Toxicon 69 (2013) 65–74

Contents lists available at SciVerse ScienceDirect

Toxicon

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

Phylogenetic analysis of β-defensin-like genes of *Bothrops*, *Crotalus* and *Lachesis* snakes

Poliana G. Correa, Nancy Oguiura*

Ecology and Evolution Laboratory – Butantan Institute, Av. Dr. Vital Brasil 1500, CEP 05503-900 – São Paulo, Brazil

ARTICLE INFO

Article history:
Received 4 November 2012
Received in revised form 11 February 2013
Accepted 19 February 2013
Available online 14 March 2013

Keywords:
Beta-defensin
Innate immunity
Brazilian pitvipers
Snakes
Gene tree
Birth-and-death evolution

ABSTRACT

Defensins are components of the vertebrate innate immune system; they comprise a diverse group of small cationic antimicrobial peptides. Among them, β-defensins have a characteristic β-sheet-rich fold plus six conserved cysteines with particular spacing and intramolecular bonds. They have been fully studied in mammals, but there is little information about them in snakes. Using a PCR approach, we described 13 β-defensin-like sequences in *Bothrops* and *Lachesis* snakes. The genes are organized in three exons and two introns, with exception of *B.atrox_defensinB_01* which has only two exons. They show high similarities in exon 1, intron 1 and intron 2, but exons 2 and 3 have undergone accelerated evolution. The theoretical translated sequences encode a pre-β-defensin-like molecule with a conserved signal peptide and a mature peptide. The signal peptides are leucine-rich and the mature β-defensin-like molecules have a size around 4.5 kDa, a net charge from +2 to +11, and the conserved cysteine motif. Phylogenetic analysis was done using maximum parsimony, maximum likelihood and Bayesian analyses, and all resulted in similar topologies with slight differences. The genus *Bothrops* displayed two separate lineages. The reconciliation of gene trees and species tree indicated eight to nine duplications and 23 to 29 extinctions depending on the gene tree used. Our results together with previously published data indicate that the ancestral β-defensin-like gene may have three exons in vertebrates and that their evolution occurred according to a birth-and-death model.

1. Introduction

A wide variety of organisms have an innate immune system that provides the first line of defense against external pathogens. Vertebrates have, among the components of this innate immune system, defensins comprising a diverse group of small cationic antimicrobial peptides. These molecules have both antimicrobial and cell signaling functions (Lai and Gallo, 2009). They are grouped into three families: alpha (α), beta (β), and theta (θ), according to the pattern of disulfide bonds between cysteine residues (Cys). β-Defensins are a subgroup of defensins that have a characteristic β-sheet-rich fold plus six conserved Cys with particular spacing and intramolecular bonds. The structure of pre-β-defensin consists of a signal sequence, a short or absent propiece, and the mature defensin (Ganz, 2003). β-Defensin-like peptides are found in the venom of diverse organisms, including sea anemones, snakes and platypus (Torres and Kuchel, 2004) as well lizards (Fry et al., 2005). Interestingly crotamine (one of four major components of the venom of the South American rattlesnake) has been shown to have a global fold and a Cys-pairing pattern similar to that of the β-defensin scaffold, although the peptides show low sequence similarity and display different biological activities (Fadel et al., 2005). Crotamine

* Corresponding author. Tel.: +55 (11) 2627 9812; fax: +55 (11) 2627 9811.
E-mail addresses: pgcorrea@ig.com.br (P.G. Correa), nancyoguiura@butantan.gov.br, naniogui@hotmail.com (N. Oguiura).

0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2013.02.013

has an antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*, as well against *Candida* spp., *Trichosporon* spp. and *Cryptococcus neoformans* (Oguiura et al., 2011; Yamane et al., 2012; Yount et al., 2009). Defensin-like peptides from the platypus also show a similar overall fold and Cys-pairing pattern as β-defensin-2, although no antimicrobial activity (Torres et al., 1999).

In vertebrates, β-defensin-like genes have been described in birds (Xiao et al., 2004), fishes (Zou et al., 2007), lizards (Dalla Valle et al., 2012), mammals and primates (Del Pero et al., 2002; Luenser and Ludwig, 2005; Luenser et al., 2005; Patil et al., 2005), platypus (Whittington et al., 2008) and rattlesnakes (Rádis-Baptista et al., 2003, 2004). The β-defensin genes are organized in a different manner in each animal group. The most common structure found in mammals is two exons and one intron (Patil et al., 2005), which also includes the platypus (Whittington et al., 2008), while there are four exons and three introns in chickens (Xiao et al., 2004). In snakes, β-defensin-like genes have three exons and two introns (Rádis-Baptista et al., 2003; 2004), as well as lizards (Dalla Valle et al., 2012) and fishes (Zou et al., 2007).

The β-defensin genes constitute a multigene family. Previous studies have shown that many multigene families, including proteins of the immune system, evolved according to a mechanism defined as the birth-and-death process (Nei and Rooney, 2005). This process was reported for mammalian β-defensin genes (Morrison et al., 2003), bovine defensin genes (Liu et al., 2009) and α-defensin genes (Das et al., 2010), and may explain the degree of diversity amongst the sequences in *Anolis carolinensis* (Dalla Valle et al., 2012). The unusually high degree of sequence variation in the mature peptide produced by the paralogous and in some cases orthologous genes implies extensive specialization and species-specific adaptation (Semple et al., 2006).

Comparative studies are important in determining patterns of evolution and function of the innate immune system. In this work, we describe new β-defensin-like genes in Brazilian pitvipers of the *Bothrops* and *Lachesis* genera, where we analyzed them phylogenetically and reconciled the species tree with gene tree to infer duplication/speciation nodes of these β-defensin-like genes.

2. Material and methods

2.1. Tissues

The snakes studied in this work were *Bothrops alternatus* (Estiva - MG, IBSP 77.198), *B. atrox* (Rio Branco – AC, IBSP 79.765), *B. diporus* (Blumenal – SC, IBSP 60.323), *B. insularis* (Queimada Grande Island – SP), *B. erythromelas* (Ibitira – BA, IBSP 79.766), *B. jararaca* (Embu Guaçu – SP), *B. jararacussu* (Ubatuba – SP), *B. leucurus* (Porto Seguro – BA, IBSP 79.100), *B. mattogrossensis* (N. Sra do Livramento – MT, IBSP 77.705), *B. neuwiedi* (Baependi – MG, IBSP 74.566), *B. pauloensis* (Frutal – MG, IBSP 71.111), *Crotalus durissus*, *Lachesis muta* (Northeast Brazil).

We used livers and scales from snakes deposited in the Tissue Collection of Alphonse Hoge Herpetological Collection at the Butantan Institute and the blood from *B. insularis* snakes, kept alive in the Ecology and Evolution Laboratory, and from *L. muta*, kept in the Herpetology Laboratory, both at the Butantan Institute.

2.2. DNA purification

The DNA was purified from liver tissues (Ausubel et al., 2000), scales (Fetzner, 1999) or blood (ZR Genomic DNA Tissue kit, ZymoResearch), which was then quantified at 260 nm using the NanoDrop ND-2000c spectrophotometer.

2.3. PCR

2.3.1. Primers

The forward and reverse primers H010 (5′-AAG CAGTCTCAGCATGAAGAT-3′) and 3′UTRas (5′-GGCACTCTC AGGTCCTTGGCCAT-3′) were designed on the basis of crotamine (Rádis-Baptista et al., 2003) and crotasin (Rádis-Baptista et al., 2004) gene sequences to amplify β-defensin-like sequences.

2.3.2. Reaction

A 50 μl reaction mix contained 100–1000 ng DNA sample, 0.1 μM each primer, 1.25 U Taq DNA Polymerase Platinum (Invitrogen), buffer with the addition of 1.5 mM $MgCl_2$, and 0.2 mM dNTPs mix. The amplification process used an initial denaturation step of 4 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 52.5, 55 or 58 °C and 45 s at 72 °C, and finally 1 min at 72 °C.

2.4. Cloning

The amplified DNA was purified, after electrophoresis on a 1% agarose gel, using the Zymoclean Gel DNA Recovery kit (ZymoResearch). The purified DNA was cloned into the

Table 1
β-Defensin-like sequence of snakes from different groups of *Bothrops* and *Lachesis* genera.

Snake	Sequence		GenBank
group *'atrox'*	*B.atrox_defensinB_01*	*DefbBa01*	KC117158
	B.leucurus_defensinB_01	*DefbBl01*	KC117166
group *'alternatus'*	*B.alternatus_sequence_01*	*SeqBal01*	KC117159
group *'jararaca'*	*B.jararaca_defensinB_01*	*DefbBj01*	KC117163
	B.jararaca_defensinB_02	*DefbBj02*	KC117164
	B.insularis_sequence_02	*SeqBi02*	KC117162
group *'jararacussu'*	*B.jararacussu_defensinB_01*	*DefbBju01*	KC117165
group *'neuwiedi'*	*B.diporus_defensinB_03*	*DefbBd03*	KC117160
	B.erythromelas_defensinB_01	*DefbBe01*	KC117161
	B.mattogrossensis_defensinB_02	*DefbBm02*	KC117167
	B.mattogrossensis_defensinB_03	*DefbBm03*	KC117168
	B.neuwiedi_defensinB_02	*DefbBn02*	KC117169
	B.pauloensis_defensinB_01	*DefbBp01*	KC117170
genus *Lachesis*	*L.muta_defensinB_01*	*DefbLm01*	KC117171
	L.muta_defensinB_02	*DefbLm02*	KC117172

The names of sequences and respective GenBank accession numbers are presented as well as the sequences are organized in the *Bothrops* groups as described in Carrasco et al. (2012).

Table 2
Sizes and organization of β-defensin-like gene of some Brazilian pitvipers.

Gene	Total length	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3
DefbBa01	2166	58	1784	$122^{*} + 202^{\&}$	–	–
DefbBal01	1658	58	1308	$83^{*} + 209^{\&}$	–	–
DefbBd03	2134	58	1758	112	153	$16^{*} + 37^{\&}$
DefbBe01	852	58	470	118	153	$16^{*} + 37^{\&}$
DefbBi02	1630	58	1281	$68^{*} + 207^{\&}$	–	–
DefbBj01	861	58	479	118	153	$16^{*} + 37^{\&}$
DefbBj02	2145	58	1762	118	154	$16^{*} + 37^{\&}$
DefbBju01	1996	58	1619	118	149	$16^{*} + 37^{\&}$
DefbBl01	2397	58	2018	118	153	$13^{*} + 37^{\&}$
DefbBm02	2001	58	1619	118	153	$16^{*} + 37^{\&}$
DefbBm03	2001	58	1619	118	153	$16^{*} + 37^{\&}$
DefbBn02	2083	58	1701	118	153	$16^{*} + 37^{\&}$
DefbBp01	1271	58	898	112	150	$16^{*} + 37^{\&}$
DefbLm01	1271	58	898	115	153	$10^{*}+37^{\&}$
DefbLm02	1909	58	1511	118	153	$10^{*} + 59^{\&}$

Total lengths (bp) correspond to sequences between PCR primers H010 and 3′UTRas. The boundaries of exons-introns were estimated according to *Crt-p1* (*C.durissus_crotamine*, GenBank: AF223646)) and Cts-p2 (*C.durissus_crotasin*, GenBank: AF250212) sequences after the alignment. * Indicates the size of codifying sequence and & the 3′UTR in the last exon.

pTZ57 R/T vector according to the manufacturer's instructions (Fermentas). Ten microliters of ligation mixture were used to transform the *E. coli* DH5α (Ausubel et al., 2000). Six clones were cultured, and the plasmids were then purified using Zyppy Plasmid Miniprep (ZymoResearch).

2.5. Sequencing

Clones were sequenced using the Big Dye Terminator V3.1 Cycle Sequence kit and fractionated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequencing was performed at the Biotechnology Center in the Butantan Institute, using the primers M13 (5′-GTAAAACGACGGCC AGT-3′) and T7 (5′-TAATACGACTCACTATAGGG -3′) to sequence the insert's boundaries, and intron-def-FWD (5′-GATTATTTCTTCCCTCCTACG-3′) and intron-def-REV (5′-GACTTCCGATTCCCTGTTGC-3′) to sequence intron 1.

2.6. Sequence analysis

The sequences were analyzed for selective pressure using the Hyphy package in the Datamonkey server at www.datamonkey.org (Pond et al., 2005). Datamonkey implements likelihood-based approaches for detecting sites under selection (Pond and Frost, 2005). Our data were analyzed using the following options: codon, universal code, SLAC (single likelihood ancestor counting) and REV model (time reversible model nucleotide substitution model to estimate the branch lengths and nucleotide substitution biases).

Sequences were aligned in MAFFT v7.017b (Katoh and Toh, 2010), strategy E–INS–i to less than 200 sequences, with multiple conserved domains and long gaps. Gene phylogenies were constructed by maximum parsimony using TNT1.1 (Goloboff et al., 2008), by maximum likelihood using TreeFinder 1.4 (Jobb et al., 2004), and by Bayesian analysis using MrBayes 3.2 (Ronquist et al., 2011). We used five partitions for the probabilistic analyses (three exons and two introns), assuming the best substitution model according to AICc using TreeFinder. The reconciliation of gene tree with species tree was done in Mesquite v2.75 (Maddison and Maddison, 2011).

3. Results and discussion

We detected 13 β-defensin-like sequences from 12 species of Brazilian Crotalinae snakes, which are listed along with GenBank accession number in Table 1, and aligned

Table 3
Similarity of β-defensin-like sequences of Brazilian pitvipers with crotamine (*Crt-p1*) and crotasin (*Cts-p2*) genes.

Genes	Exon 1		Intron 2		Exon 2		Intron 2		Exon 3	
	Crt-p1	*Cts-p2*	*Crt-p1*	*Cts-p2*	*Crt-p1*	*Cts-p2*	*Crt-p1*	*Cts-p2*	*Crt-p1*	*Cts-p2*
DefbBa01	96.6	93.1	92.8	93.6	63.6	66.9	96.6	97.4	75.0	68.8
DefbBd03	96.6	93.1	92.8	94.2	62.5	66.9	95.9	96.7	68.8	62.5
DefbBe01	96.6	93.1	92.6	90.4	63.6	67.8	97.2	98.0	68.8	62.5
DefbBj01	96.6	93.1	93.9	92.0	63.6	67.8	97.2	98.0	68.8	62.5
DefbBj02	96.6	93.1	89.1	91.1	67.8	72.0	95.9	96.7	75.0	75.0
DefbBju01	96.6	93.1	92.1	92.7	70.3	69.5	97.2	97.9	81.3	81.3
DefbBl01	94.8	98.3	91.9	91.2	68.1	71.6	95.9	96.7	75.0	75.0
DefbBm02	96.6	93.1	91.9	92.3	64.4	66.9	96.6	97.4	81.3	81.3
DefbBm03	96.6	93.1	91.9	92.2	63.6	67.8	96.6	97.4	75.0	75.0
DefbBn02	96.6	93.1	89.5	91.1	67.8	71.2	95.9	96.7	75.0	75.0
DefbBp01	94.8	91.4	92.7	93.6	62.5	71.4	96.5	97.3	68.8	62.5
DefbLm01	96.6	93.1	92.6	94.5	63.5	69.6	95.9	96.7	90.0	70.0
DefbLm02	96.6	93.1	92.7	93.4	69.5	57.6	96.6	97.4	80.0	60.0

The values presented here are percentage of nucleotide identity. Contiguous deletions or insertions were counted as one event.

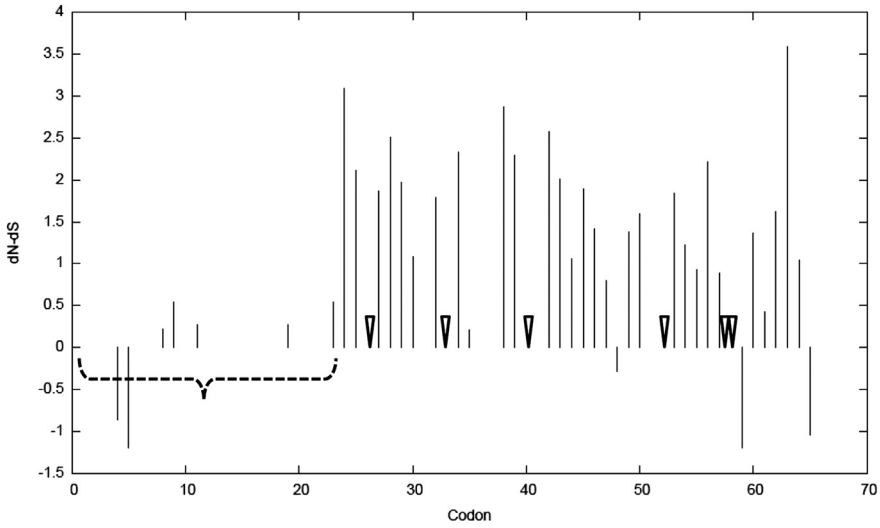

Fig. 1. Analysis of selective pressure of β-defensin-like sequences in Brazilian pitvipers. The ω values (dN-dS) are shown on *Y*-axis, and the codons of exon 1, 2 and 3 sequences on *X*-axis. The dashed brace indicates the peptide signal region. Triangles indicate the positions of cysteines.

sequences are shown in Supplementary Material 1. Despite the similarity of the nucleotide sequences, mutations in *B.alternatus_sequence_01* and *B.insularis_sequence_02* caused the loss of Cys which resulted in the loss of β-defensin structure and a change or loss of function. Although the sequence *B.atrox_defensinB_01* showed a premature stop codon, this occurred after the sixth Cys, which did not compromise the β-defensin scaffold. *B.atrox_defensinB_01* may maintain its antimicrobial function with a short C-terminal. The gene sizes varied from 852 to 2397 bp, and they were organized in three exons and two introns (Table 2), except the *DefbBa01* sequence which had only two exons. Interestingly, Oguiura et al. (2009) also described two sequences of crotamine genes without intron 2 in two rattlesnakes, indicating the possible occurrence of a minor gene structure with two exons and one intron. Intron 1 was long and in phase 1, dividing the codon between the first and second nucleotides, and intron 2 was short and in phase 2, dividing the codon between the second and third nucleotides as well as crotamine and crotasin genes (Rádis-Baptista et al., 2003, 2004). The variation in gene size was mainly due to the size variation of intron I, a region where insertions or deletions as well duplication were detected. The similarity of new sequences was analyzed in relation to the previous published rattlesnake β-defensin-like sequences, crotamine (*Crt-p1*) and crotasin (*Cts-p2*) (in Table 3, we did not compare

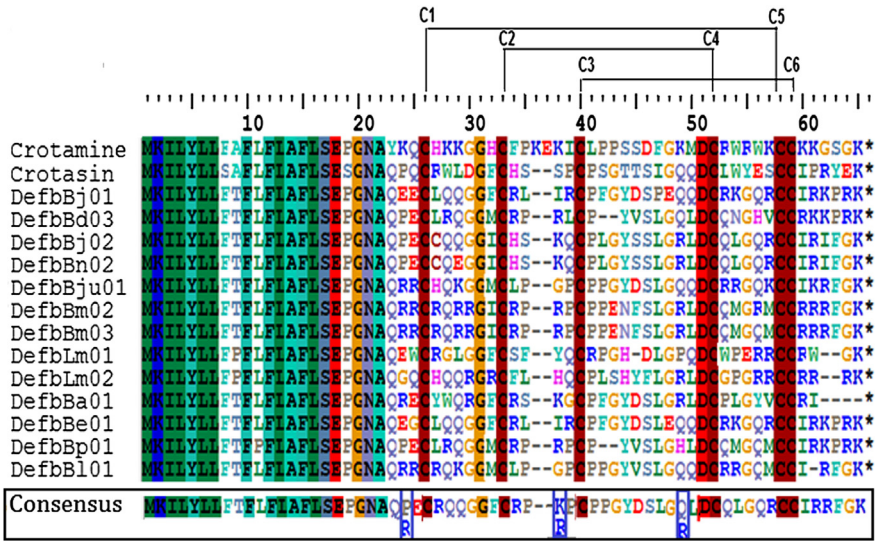

Fig. 2. Alignment of β-defensin-like amino acid sequences in Brazilian pitvipers. The deduced amino acid sequences were aligned using Muscle in the BioEdit v7.1.3 program. Green indicates hydrophobic amino acids, red the negatively charged amino acids, blue the positively charged amino acids and brown the other amino acids including cysteines, glycines and prolines. Above the figure, black lines indicate the pattern of cysteine bridges. Below the figure is the consensus sequence where the most frequent residues is annotated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Theoretical characteristics of deduced amino acid sequences of β-defensin-like of Brazilian pitvipers.

β-Defensin-like molecules	Snakes	p*I*	Net charge	Hidrofobicity (kcal mol $^{-1}$)	MW (kDa)
DefbBa01	*B. atrox*	8.3	+3	+26.1	4.3
DefbBd03	*B. diporus*	9.1	+6	+37.1	4.5
DefbBe01	*B. erythromelas*	8.8	+5	+42.6	4.8
DefbBj01	*B. jararaca*	8.5	+4	+45.1	4.9
DefbBj02	*B. jararaca*	10.4	+9	+36.5	4.5
DefbBju01	*B. jararacussu*	9.5	+7	+43.9	4.6
DefbBl01	*B. leucurus*	9.6	+7	+33	4.5
DefbBm02	*B. mattogrossensis*	12.0	+11	+40	5
DefbBm03	*B. mattogrossensis*	11.9	+10	+39	5
DefbBn02	*B. neuwiedi*	7.9	+2	+33.7	4.5
DefbBp01	*B. pauloensis*	8.8	+5	+32.8	4.5
DefbLm01	*L. muta*	8	+2	+33.7	4.5
DefbLm02	*L. muta*	10.4	+8	+36.9	4.7
Crotasin	*C. d.terrificus*	5.3	−1	+28.9	4.7
Crotamine	*C. d.terrificus*	9.6	+7	+45.1	4.8

Isoeletric point (p*I*), net charge at neutral pH, hydrophobicity (Wimley–White scale) and molecular weight (MW) were calculated on PepDraw software at http://www.tulane.edu/~biochem/WW/PepDraw/index.html.

the non-β-defensin-like sequences). Exon 1 and introns 1 and 2 displayed more than 90% identity, and curiously, intron 1 had high similarity despite the wide variation in its size. Also high similarity in exon 1 was expected because it codes for the signal peptide, which needs to be preserved to correctly address the protein in the cell. Fig. 1 shows the selective pressure analysis of exonic sequences of snake β-defensin-like genes: the proportion of dN-dS in signal

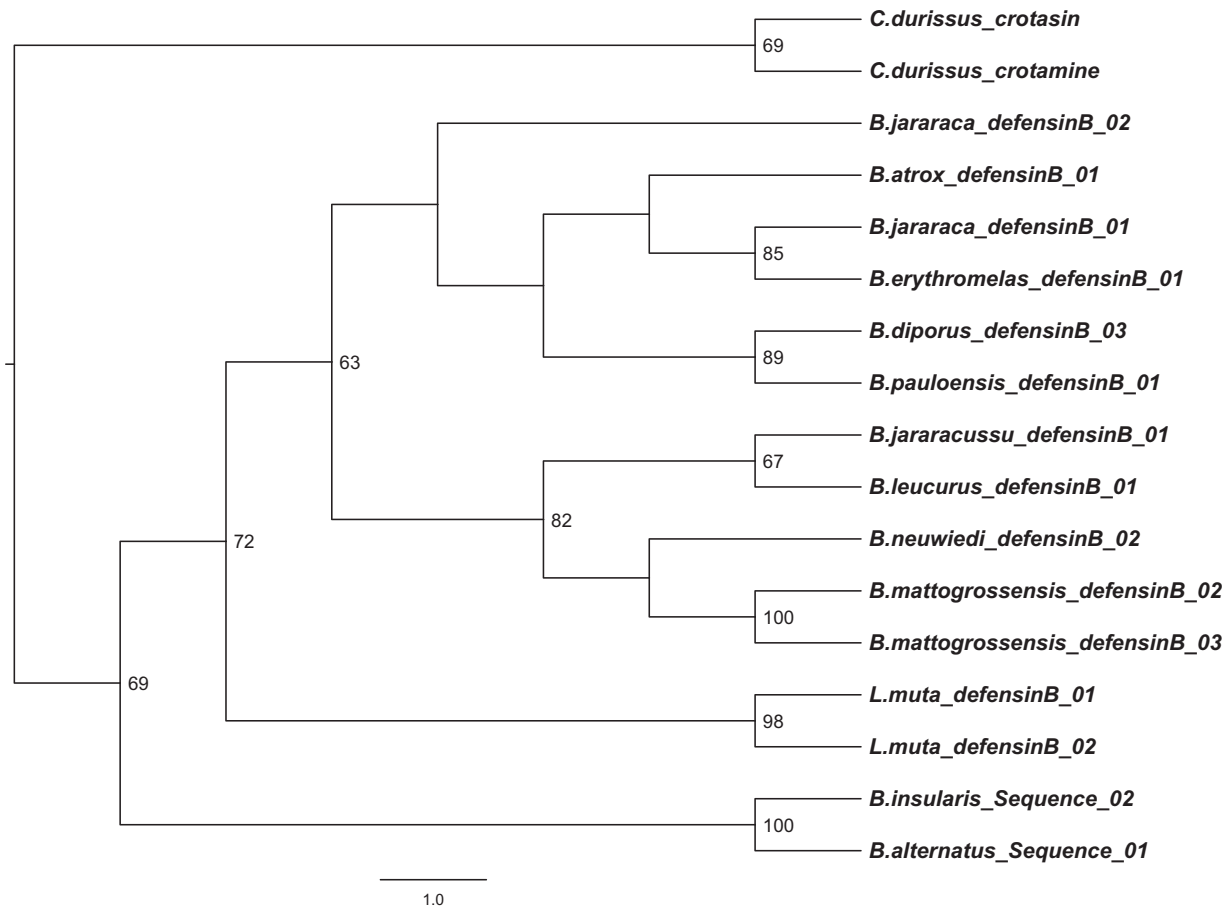

Fig. 3. Maximum parsimony cladogram of β-defensin-like sequences of Brazilian pitvipers. Cladogram of the most parsimonious trees using TNT1.1. Of 2955 nt, 1869 were conserved, 515 variable and 264 parsimony informative. Only bootstrap values greater than 50 are shown. The cladogram was rooted in *Crotalus* sequences. Scale bar below the tree measures evolutionary distances in substitutions per site.

peptide indicated a conserved sequence ($\omega < 1$, 0 or negative in general). On the other hand, ω value for exons 2 and 3 were higher (more than 1 in general) indicating positive selection, except in the Cys codons, which were conserved ($\omega = 0$). Introns were not analyzed, because we considered that these non-coding sequences were only subject to neutral evolution. Exons 2 and 3, which encode the mature protein, underwent an accelerate evolution as other snake toxins and defensins. Accelerated amino acid substitutions have been reported to occur not only in toxins but also in such proteins as antigen recognition sites of the MHC molecules and other antimicrobial peptides.

The analysis of deduced amino acid sequences by Signal P 4.0 (Petersen et al., 2011) indicated the β-defensin-like precursors consisted of signal peptide (SP) and mature peptide (MP), and lacked the anionic propiece between the SP and MP, which is common in mammalian α-defensins and can be shorter or absent in β-defensins (Ganz, 2003). The signal peptides were hydrophobic and Leu-rich (five Leu and two Ile in 22 aa) as in other immature β-defensins (Luenser et al., 2005; Patil et al., 2005). Despite the accelerated evolution, the deduced amino acid sequences (Fig. 2) exhibited the consensus pattern of mature β-defensins. The consensus sequence of mature peptide is X_3-C-X_6-C-X_{4-6}-C-X_{9-11}-C-X_5-CC-X_{4-6} with a high proportion of basic amino acids in carboxy-terminal region. Between the second and third Cys, crotamine has six amino acid residues instead of four in crotasin and other snake β-defensin-like sequences. Also, the first amino acid of the N-terminus of mature peptide of crotamine is Tyr instead of Gln in crotasin, and the newly described β-defensin-like molecules. Like crotamine and crotasin, the β-defensin-like sequences of *Bothrops* and *Lachesis* snakes have Gly at position 9 and Asp at position 29 of mature peptide. Also, all sequences have a terminal Lys, but we do not know if they are removed after post-translational processing as occurs in crotamine. All sequences described exhibited the characteristics of the β-defensin family, namely the six conserved Cys motif, small size (about 5 kDa), positive net charge, and high hydrophobicity (Table 4).

We analyzed three data sets by maximum parsimony: intronic sequences only, exonic sequences only, and the whole genes. In the case of snake β-defensin-like sequences, the best phylogenetic signal was obtained using the

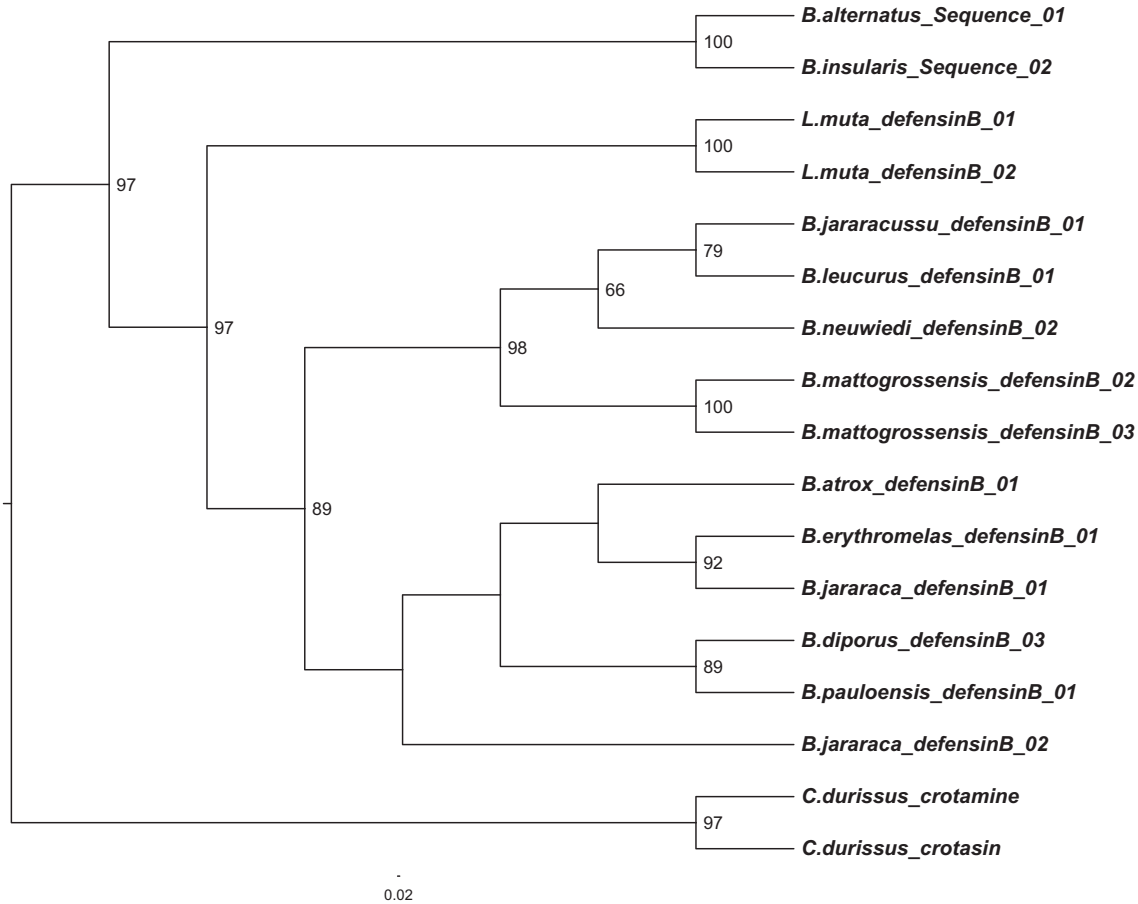

Fig. 4. Maximum likelihood cladogram of β-defensin-like sequences of Brazilian pitvipers. Sequences were analyzed using TreeFinder based on the HKY model to partitions exons 1, 2, 3 and intron 2, and TVM model for intron 1. *Crotalus* sequences were used as outgroup. The bootstrap values are shown at each node. Scale bar below the tree measures evolutionary distances in substitutions per site.

concatenated exonic and intronic sequences. In contrast, Luenser et al. (2005) analyzed caprine and ovine β-defensin-like sequences and found a phylogenetic signal only when intronic sequences were used to construct the phylogenetic tree. Phylogenetic analyses were done using parsimony and probabilistic approaches obtaining three topologies (Figs. 3–5). The best substitution model obtained using TreeFinder resulted in two models, TVM for intron 1 and HKY for the other partitions (intron 2, exons 1, 2 and 3) and they were used for both maximum likelihood and Bayesian analyses. All topologies showed three branches including non-β-defensins and β-defensin-like sequences of *Crotalus* and *Lachesis* and two lineages of *Bothrops*. The lineages were jararaca (*B.jararaca_defensinB_01* and *_02*, *B.atrox_defensinB_01*, *B.erythromelas_defensinB_01*, *B.pauloensis_defensinB_01*, *B.diporus_defensinB_03*) and jarara cussu (*B.jararacussu_defensinB_01*, *B.leucurus_defensinB_01*, *B.neuwiedi_defensinB_02*, *B.mattogrossensis_defensinB_02* and *03*), and the β-defensin-like genes of *'neuwiedi'* (*B. erythromelas*, *B. pauloensis*, *B. diporus*, *B. neuwiedi* and *B. mattogrossensis*) and *'atrox'* (*B. atrox* and *B. leucurus*) groups were recovered in both branches. Maximum parsimony and Bayesian analyses recovered *B.neuwiedi_defensinB_02* together with *B.matogrossensis_defensinB_01* and *02*, both of the '*neuwiedi*' group, though without support. The lineage jararaca which showed polytomy in Bayesian analysis, had low support in other analyses. The two paralogous β-defensin-like genes jararaca_01 and jararaca_02 may have duplicated before the speciation of the *'neuwiedi'*, *'jararacussu'* and *'jararaca'* groups. The sequences *B.mattogrossensis_defensinB_02* and *_03* seem to be polymorphic sequences and not duplicated genes. In all trees, the low support of branches was probably due to lack of sequence sampling from other snake species groups as well in the same species and due to gene duplications. Thus, an increase in the number of sequences of the same species, and also a larger sampling in β-defensin-like sequences from other snake species, may improve the tree topology and branch support in future studies. The great number of gaps and only one sequence in that gap did not seem to affect the parsimony or Bayesian analyses but it seemed to be spurious in likelihood analysis.

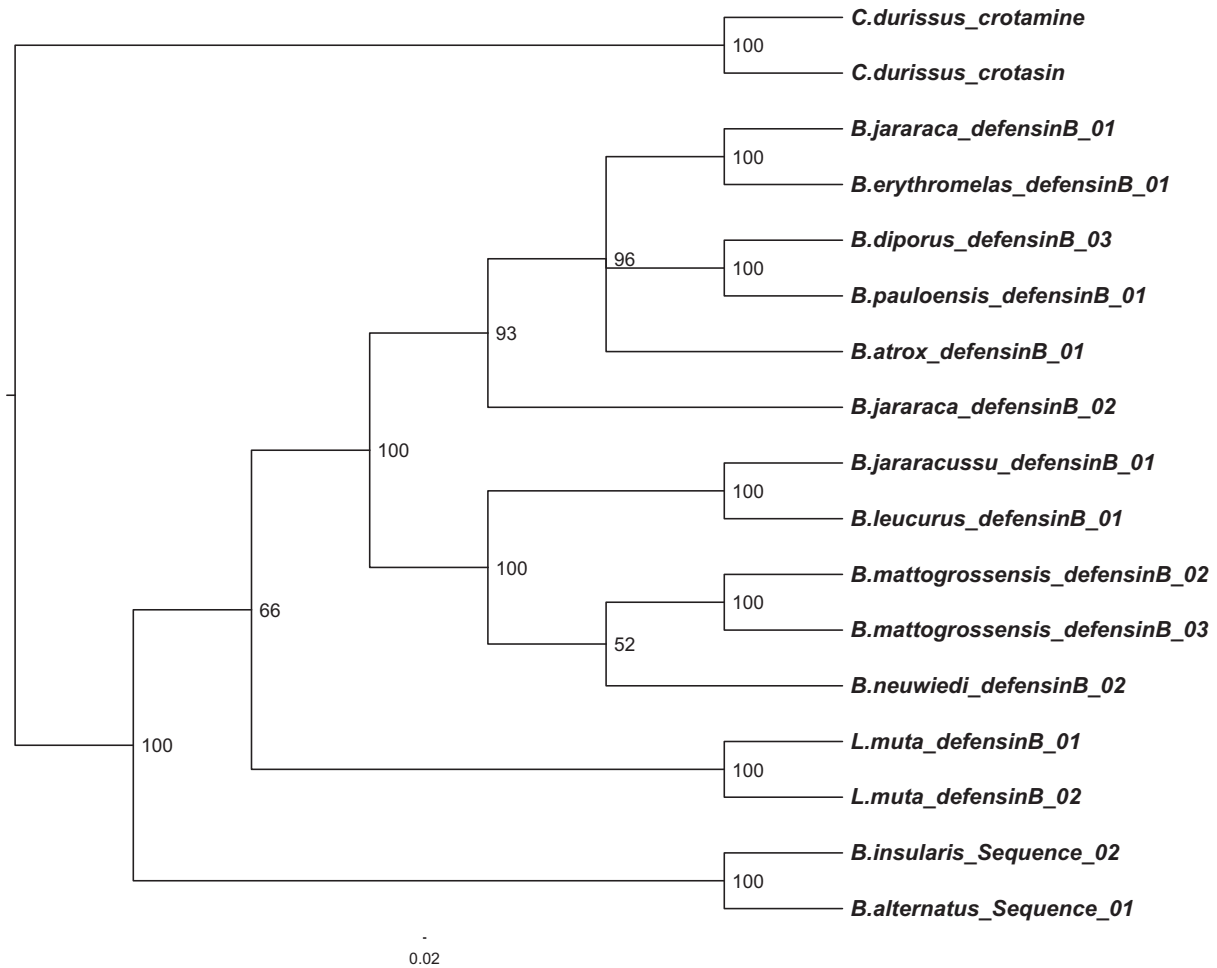

Fig. 5. Bayesian cladogram of β-defensin-like sequences of Brazilian pitvipers. Bayesian Markov Chain Monte Carlo consensus cladogram derived from an analysis based on the HKY model to partitions exons 1, 2, 3 and introns 2, and TVM model for intron 1. The posterior probabilities are shown at each node. Scale bar below the tree measures evolutionary distances in substitutions per site.

To construct the evolutionary history of the β-defensin genes in the snakes studied, all topologies were used to reconcile the gene tree with species tree. The reconciliation with the maximum parsimony gene tree resulted in eight duplications and 24 extinctions (Fig. 6), while the Bayesian gene tree showed eight duplications and 23 extinctions and maximum likelihood gene tree nine duplications and 29 extinctions. These events of duplication and differentiation of the genes occurred over a period of about 22 million years, the timeframe for the evolution of viperid snakes in the New World (Wüster et al., 2008). The high number of extinctions may be due to the lack of other β-defensin-like genes from the same species as well as from other *Bothrops* snakes. The evolution of these genes occurred according to the birth-and-death model, as for β-defensin genes and other multigene families in vertebrates (Nei and Rooney, 2005) and as suggested for the crotamine and crotasin genes (Oguiura et al., 2009).

We amplified β-defensin-like sequences of several snakes and we noticed that their genes have the same organization as the crotamine and crotasin genes as well other β-defensin-like genes of lizards and fishes. The evolution of genes is dynamic, where not only do substitutions occur but also intron gains and losses (Babenko et al., 2004). Coulombe-Huntington and Majewski (2007) observed a trend toward intron losses in mammals; furthermore, they observed that intron losses occurred more frequently in those smaller than 150 bp. We proposed that the structure of three exons and two introns is a squamate characteristic, because it is found in snakes and lizards, whereas the feature of two exons is characteristic for mammals (Patil et al., 2005) and four exons for birds (Xiao et al., 2004). All β-defensin-like sequences that have been described show a common main gene organization in a particular group of animals, but also one or more sequences with a different structure: our *DefbBa01* has only two exons, some in lizards have four exons (Dalla Valle et al., 2012), and mammals also have genes with more than two exons (Patil et al., 2005). In summary, all animals possess two or more gene structures, but with the predominance of one. As the β-defensin-like genes of zebrafish are organized in three exons and two introns (the first in phase 1 and the second in phase 2; Zou et al., 2007), and the ray finned fishes are the basis of the species tree (Shen et al., 2011), we speculate that the ancestral gene had this gene structure. After the speciation of mammals, the copies with two exons duplicated, and sometime after the speciation of the squamates and birds/turtles/crocodilians group, intron insertions occurred in the β-defensin-like genes, and this different arrangement duplicated more than that with three exons. Only studies of β-defensin-like genes in other animals including turtles and crocodilians and also amphibians and other fishes can further elucidate gene evolution in vertebrates.

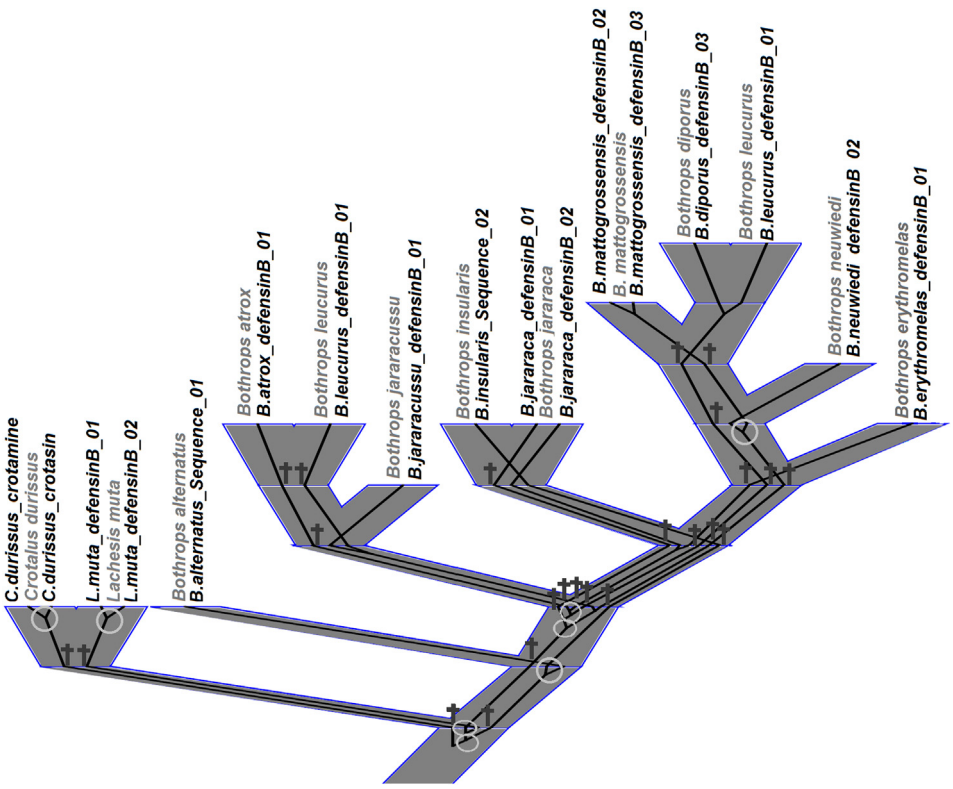

Fig. 6. Gene and species reconciliation tree of β-defensin-like sequences and snake species trees using maximum parsimony topology. Mesquite v.2.7.5 software was used to reconcile the gene (shown in Fig. 3) and the species trees. The species tree, in gray, is based on the topologies described in Castoe and Parkinson (2006), Fenwick et al. (2009) and Wüster et al. (2002). The black line represents the gene tree, the light gray circles the gene duplications, and the black crosses the gene extinctions.

Ethical statement

I declare that this work was conducted in accordance with the rules governing the scientific procedures, ethical conduct and protection to flora and fauna of Brazil.

Conflict of interest statement

I declare that there is no conflict of interest related to this publication.

Acknowledgments

We thank the following: Taís Machado, José S.L. Patané, and Hebert Ferrarezzi of the Ecology and Evolution Laboratory (Butantan Institute) for their assistance and support with the phylogenetic analysis; Valdir J. Germano, Daniela P.T. Gennari, and Kathleen F. Grego of the Herpetology Laboratory (Butantan Institute); Taís Machado and Rogério L. Zacariotti of the Ecology and Evolution Laboratory (Butantan Institute) for their assistance in obtaining the snake tissues or blood; and also Paulo L. Ho and Leonardo S. Kobashi of the Biotechnology Laboratory (Butantan Institute) for sequencing of the DNA samples. This work was supported by FAPESP 2010/08580-8 and INCTTOX.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2013.02.013.

References

Ausubel, F.M., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., Struhl, K. (Eds.), 2000. Current Protocols in Molecular Biology [CD-ROM]. John Wiley & Sons, Inc., New York.

Babenko, V.N., Rogozin, I.B., Mekhedov, S.L., Koonin, E.V., 2004. Prevalence of intron gain over intron loss in the evolution of paralogous gene families. Nucleic Acids Res. 32, 3724–3733.

Carrasco, P.A., Mattoni, C.I., Leynaud, G.C., Scrocchi, G.J., 2012. Morphology, phylogeny and taxonomy of South American bothropoid pitvipers (Serpentes, Viperidae). Zool. Scr. 41, 109–124.

Castoe, T.A., Parkinson, C.L., 2006. Bayesian mixed models and the phylogeny of pitvipers (Viperidae: Serpentes). Mol. Phylogenet. Evol. 39, 91–110.

Coulombe-Huntington, J., Majewski, J., 2007. Characterization of intron loss events in mammals. Genome Res. 17, 23–32.

Dalla Valle, L., Benato, F., Maistro, S., Quinzani, S., Alibardi, L., 2012. Bioinformatic and molecular characterization of beta-defensins-like peptides isolated from the green lizard *Anolis carolinensis*. Dev. Comp. Immunol. 36, 222–229.

Das, S., Nikolaidis, N., Goto, H., Mccallister, C., Li, J., Hirano, M., Cooper, M.D., 2010. Comparative genomics and evolution of the alpha-defensin multigene family in primates. Mol. Biol. Evol. 27, 2333–2343.

Del Pero, M., Boniotto, M., Zuccon, D., Cervella, P., Spanò, A., Amoroso, A., Crovella, S., 2002. β-Defensin 1 gene variability among non-human primates. Immunogenetics 53, 907–913.

Fadel, V., Bettendorff, P., Herrmann, T., de Azevedo Jr., W.F., 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.

Fenwick, A.M., Gutberlet Jr., R.L., Evans, J.A., Parkinson, C.L., 2009. Morphological and molecular evidence for phylogeny and classification of South American pitvipers, genera *Bothrops, Bothriopsis*, and *Bothrocophias* (Serpentes: Viperidae). Zool. J. Linn. Soc. 156, 617–640.

Fetzner, J.R., 1999. Extracting high-quality DNA from shed reptile skins: a simplified method. BioTechniques 26, 1052–1054.

Fry, B.G., Vidal, N., Norman, J.A., Vonk, F.J., Scheib, H., Ramjan, S.F.R., Kuruppu, S., Fung, K., Hedges, S.B., Richardson, M.K., Hodgson, W.C., Ignjatovic, V., Summerhayes, R., Kochva, E., 2005. Early evolution of the venom system in lizards and snakes. Nature 439, 584–588.

Ganz, T., 2003. Defensins: antimicrobial peptides of innate immunity. Nat./Rev./Immunol. 3, 710–720.

Goloboff, P., Farris, J., Nixon, K., 2008. TNT, a free program for phylogentic analysis. Cladistics 24, 774–786.

Jobb, G., von Haeseler, A., Strimmer, K., 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4, 18. http://dx.doi.org/10.1186/1471-2148-4-18.

Katoh, K., Toh, H., 2010. Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics 26, 1899–1900.

Lai, Y., Gallo, R.L., 2009. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. Trends Immunol. 30, 131–141.

Liu, G.E., Ventura, M., Cellamare, A., Chen, L., Cheng, Z., Zhu, B., Li, C., Song, J., Eichler, E.E., 2009. Analysis of recent segmental duplications in the bovine genome. BMC Genomics 10, 571. http://dx.doi.org/10.1186/1471-2164-10-571.

Luenser, K., Ludwig, A., 2005. Variability and evolution of bovine β-defensin genes. Genes Immun. 6, 115–122.

Luenser, K., Fickel, J., Ludwig, A., 2005. Evolution of caprine and ovine β-defensin genes. Immunogenetics 57, 487–498.

Maddison, W.P., Maddison, D.R., 2011. Mesquite: A Modular System for Evolutionary Analysis. Version 2.75. http://mesquiteproject.org.

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.

Nei, M., Rooney, A.P., 2005. Concerted and birth-and-death evolution of multigene families. Annu. Rev. Genet. 39, 121–152.

Oguiura, N., Collares, M.A., Furtado, M.F.D., Ferrarezzi, H., Suzuki, H., 2009. Intraspecific variation of the crotamine and crotasin genes in *Crotalus durissus* rattlesnakes. Gene 446, 35–40.

Oguiura, N., Boni-Mitake, M., Affonso, R., Zhang, G., 2011. In vitro antibacterial and hemolytic activities of crotamine, a small basic myotoxin from rattlesnake *Crotalus durissus*. J. Antibiot. 64, 327–331.

Patil, A.A., Cai, Y., Sang, Y., Blecha, F., Zhang, G., 2005. Cross-species analysis of the mammalian β-defensin gene family: presence of syntenic gene clusters and preferential expression in the male reproductive tract. Physiol. Genomics 23, 5–17.

Petersen, T.N., Brunak, S., Von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Pond, S.L.K., Frost, S.D.W., 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 21, 2531–2533.

Pond, S.L.K., Frost, S.D.W., Muse, S.V., 2005. HyPhy: hypothesis testing using phylogenies. Bioinformatics 21, 676–679.

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., 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.

Ronquist et al., 2011. Online manual at mrbayes.sourgefourge.net/manual.php.

Semple, C.A., Gautier, P., Taylor, K., Dori, J.R., 2006. The changing of the guard: molecular diversity and rapid evolution of β-defensins. Mol. Divers. 10, 575–584.

Shen, X.-X., Liang, D., Wen, J.-Z., Zhang, P., 2011. Multiple genome alignments facilitate development of NPCL markers: a case study of tetrapod phylogeny focusing on the position of turtles. Mol. Biol. Evol. 28, 3237–3252.

Torres, A.M., Kuchel, P.W., 2004. The β-defensin-fold family of polypeptides. Toxicon 6, 581–588.

Torres, A.M., Wang, X., Fletcher, J.I., Alewood, D., Alewood, P.F., Smith, R., Simpson, R.J., Nicholson, G.M., Sutherland, S.K., Gallagher, C.H., King, G.F., Kuchel, P.W., 1999. Solution structure of a defensin-like peptide from platypus venom. Bioch. J. 341, 785–794.

Whittington, C.M., Papenfuss, A.T., Bansal, P., Torres, A.M., Wong, E.S.W., Deakin, J.E., Graves, T., Alsop, A., Schatzkamer, K., Kremitzki, C., Ponting, C.P., Temple-Smith, P., Warren, W.C., Kuchel, P.W., Belov, K., 2008. Defensins and the convergent evolution of platypus and reptile venom genes. Genome Res. 6, 986–994.

Wüster, W., Salomão, M.G., Quijada-Mascareñas, J.A., Thorpe, R.S., et al., 2002. Origins and evolution of the South American pit viper fauna: evidence from mitochondrial DNA sequence analysis. In: Schuett, G.W., Höggren, M., Douglas, M.E., Greene, H.W. (Eds.), Biology of the Vipers. Eagle Mountais Publishing, Utah, pp. 111–128.

Wüster, W., Peppin, L., Pook, C.E., Walker, D.E., 2008. A nesting of vipers: phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes). Mol. Phylogenet. Evol. 49, 445–459.

Xiao, Y., Hughes, A.L., Ando, J., Matsuda, Y., Cheng, J.-F., Skinner-Noble, D., Zhang, G., 2004. A genome-wide screen identifies a single β-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian. BMC Genomics 5, 56. http://dx.doi.org/10.1186/1471-2164-5-56.

Yamane, E.S., Bizerra, F.C., Oliveira, E.B., Rajabi, M., Nunes, G.L.C., De Souza, A.O., da Silva, I.D.C.G., Yamane, T., Karpel, R.L., Silva Jr., P.I., Hayashi, M.A.F., 2012. Unraveling the antifungal activity of a South American rattlesnake toxin crotamine. Biochimie. http://dx.doi.org/10.1016/j.biochi.2012.09.019.

Yount, N.Y., Kupferwasser, D., Spisni, A., Dutz, S.M., Ramjan, Z.H., Sharma, S., Waring, A.J., Yeaman, M.R., 2009. Selective reciprocity in antimicrobial activity versus cytotoxicity of hBD-2 and crotamine. Proc. Natl. Acad. Sci. U.S.A. 106, 14972–14977.

Zou, J., Mercier, C., Koussounadis, A., Secombes, C., 2007. Discovery of multiple beta-defensin like homologues in teleost fish. Mol. Immunol. 44, 638–647.