Gene 446 (2009) 35–40

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Intraspecific variation of the *crotamine* and *crotasin* genes in *Crotalus durissus* rattlesnakes

Nancy Oguiura [a,*], Maíra A. Collares [a], M. Fátima D. Furtado [b], Hebert Ferrarezzi [a], Hana Suzuki [c]

[a] *Laboratório Especial de Ecologia e Evolução - Instituto Butantan, Av. Dr. Vital Brasil, 1500 – CEP 05503-900 – São Paulo – Brazil*
[b] *Laboratório de Herpetologia - Instituto Butantan, Av. Dr. Vital Brasil, 1500 – CEP 05503-900 – São Paulo – SP - Brazil*
[c] *Laboratório de Biologia Celular - Instituto Butantan, Av. Dr. Vital Brasil, 1500 – CEP 05503-900 – São Paulo – SP - Brazil*

ARTICLE INFO

Article history:
Received 17 March 2009
Received in revised form 12 May 2009
Accepted 27 May 2009
Available online 10 June 2009

Received by J.G. Zhang

Keywords:
Rattlesnake
Beta-defensin
Crotamine
Gene copy number
Molecular evolution
Gene expression

ABSTRACT

Crotamine is a small basic myotoxin peptide of *Crotalus durissus* venom, with β-defensin scafold and variable concentration in individual venoms. The *crotamine* gene was mapped to the end of chromosome 2 and the signal intensity differed significantly between the two homologues. In contrast to *crotamine*, the paralogous *crotasin* gene is scarcely expressed in the venom glands. In this study, we analyzed the crotamine concentrations in the venoms of a total of 23 rattlesnakes from diverse Brazilian localities by ELISA as well as the copy number of both crotamine and crotasin genes by real-time PCR. Crotamine was found to constitute 5–29% of venom proteins varying greatly among individual animals. The *crotamine* gene exists from 1 to 32 copies per haploid genome, whereas the *crotasin* gene is present from 1 to 7 copies. Furthermore, we observed that the crotamine concentration and *crotamine* gene copy number are positively correlated ($r^2 = 0.68$), implying the variation of crotamine in venom results from the variation of the gene copy number. Sequencing of 50 independent copies of *crotamine* and *crotasin* genes from four different rattlesnakes revealed the presence of six crotasin isoforms with a single amino acid difference from the original crotasin sequence, whereas only two additional crotamine isoforms were observed. Taken together, our results suggested that after duplication from a common ancestor gene, crotamine and crotasin may have diverged in such a way that the *crotamine* gene underwent repetitive duplication to increase its copy number, whereas the *crotasin* gene diversified its sequence.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Crotalus durissus has a wide and discontinuous distribution in South America, from Colombia to Argentina, in all mainland countries except for Ecuador and Chile (Campbell and Lamar, 2004). Its venom is a mixture of various compounds and crotamine is the toxin which induces myonecrosis and spastic paralysis in the hind limbs of mice. This small myotoxin was first observed in *C. durissus* venoms from South America (Gonçalves and Polson, 1947; Gonçalves and Vieira, 1950) and, later, in other species of the genus *Crotalus* from North America (Bieber and Nedelkov, 1997). Recently, new activities were described to crotamine such as anti-leishmanial activity *in vitro* (Passero et al., 2007) and DNA carrier property *in vitro* and *in vivo* (Nascimento et al., 2007). Crotamine has 42 aa (Laure, 1975), three disulfide bridges and β-defensin scaffold (Fadel et al., 2005; Siqueira et al., 2002). The first polymorphism described for rattlesnake venoms were the crotamine-positive and crotamine-negative varieties (Gonçalves and Arantes, 1956), subsequently associated to specific geographic areas (Schenberg, 1959). Different crotamine isoforms and concentrations occurring in the individual venoms have also been described (Oguiura et al., 2005).

The *crotamine* (*Crt-p1*) gene (Rádis-Baptista et al., 2003) has 1.8 kbp organized in three exons and two introns and codifies the crotamine described by Laure (1975). The exon 1 includes the 5′-*UTR* and encodes the first 19 aa of the signal peptide. The exon 2 encodes 42 aa, three belonging to the remaining signal peptide and 39 to the mature crotamine, whereas the exon 3 encodes the last three aa of the mature toxin. *In situ* hybridization mapping revealed that the *crotamine* gene is located at the end of the long arm of chromosome 2, with the intensity of signals differing between the homologues.

The *crotasin* gene (*Cts-p2*), described by Rádis-Baptista et al. (2004), was discovered in crotamine-negative rattlesnakes by its similarity to *crotamine* sequence. *Cts-p2* is expressed highly in pancreas and scarcely in the venom glands. *Cts-p2* spans 2.5 kbp consisting of three exons and two introns. The first exon encodes the signal peptide; the exon 2 encodes the majority of the mature peptide and the third exon the C-terminal region. The similarity between *Crt-p1* and *Cts-p2* is of 96.9% in the signal peptide, 59.5% in the mature

Abbreviations: *Crt-p1*, crotamine gene; *Cts-p2*, crotasin gene; PBS, phosphate buffered saline; dNTP, dATP + dCTP + dGTP + dTTP; TNT, Tree analysis using New Technology; LD_{50}, Lethal Dose 50%; crotamine-ser 12, crotamine with serine at position 12; crotamine-ile19, crotamine with isoleucine at position 19.

* Corresponding author. Tel.: +55 11 3726 7222x2073; fax: +55 11 3726 7222x2014.
E-mail address: nancyoguiura@butantan.gov.br (N. Oguiura).

0378-1119/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2009.05.015

peptide, 93.4% in intron sequences, 93.4% in 5′-*UTR* and 100% in 3′-*UTR*. The intron 1 of *Cts-p2* has a sequence duplication of 0.7 kbp. Crotasin, like crotamine, is a peptide with presumed antibiotic activity since it exhibits the β-defensin scaffold.

The *crotamine* and *crotasin* genes are paralogous evolved through an accelerated evolution of exon 2 (Rádis-Baptista et al., 2004). The first is found only in crotamine-positive rattlesnakes, whereas the latter occurs in all of them.

Considering the variation of crotamine concentration in rattlesnake venom (Oguiura et al., 2000) and the difference in signal intensities of the *crotamine* gene in both homologous chromosomes (Rádis-Baptista et al., 2004), we sought to determine the relationship between the crotamine concentration in the venom and the gene copy number. We also sequenced the exon 2, intron 2 and exon 3 of *crotamine* and *crotasin* genes of rattlesnakes from different localities in order to investigate their genetic variability and the evolutionary relationships of the sequences.

2. Materials and methods

Venom and liver tissue were collected from 23 snakes captured from various localities in Brazil (Table 1). The snakes were sexed, their snout–vent length measures and weights were taken, and the specimens were deposited in the Herpetological Collection "Alphonse Richard Hoge" of the Instituto Butantan. The venoms were centrifuged at 3000 rpm for 30 min at 4 °C, lyophilized and stored at −20 °C. The liver tissues were stored at −20 °C.

2.1. Determination of crotamine concentration

The venoms were diluted in PBS for ELISA tests. Crotamine was quantified in total venom by ELISA and the total venom proteins by folin phenol method (Lowry et al., 1951). Each sample was diluted in triplicates and then submitted to serial dilutions. The trendline was obtained with the averages of each dilution, and the crotamine concentration was estimated by the parallel-line described in Oguiura et al. (2000). The crotamine concentration is presented as % of total venom protein.

2.2. DNA purification and quantification

The genomic DNA was extracted from the liver tissues using SDS and proteinase K in Tris.HCl buffer with 2.5% sucrose, precipitated with NaCl/ethanol (Rocha, 2001), and purified with GFX PCR and Gel Band Purification Kit (Amersham Biosciences).

The DNA was quantified using SYBR Green I Nucleic Acid Gel Stain (Invitrogen) that is selective for double-stranded DNA. The fluorescence measurements were taken at room temperature using the Thermo Spectronic Series 2 spectrofluorometer (Aminco–Bowman). The excitation and emission wavelengths used were 497 nm and 520 nm, respectively.

The calibration curve was established using λ DNA from 1.25 to 20 ng/mL (Roche). Each point plotted was an average of the triplicate fluorescence values. The concentrations of DNA samples were estimated based on the linear regression of the calibration curve (Rengarajan et al., 2002).

2.3. Quantification of crotamine and crotasin gene

The copy numbers of *crotamine* and *crotasin* genes were determined by absolute quantitative real-time PCR (Lee et al., 2006) in the PTC-200 Peltier Thermal Cycler/Chromo 4 System (MJ Research), using the Opticon Monitor 2 software. Briefly, gene copy numbers were estimated based on calibration curve of absolute number of plasmids containing the *crotamine* cDNA (KA 19, Rádis-Baptista et al., 1999), or the *crotasin* cDNA (pBAD-cts was provided by Dr G. Rádis-Baptista). The gene copy numbers were the average of triplicates of each sample.

The real-time qPCR for the *crotamine* gene was performed using the mixture of 20 μL with ABsolute™ QPCR SYBR Green Mix kit (AB Gene), 13 ng of genomic DNA, 140 nM of each primer 5-crot (CAGTGTCATAAGAAAGGAGG) and 3-crot (CATCTCCATCGACAGTCCAT), and the following thermal cycling protocol: 15 min at 95 °C followed by 30 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. After the amplification, the melting curve analysis was performed with a temperature gradient from 55 to 90 °C at 1 °C/min. The real-time qPCR for the *crotasin* gene was performed similarly as described

Table 1
Locality of capture, crotamine concentration and copy number (average ± standard deviation) of haploid genome of *crotamine* (*Crot*) and *crotasin* (*Cts*) genes.

Rattlesnakes	Locality of capture (State)	Coordinates	Crotamine concentration	Gene copy number[a]	
				Crot	*Cts*
Cd172	Itumbiara (GO)	18°25'09 S 49°12'55 W	9	7 ± 0.1	ND[b]
IBSP 60277	Itarumã (GO)	18°46'09 S 51°20'53 W	8	9 ± 1.1	7 ± 0.1
IBSP 60276	Itarumã (GO)	18°46'09 S 51°20'53 W	19	25 ± 5.5	ND[b]
IBSP 60115	Guarantã (SP)	21°53'42 S 49°35'23 W	29	32 ± 4.9	4 ± 1.0
IBSP 75906	Pompéia (SP)	22°06'31 S 50°10'18 W	12	24 ± 2.3	ND[b]
Cd106	Anaurilândia (MS)	22°11'15 S 52°43'04 W	8	14 ± 1.5	ND[b]
IBSP 61191	Diamante do Norte (PR)	22°39'23 S 52°51'35 W	17	18 ± 0.4	2 ± 0.2
Cd206	Pardinho (SP)	23°04'52 S 48°22'25 W	0	0	1 ± 0.3
IBSP 75908	Pardinho (SP)	23°04'52 S 48°22'25 W	10	15 ± 2.5	ND[b]
IBSP 74941	Jaguapitã (PR)	23°06'46 S 51°31'55 W	11	5 ± 1.5	5 ± 0.6
IBSP 74942	Jaguapitã (PR)	23°06'46 S 51°31'55 W	12	5 ± 0.9	5 ± 0.5
IBSP 75023	Ribeirão Claro (PR)	23°11'39 S 49°45'29 W	14	8 ± 0.5	3 ± 1.0
IBSP 74926	Ribeirão Claro (PR)	23°11'39 S 49°45'29 W	0	0	2 ± 0.5
IBSP 74927	Ribeirão Claro (PR)	23°11'39 S 49°45'29 W	0	0	1 ± 0.2
IBSP 61201	Marialva (PR)	23°29'06 S 51°47'30 W	7	3 ± 0.04	ND[b]
IBSP 61200	Marialva (PR)	23°29'06 S 51°47'30 W	14	24 ± 1.5	ND[b]
IBSP 61807	Cianorte (PR)	23°39'48 S 52°36'18 W	9	1 ± 0.2	ND[b]
IBSP 61808	Cianorte (PR)	23°39'48 S 52°36'18 W	16	14 ± 2.4	ND[b]
IBSP 61322	Tapiraí (SP)	23°57'49 S 47°30'26 W	5	4 ± 1.2	ND[b]
IBSP 56468	Igautemi (MS)	24°38'10 S 54°05'47 W	6	1 ± 0.2	ND[b]
IBSP 56469	Igautemi (MS)	24°38'10 S 54°05'47 W	7	7 ± 0.9	2 ± 0.7
IBSP 61275	Cascavel (PR)	24°57'21 S 53°27'19 W	14	8 ± 1.8	ND[b]
IBSP 75714	São Miguel do Iguaçu (PR)	25°22'25 S 54°12'59 W	20	19 ± 1.9	ND[b]

[a] The *C. d. terrificus* haploid genome is 1.3 pg according to Olmo, 1981.
[b] ND = not determined.

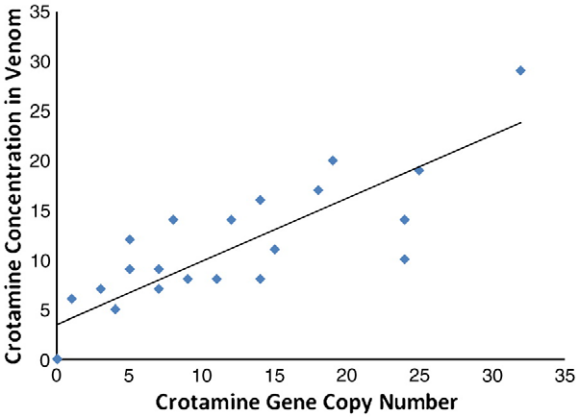

Fig. 1. Relationship between crotamine concentration and gene copy number.

for the *crotamine* gene using the primers (cts-1F, CCACAGTGTCGTTGGCTAGA; cts-1R, TGCAGCAGCTCTCATACCAC) at 200 nM each. The annealing temperature in the thermal cycling protocol was set at 55 °C.

The real-time qPCR was optimized for DNA and primers concentrations and for annealing temperature. A single peak at the predicted melt temperature indicated the specificity of PCR products.

2.4. Cloning and sequencing of PCR fragments

PCR reactions were performed in PTC-100 (MJ Research). The PCR mixture for the *crotamine* gene amplification consisted of 30 μL with 200 ng of template DNA, 200 nM of dNTP, 200 nM of each 5-crot and 3-UTRas (GGCACTCTCAGGTCCTTGGCCAT) primers, and 1 U of Ultratools DNA polymerase (Biotools) in standard reaction buffer (with 2 mM $MgCl_2$). The thermal cycling protocol was 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C. The PCR for the *crotasin* gene amplification was performed similarly as described for the *crotamine* gene using the primers cts-1F and 3-UTRas, and 55 °C as annealing temperature.

The PCR fragment was cloned using the pMOS*Blue* blunt ended cloning kit (Amersham Pharmacia Biotech) and nine clones were purified with GFX Micro Plasmid Prep kit (Amersham Bioscience). The clones were sequenced using the Big Dye Terminator V 3.1 Cycle Sequencing kit on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequences were analyzed using BioEdit 7.0.5.3 (Hall, 1999).

2.5. Phylogenetic analysis

Phylogenetic analyses of *crotamine* and *crotasin* genes were performed using sequences obtained in the present study and the retrieved from GenBank (AF408396, AF250212, AF223947, AF223946, AF044674, AF053075, and AF055988). Maximum Parsimony trees were generated using TNT (Goloboff et al., 2008), arbitrarily rooted with AF408396. The matrix consisted of a 3 141 bp alignment of the full-length GenBank sequences including 231 variable and 101 parsimony informative positions. The gaps were read as one additional state, independently of the gap extension.

3. Results

Table 1 shows that crotamine concentrations varied from 5 to 29% of total venom protein, the *crotamine* genes from 1 to 32 copies per haploid genome, and the *crotasin* gene from 1 to 7 copies among 23 rattlesnakes analyzed. The crotamine concentration and *crotamine* gene copy number per haploid genome were well correlated ($r^2 = 0.68$ and $p < 0.001$) (Fig. 1), suggesting the variation of crotamine in venom results from the variation of the gene copy number.

Four rattlesnakes from different localities had the *crotamine* and *crotasin* genes sequenced: IBSP60277 from Itarumã (GO), IBSP60115 from Guarantã (SP), IBSP61191 from Diamante do Norte (PR), and IBSP56469 from Igautemi (MS). PCR using the 5-crot and 3-UTRas primers resulted in *crotamine* amplicons of about 0.3 kbp. After the *crotamine* cloning in pMOSblue vector, nine aleatory transformants were taken and the recombinant plasmids sequenced. The GenBank accession number and the deduced aa sequence are shown in Table 2. The analyzed rattlesnakes had multiple sequences encoding mainly the crotamine described by Laure (1975) and few, such as EU488730 and EU488732, encoding crotamine-ser12 and crotamine-ile19 (Santos et al., 1993), respectively. The sequences EU488732 and EU488731 do not have introns, and in general most of the mutations occurred in non-translated regions (Fig. 2).

PCR using the cts-1F and 3-UTRas primers resulted in *crotasin* amplicons of about 0.3 kbp. The GenBank accession number and the deduced aa sequence are shown in Table 3. The analyzed rattlesnakes had multiple sequences codifying not only the crotasin described by Rádis-Baptista et al. (2004) but also others peptides modified by just one amino acid. The majority of the mutations occurred in the exon 3 and 3′ *UTR* (Fig. 3). The observed diversity of *crotamine* and *crotasin* sequences indicates they comprise multigene families.

Fig. 4 shows the phylogenetic trees of *crotamine* and *crotasin* sequences, strict consensus tree (left) and two of the 74 original trees selected to show relative branch lengths, which in the case of *crotasin* comprise all the possibilities recovered (right). The trees show that *crotamine* and *crotasin* multigene families are distinctly separated in 2 major lineages, both with multiple paralogous copies, with the branch lengths indicating more differentiation in the *crotamine* copies. The original trees with relative branch lengths also show that the

Table 2
GenBank accession number of *crotamine* sequences and translation of exons.

Rattlesnake	Sequences	GenBank accession number	Translation of nt sequence
IBSP 60277	13B	EU488733	HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
	14B	EU488734	HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
	8B	EU488732	HCFPKEKIC**I** PPSSDFGKMD CRWRWKCCKK GSGK
	7B, 9B, 10B, 12B		HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
IBSP 60115	4K	EU488728	HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
	7K	EU488729	HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
	1E, 3E, 8E, 3K		HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
IBSP 61191	17	EU488727	HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
	15, 12, 13F, 14, 8A		HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
IBSP 56469	3A	EU488730	HC**S**PKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
	6B	EU488731	HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK
	6, 5F, 4, 4F, 9		HCFPKEKICL PPSSDFGKMD CRWRWKCCKK GSGK

The amino acid substitutions are highlighted.

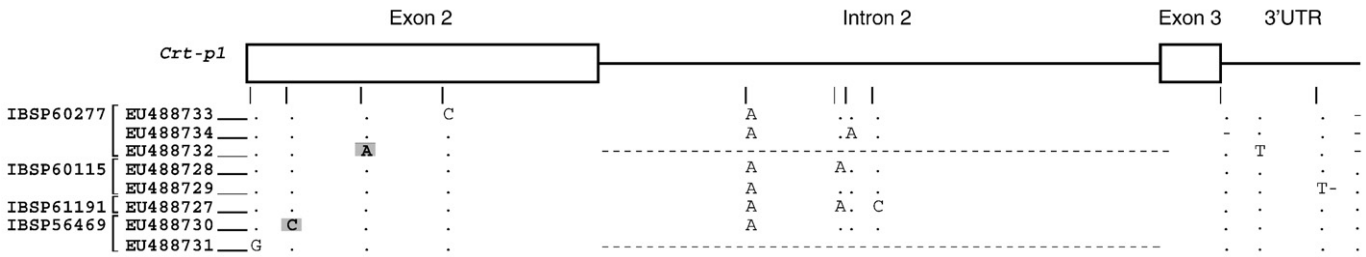

Fig. 2. Diagram of substitution pattern in comparison to *Crt-p1* (Radis-Baptista et al., 2003). Non-synonymous mutations are highlighted, nucleotide identity indicated by dots, and deletion indicated by dashes.

sequence EU828607 remained identical to most recent common ancestral *crotasin* gene.

4. Discussion

Schenberg (1959) observed a geographic distribution of crotamine-positive and crotamine-negative rattlesnake varieties in a limited area of longitudes 46 W to 52 W and latitudes 20S to 24S. He found that the crotamine-positive varieties concentrate in the northwest, whereas the crotamine-negative varieties predominate in the southeast of this area. On the contrary, we found both varieties in his crotamine-negative predominant area, namely IBSP-75908 (Pardinho - SP), IBSP-74926 and IBSP-74927 (Ribeirão Claro - PR). This fact may be because ELISA is a more sensitivity method than the intraperitoneal injection of venom in mice used by Schenberg (1959). Interestingly, most of the venoms from localities adjacent to the crotamine-positive area had crotamine concentrations higher than 10%, in contrast to those from the other areas, in which the majority had less than 10% of crotamine.

Correlations between protein concentration and gene copy number have been observed in many groups. In humans, such correlation is known for α-defensins (Linzmeier and Ganz, 2005), amylase gene (Perry et al. 2007), and an indirectly relation was described between the mRNA level and β-defensin genes (Hollox et al., 2003). To our knowledge, the present study is the first report of variations of toxin concentrations related to gene copy number in snakes.

Some slight deviations in the crotamine concentration and copy number correlation might be explained by the gene quantification assay used or by non-translated sequence alterations. The set of primers used in real-time qPCR did not distinguish the functional gene from pseudogene since they hybridize in exon 2; therefore, the intronless sequences EU488732 (IBSP60277) and EU488731 (IBSP56469) (Fig. 2) could increase the gene copy number but not the crotamine expression. Besides, crotamine concentration might be affected by mutations in 3′ *UTR* sequences as in EU488734 and EU488729 sequences (Fig. 2). In such cases, mutations could change the efficiency of mRNA ligation to ribosomes or mRNA stability (Kozak, 1997; Preiss and Hentze, 1999).

Snake venom is a complex mixture of toxins and other components, primarily with the function of immobilizing and digesting prey, and also having a direct toxic effect upon bacteria in the prey's gut (Chippaux et al., 1991, Thomas and Pough, 1979). Crotamine is a minor component of the rattlesnake venom which induces myonecrosis and spastic paralysis in the hind limbs of mice, with the β-defensin scaffold suggesting antibiotic activity. The crotamine-negative variety, lacking the gene, presents the same toxicity, in terms of DL50, as the crotamine-positive variety. This polymorphism in *Crotalus durissus* may be important for facing environmental changes and the variability in such a minor components causing no loss in toxicity may be under the influence of genetic drift (Chippaux et al., 1991).

The high copy number of crotamine gene, and most being identical in the analyzed stretch might suggest concerted evolution as described for sea anemone neurotoxin (Moran et al., 2008). However, this model does not fit because of the occurrence of pseudogenes with 5′ UTR mutations, described by Smith and Schmidt (1990) and Radis-Baptista et al. (1999). The birth-and-death model (Nei and Rooney, 2005) also might explain our results, however gene information of

Table 3
GenBank accession number of crotasin sequences and translation of exons.

Rattlesnake	Sequences	GenBank accession number	Translation of nt sequence
IBSP 60277	6Z	EU828613	GFCHSSPCPS GTTSIGHQDC LWYERCCIPR YEK
	7Z	EU828609	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR DEK
	3Z	EU828608	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK
	1Z–2Z	EU828607–EU828611	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR CEK
	5Z–9Z	EU828610–EU828612	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK
IBSP 60115	5V	EU828603	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK
	8V	EU828604	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK
	7V	EU828605	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR DEK
	9V	EU828606	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK
IBSP 61191	2X	EU828599	GFCHSSPCPS GTTSIVQQDC LWYERCCIPR YEK
	8X	EU828600	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR DEK
	3X–5X	EU828601–EU828602	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK
IBSP 56469	8Y	EU828614	GFCHSSPCPS GTTSIGHQDC LWYERCCIPR YEK
	4Y	EU828615	GFCHSSPCPS GTTSIGQQDC LWYEGCCIPR CEK
	6Y	EU828616	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK
	5Y–7Y	EU828617–EU828618	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR DEK
	1Y–2Y–3Y–9Y	EU828619–EU828620–EU828621–EU828622	GFCHSSPCPS GTTSIGQQDC LWYERCCIPR YEK

The amino acid substitutions are highlighted.

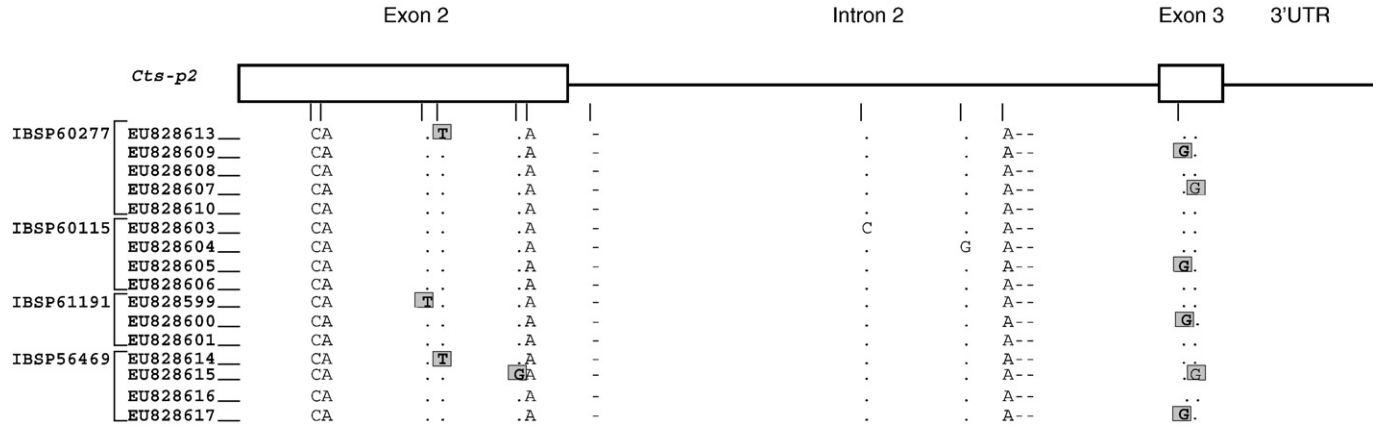

Fig. 3. Diagram of substitution pattern in comparions to *Cts-p2* (Radis-Baptista et al., 2004). Non-synonymous mutations are highlighted, nucleotide identity indicated by dots, and deletion indicated by dashes. Substitutions in 3′ UTR are not shown.

related species is needed for consistent conclusion about the evolutionary model. The subtelomeric localization of crotamine gene might explain the variability of the copy number because it was observed an accelerated rate of duplication and rearrangement in regions near the telomeres and centromeres of primate genomes, these events may result in large segments of nearly identical sequences (Samonte and Eichler, 2002).

According to Rádis-Baptista et al. (2004), *crotamine* and *crotasin* genes have evolved through an accelerated evolution of the exon 2, and these multigene families appeared as discrete lineages in the

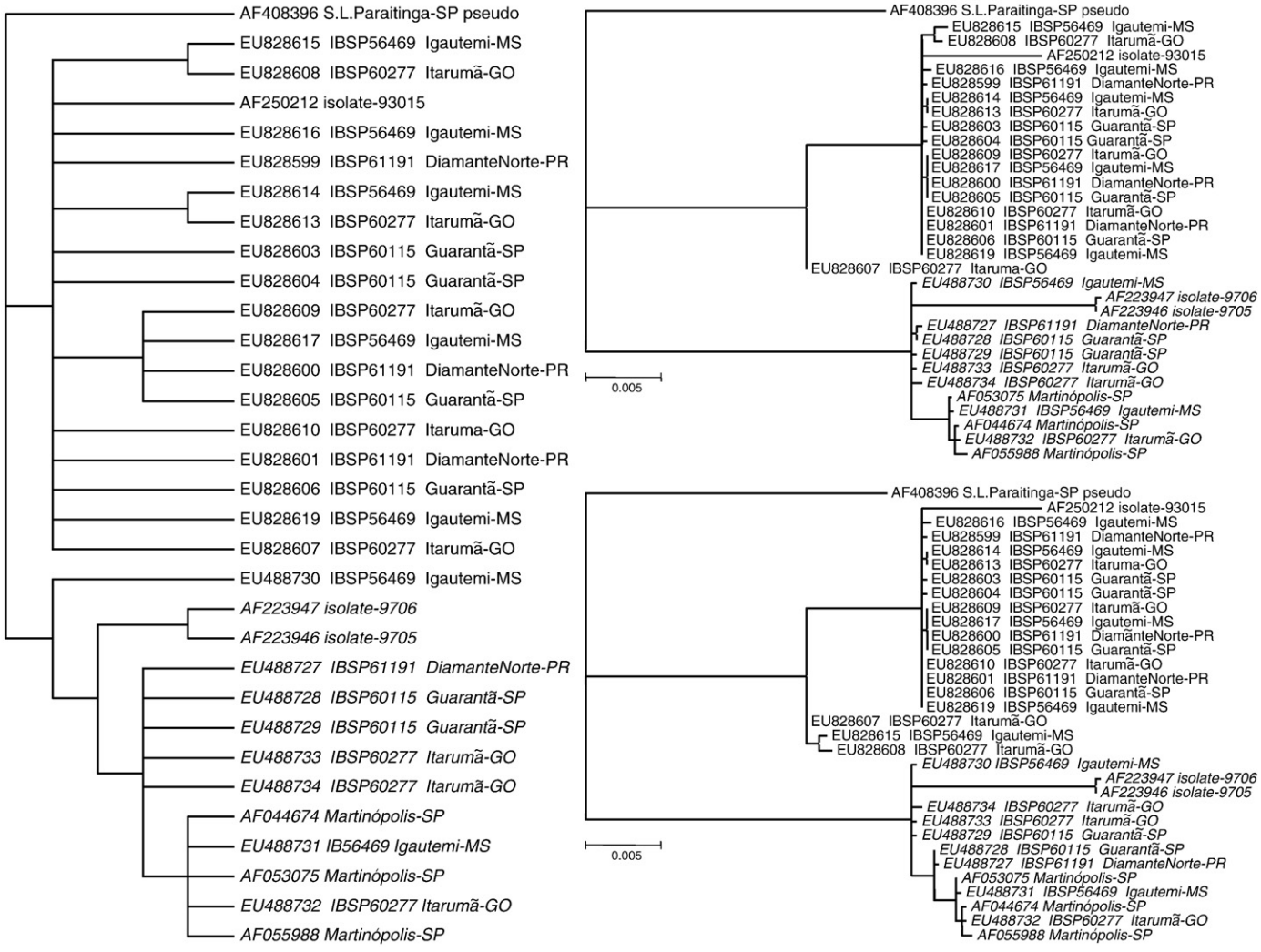

Fig. 4. Evolutionary relationships of the crotasin and crotamin sequences, named by their GenBank accession number, respective voucher specimens (if available) and locality of capture. Best score = 304 steps; consistency index = 0.99; retention index = 0.995). Crotamine sequences are shown in italic type.

phylogenetic trees. Despite of sequences of *crotamine* being more differentiated than *crotasin* ones, as indicated in the phylogenetic trees, these latter codify a great number of distinct peptides (Table 3). The rattlesnakes IBSP60277, IBSP60115, and IBSP61191 present only one crotamine sequence and IB56469 produces two isoforms, but regarding *crotasin*, they present two (IB60115), three (IB61191) and four (IB54469, IB60277) isoforms This fact may suggest they are in accelerated evolution like other defensin genes of rodents (Morrison et al., 2003) and humans (Semple et al., 2003).

5. Conclusions

The concentration of crotamine in rattlesnake venoms depends on the copy number of its gene, since an increase in gene copy number is positively correlated to an increase in crotamine concentration.

After the duplication from a common ancestor gene, *crotamine* gene may have undergone repetitive duplication in order to increase its copy number, whereas the *crotasin* gene has diversified its sequences, forming two separate evolutionary lineages.

Acknowledgements

We acknowledge Centro de Biotecnologia, Instituto Butantan for the sequencing and facilities, Laboratório de Imunogenética, Instituto Butantan for the Real Time PCR instrument, and FUNDAP for the fellowship to M.A.C. We are grateful to the anonymous reviewers for the valuable suggestions which improved this manuscript. TNT program is sponsored by Willi Hennig Society. This work was supported by FAPESP 04/09664-0.

References

Bieber, A.L., Nedelkov, D., 1997. Structural, biological and biochemical studies of myotoxin *a* and homologous myotoxins, J. Toxicol. Toxin. Rev. 16, 33–52.
Campbell, J.A., Lamar, W.W., 2004. Pitvipers, family Viperidae — rattlesnakes. The Venomous Reptiles of the Western Hemisphere, vol. 2. Cornell University Press, New York, pp. 477–616.
Chippaux, J.-P., Williams, V., White, J., 1991. Snake venom variability: methods of study results and interpretation. Toxicon 29, 1279–1303.
Fadel, V., Bettendorff, P., Herrmann, T., Azevedo Jr., W.F., de Oliveira, E.B., Yamane, T., Wüthrich, K., 2005. Automated NMR structure determination and disulfide bond identification of the myotoxin crotamine from *Crotalus durissus terrificus*. Toxicon 46, 759–767.
Goloboff, P., Farris, J., Nixon, K., 2008. TNT, a free program for phylogentic analysis. Cladistics 24, 774–786.
Gonçalves, J.M., Arantes, E.G., 1956. Estudos sobre venenos de serpentes brasileiras. III - Determinação quantitativa de crotamina no veneno de cascavel brasileira. An. Acad. Bras. Cienc 28, 369–371.
Gonçalves, J.M., Polson, A., 1947. The electrophoretic analysis of snake venoms. Arch. Biochem 13, 253–259.
Gonçalves, J.M., Vieira, L.G., 1950. Estudos sobre venenos de serpentes brasileiras. I - Análise eletroforética. An. Acad. Bras. Cienc 22, 141–150.
Hall, T.A., 1999. BioEdit: an user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser 41, 95–98.
Hollox, E.J., Armour, J.A.L., Barber, J.C.K., 2003. Extensive normal copy number variation of a β-defensin antimicrobial-gene cluster. Am. J. Hum. Genet. 73, 591–600.
Kozak, M., 1997. Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. EMBO J. 16, 2482–2492.
Laure, C.J., 1975. Die primärstruktur des crotamins. Hoppe-Seyler's Z. Physiol. Chem. 356, 213–215.
Lee, C., Kim, J., Shin, S.G., Hwang, S., 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. 123, 273–280.
Linzmeier, R.M., Ganz, T., 2005. Human defensin gene copy number polymorphisms: comprehensive analysis of independent variation in α- and β-defensin regions at 8p22–p23. Genomics 86, 423–430.
Lowry, O.H., Rosenbourgh, N.J, Farr, J.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.
Moran, Y., Weinberger, H., Sullivan, J.C., Reitzel, A.M., Finnerty, J.R., Gurevitz, M., 2008. Concerted evolution of sea anemone neurotoxin genes is revealed through analysis of the *Nematostella vectensis* genome. Mol. Biol. Evol. 25, 737–747.
Morrison, G.M., Semple, C.A.M., Kilanowski, F.M., Hill, R.E., Dorin, J.R., 2003. Signal Sequence conservation and mature peptide divergence within subgroups of the murine β-defensin gene family. Mol. Biol. Evol. 20, 460–470.
Nascimento, F.D., Hayashi, M.A.F., Kerkis, A., Oliveira, V., Oliveira, E.B., Rádis-Baptista, G., Nader, H.B., Yamane, T., Tersario, I.L.S., 2007. Crotamine mediates gene delivery into cells through the binding to heparan sulfate proteoglycans. J. Biol. Chem. 282, 21349–21359.
Nei, M., Rooney, A.P., 2005. Concerted and birth-and-death evolution of multigene families. Annu. Rev. Genet. 39, 121–152.
Oguiura, N., Boni-Mitake, M., Rádis-Baptista, G., 2005. New view on crotamine, a small basic polypeptide myotoxin from South American rattlesnake venom. Toxicon 46, 363–370.
Oguiura, N., Camargo, M.E., Da Silva, A.R.P., Horton, D.S.P.Q., 2000. Quantification of crotamine, a small basic myotoxin, in South American rattlesnake (*Crotalus durissus terrificus*) venom by enzyme-linked immunosorbent assay with parallel-lines analysis. Toxicon 38, 443–448.
Olmo, E., 1981. Evolution of genome size and DNA base composition in reptiles. Genetica 57, 39–50.
Passero, L.F.D., Tomokane, T.Y., Corbett, C.E.P., Laurenti, M.D., Toyama, M.H., 2007. Comparative studies of the anti-leishmanial activity of three *Crotalus durissus* ssp. venoms. Parasitol. Res. 101, 1365–1371.
Perry, G.H., Dominy, N.J., Claw, K.G., Lee, A.S., Fiegler, H., Redon, R., Werner, J., Villanea, F.A., Mountain, J.L., Misra, R., Carter, N.P., Lee, C., Stone, A.C., 2007. Diet and the evolution of human amylase gene copy number variation. Nat. Genet. 39, 1256–1260.
Preiss, T., Hentze, M.W., 1999. From factors to mechanism: transition and translational control in eukaryotes. Curr. Opin. Genet. Dev. 9, 515–521.
Rádis-Baptista, G., Kubo, T., Oguiura, N., Da Silva, A.R.B.P., Hayashi, M.A.F., Oliveira, E.B., Yamane, T., 2004. Identification of crotasin, a crotamine-related gene of *Crotalus durissus terrificus*. Toxicon 43, 751–759.
Rádis-Baptista, G., Kubo, T., Oguiura, N., Svartman, M., Almeida, T.M.B., Batistic, R.F., Oliveira, E.B., Vianna-Morgante, A.M., Yamane, T., 2003. Structure and chromosomal localization of the gene for crotamine, a toxin from the South American rattlesnake, *Crotalus durissus terrificus*. Toxicon 42, 747–752.
Rádis-Baptista, G., Oguiura, N., Hayashi, M.A.F., Camargo, M.E., Grego, K.F., Oliveira, E.B., Yamane, T., 1999. Nucleotide sequence of crotamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*). Toxicon 37, 973–984.
Rengarajan, K., Cristol, S.M., Mehta, M., Nickerson, J.M., 2002. Quantifying DNA concentrations using fluorometry: a comparison of fluorophores. Mol. Vis. 8, 416–421.
Rocha, L.S., 2001. Espécies crípticas do complexo *Anastrepha fraterculus* (Diptera, Tephtitidae): estudo sobre o DNA repetitivo. Dissertação, grau Mestre, Biologia / Genética - Instituto de Biociências, Universidade de São Paulo, São Paulo, 61p.
Samonte, R.V., Eichler, E.E., 2002. Segmental duplications and the evolution of the primate genome. Nat. Rev. Genet. 3, 65–72.
Santos, M.C., dos Morhy, L., Ferreira, L.C.L, Oliveira, E.B., 1993. Purification and properties of a crotamine analog from *Crotalus durissus ruruima* venom. Toxicon 31, 166.
Schenberg, S., 1959. Geographical pattern of crotamine distribution in the same rattlesnake subspecies. Science 129, 1361–1363.
Semple, C.A.M., Rolfe, M., Dorin, J.R., 2003. Duplication and selection in the evolution of primate β-defensin genes. Genome Biol. 4, R31.
Siqueira, A.M., Martins, N.F., De Lima, M.E., Diniz, C.R., Cartier, A., Brown, D., Maigret, B., 2002. A proposed 3D structure for crotamine based on homology building, molecular simulations and circular dichroism. J. Mol. Graph. Model 20, 389–398.
Smith, L.A., Schmidt, J.J., 1990. Cloning and nucleotide sequences of crotamine genes. Toxicon 28, 575–585.
Thomas, R.G., Pough, F.H., 1979. The effect of rattlesnake venom on digestion of prey. Toxicon 17, 221–228.