold of Elapidae toxins, also characterized in a Colubridae venom [9] and recently described in a Viperidae transcriptome [6]. The low toxin diversity found in the transcriptome of *P. olfersii* is in agreement with the venom 2D gel (Fig. 3), which is much less complex than that of Viperidae and Elapidae venoms [2,4]. Nevertheless, other unknown classes of toxins may be present in *P. olfersii* venom gland but were not found by similarity searches (perhaps they are among the suggested ones in Supplementary Table 2).

The Duvernoy's gland secretions have been generally considered to resemble those of other medically important snake families [25]. However, the low complexity of *P. olfersii* venom demonstrated in this study might suggest a simplification of its toxin repertoire, in parallel with its venom inoculation apparatus. Since the venom properties should be more dependent on the different adaptation behaviors associated with the capture, swallowing and digestion of the prey [8] than to the snake phylogenetic positioning, this profile may be reflecting the particular arsenal of *P. olfersii*, and not necessary that of other Colubridae. The Colubridae family alone has more species (~1850), body shapes, dentitions and inhabits more distinct places than all the traditional families of venomous snakes together (~600 species of Viperidae, Elapidae, Atractaspidae) [26]. So, very discrepant venom transcriptomes/proteomes should be found in other species, making interesting to carry

out this kind of approach also to characterize the diversity of toxins from other Colubridae snakes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.07.010.

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