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Short communication

Beta-defensin genes of the Colubridae snakes *Phalotris mertensi*, *Thamnodynastes hypoconia*, and *T. strigatus*

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ABSTRACT

β-Defensins are cationic antimicrobial peptides showing little sequence similarity but highly conserved tertiary structure stabilized by a six-cysteines-motif. Using a PCR approach, we described β-defensin sequences with two exons in three species of Colubridae snakes with high sequence similarity between them. The deduced amino acid sequence presented the characteristics of β-defensin family. The phylogenetic analysis using β-defensin coding sequences of different snakes grouped them in two main branches: genes organized in three or two exons.

β-Defensins comprise a class of cationic antimicrobial peptides of innate immunity which perform functions in adaptive immunity and in non-immunological processes. β-Defensin family members have little sequence similarity, but a high degree of similarity in their tertiary structure: three β-strands arranged in an antiparallel sheet held together by the three intramolecular cysteine disulfide bonds: Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6 (Machado and Ottolini, 2015). The structure of pre-β-defensin consists of a signal sequence, a short or absent propiece, and the mature defensin (Ganz, 2003). The first β-defensin described in snakes was crotamine, a small basic myotoxin from the venom of the rattlesnake *Crotalus durissus terrificus* (Oguiura et al., 2005; Coronado et al., 2013).

In reptiles, β-defensin-like genes have been described in snakes (Corrêa and Oguiura, 2013; Rádis-Baptista et al., 2004, 2003), and lizards (Dalla Valle et al., 2012) having three exons and two introns. In snake sequences, introns 1 and 2 are, respectively, of phase 1 (intron split a codon after the first nucleotide) and 2 (intron split a codon after the second nucleotide). The phase 1 intron after the signal peptide seems be usual to β-defensin genes (Zhu and Gao, 2013) and to proteins that will be exported (Sanz and Calvete, 2016).

The evolutionary relationship between defensins is still unclear (Zhu and Gao, 2013); to understand the evolution of these genes in snakes and consequently in vertebrates, we analyzed β-defensin genes in the colubrid snakes *Phalotris mertensi*, *Thamnodynastes hypoconia* and *T. strigatus* using PCR, an approach similar to that used by Corrêa and Oguiura (2013), and Schutte and Mccray (2002).

The β-defensin genes of colubrid snakes were obtained by PCR amplification using primers designed based on cDNA sequences of Duvernoy glands from *T. strigatus* and *P. mertensi* (Ching et al., 2012; Campos et al., 2016). Genomic DNA was purified from liver obtained from the Tissue Bank of Herpetological Collection Romano Hoge of the Butantan Institute (*T. hypoconia*, Itú-SP, IBSP 83.377; *T. strigatus*, Itatiba-SP, IB-SP 83.628; and *P. mertensi*, Sumaré-SP, IBSP-82.259) using Chelex (Walsh et al., 1991). A 20-μl reaction mix contained 60–500 ng DNA sample, 0.1 mM of each primer, 0.5 U Taq DNA Polymerase Platinum (Invitrogen), buffer with the addition of 2.5 mM $MgCl_2$, and 0.2 mM dNTP mix. The amplification process consisted of an initial denaturation step of 4 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 58–65 °C and 45 s at 72 °C, and finally 1 min at 72 °C.

The amplicon was purified, after electrophoresis on a 1% agarose gel, using the Zymoclean Gel DNA Recovery kit (ZymoResearch). The purified DNA was cloned into the pTZ57 R/T vector according to the manufacturer's instructions (Fermentas). Ten microliters of ligation mixture were used to transform the *E. coli* DH5a (Ausubel et al., 2000). Clones were sequenced using the Sanger method and fractionated on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). Sequencing was performed at the Biotechnology Center in the Butantan Institute.

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We analyzed seven sequences of *P. mertensi*, eight of *T. hypoconia*, and seven of *T. strigatus*. The sequences are showed in Supplementary Material-1. These genes showed two exons. Like the other β-defensin genes of snakes and other animals, the exons 1 are interrupted after the first nucleotide of the last codon (phase 1 intron). The exon 1 codifies the signal peptide and exon 2 the last three amino acids of signal peptide and the mature peptide. Other snake β-defensin genes also codify the signal peptide in exon 1 and the last three amino acid of signal peptide and mature peptide in exons 2 and 3. Exons 1 were conserved while the second exon showed lower similarity between genes of *P. mertensi*, all sequences showed the six-cysteine motif, which is characteristic of β-defensins. The signal peptide was hydrophobic and leucine-rich. Signal P (Petersen et al., 2011) indicated that the cleavage site occurs after GNA and the first amino acid of mature peptide is Q in all β-defensins analyzed similar to crotasin and other Brazilian pit vipers (Corrêa and Oguiura, 2013). The mature peptides had a predicted positive net charge at pH 7 and showed the consensus X_{3-4}-C-X_6-C-X_4-C-X_{11}-C-X_5-C-C-X_2 similar to that of other vertebrate β-defensins. Unlike the other described β-defensins of snakes, the carboxy-terminal tails of these colubrid snake defensins were short and those of *P. mertensi* were not positively charged (Fig. 1). The function of β-defensins of snakes are unknown but crotamine has antimicrobial activities (Yamane et al., 2013; Oguiura et al., 2011; Yount et al., 2009) in addition to the myotoxic activity. These activities are due to its capacity to pore formation in artificial membrane of bacteria (Costa et al., 2014) and interaction with potassium channels (Peigneur et al., 2012). About the β-defensins studied here, we only know that synthetic linear peptides Defb_Ts and Defb_Bj-01 have some antimicrobial activity only against *Micrococcus luteus* (data not shown). It is necessary a specific investigation using a folded peptide in order to determine their function because the β-defensins can have multiple functions in innate immunity in addition to the antimicrobial activity (Lai and Gallo, 2009).

The seven *P. mertensi* genomic sequences coded for three different mature peptides but different to transcript previously described (Campos et al., 2016). Eight analyzed sequences of *T. hypoconia* coded for two sequences with one-amino acid difference and different compared to *T. strigatus*. Among the seven sequences of *T. strigatus*, six mature peptides were identical to the cDNA described before (Ching et al., 2012). We observed that Defb_Pm-06 and _Pm-07 showed a signal peptide identical to *Thamnodynastes* sequences, but diverse of one amino acid (E_{18} instead Q_{18}) in relation to other *Phalotris* signal sequences. The greatest differences of both sequences occurred in mature peptides. On the other hand, *Thamnodynastes* sequences were more conserved in both regions. The variation between introns are mainly because of size variation (Table 1). The *Thamnodynastes* sequences seem to be more conserved than other defensin sequences of snakes.

Table 1
Sequence similarities of β-defensin genes of colubrid snake and *Bothrops jararaca* snake.

Sequence	GenBank	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3
Defb_Pm-01	KX664436	–	–	–		
Defb_Pm-03	KX664441	100%	99.5%	98.4%		
Defb_Pm-04	KX664437	100%	92.7%	98.4%		
Defb_Pm-05	KX664440	100%	99.3%	98.4%		
Defb_Pm-06	KX664439	98.3%	85.7%	68.0%		
Defb_Pm-07	KX664442	96.6%	93.0%	70.5%		
Defb_Pm-08	KX664438	100%	99.5	97.5%		
Defb_Th-01	KX664421	–	–	–		
Defb_Th-02	KX664422	100%	96.3%	100%		
Defb_Th-03	KX664422	100%	99.5%	100%		
Defb_Th-04	KX664424	100%	99.5%	99.2%		
Defb_Th-05	KX664425	100%	99.5%	100%		
Defb_Th-06	KX664426	100%	99.0%	100%		
Defb_Th-07	KX664427	100%	89.0%	99.2%		
Defb_Th-08	KX664428	100%	99.4%	100%		
Defb_Ts-01	KX664429	–	–	–		
Defb_Ts-05	KX664435	100%	86.2%	100%		
Defb_Ts-06	KX664430	100%	85.9%	99.2%		
Defb_Ts-07	KX664433	100%	86.4%	100%		
Defb_Ts-08	KX664432	100%	83.9%	100%		
Defb_Ts-10	KX664431	100%	85.9%	100%		
Defb_Ts-21	KX664434	100%	86.3%	100%		
Defb_Bj-04	MG833857	–	–	–	–	–
Defb_Bj-05	MG833858	100%	99.8%	100%	100%	100%
Defb_Bj-02	KC117164	100%	77.4%	76.3%	97.4%	93.8%
Defb_Bj-06	MG833859	100%	96.2%	100%	100%	100%
Defb_Bj-07	MG833860	100%	96.0%	100%	100%	100%
Defb_Bj-01	KC117163	100%	24.8%	80.5%	98.7%	75.0%
Defb_Bj-03	MG833861	100%	99.6%	100%	100%	100%

We used Geneious 6.0.6 (Kearse et al., 2012) to analyze the sequences based on Muscle alignment. Abbreviations are defined in Fig. 1. *Defb-Pm* were compared to *Defb-Pm01*; *Defb-Th* to *Defb-Th01*; *Defb-Ts* to *Defb-Ts01*; and *Defb_Bj* to *Defb_Bj-04*. The sequences *Defb_Bj* of *B. jararaca* snake were obtained as described in Corrêa and Oguiura (2013). The similarity is presented as percentage of identity.

To identify possible differences in the diversification patterns between the similarities of β-defensin genes with two or three exons, we also analyzed seven other genomic sequences of *Bothrops jararaca* (Supplementary material-2). We could observe a greater variation between the sizes of intron 1 and the mature peptide with the same signal peptide in pit viper sequences (Table 1).

Since the majority of β-defensin genes in mammals have two exons (Patil et al., 2005) and four exons in birds (Xiao et al., 2004), we supposed that in snakes the major structure would have three exons (Corrêa and Oguiura, 2013) as in lizards (Dalla Valle et al., 2012). Surprisingly, in the colubrid snake sequences analyzed herein, the β-defensin genes showed two exons, not three like the Brazilian pit viper snakes. This characteristic is not specific to

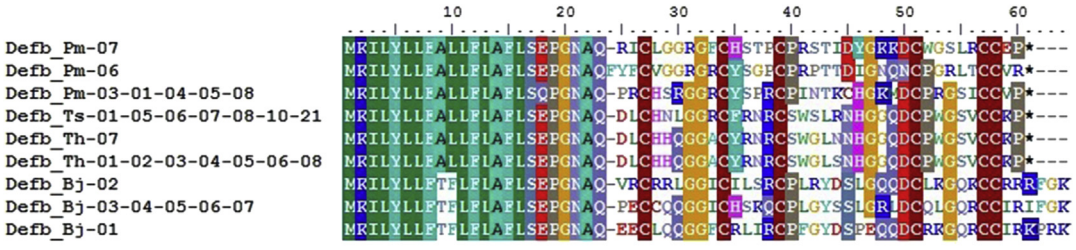

Fig. 1. Alignment of propeptide of β-defensin-coding sequences described in this work. Sequences were aligned using Muscle at Geneious 6.0.6 (Kearse et al., 2012) and the graphic view prepared using BioEdit (Hall, 1999). *Defb_Pm*, β-defensin genes of *Phalotris mertensi*; *Defb_Th*, β-defensin genes of *Thamnodynastes hypoconia*; *Defb_Ts*, β-defensin genes of *T. strigatus*. The green color indicates the polar amino acids, blue color the basic amino acids, red color the acidic amino acids, and brown color the cysteines. The predicted positive net charge at pH 7 and pI were calculated using PepDraw (http://pepdraw.com/by Thomas C. Freeman, Jr.): Pm-07 (+3, 8.3); Pm-06 (+4, 8.5); Pm-03 (+6, 8.9); Ts-01 (+3, 8.3); Th-07 (+1, 7.6); Th-01 (+1, 7.6); Bj-02 (+9, 10.4); Bj-03 (+3, 8.2); Bj-01 (+4, 8.5).

Colubridae snakes. The availability of genomic sequences from different snakes, namely *Python bivitatus* (Castoe et al., 2011b), *Ophiophagus hannah* (Vonk et al., 2013) and *Thamnophis sirtalis* (Castoe et al., 2011a), made it possible to determine β-defensin sequences in these species, and we found that they also showed two exons. It is not possible to know if it occurred an insertion or a deletion of the second intron in snake genomes. Coulombe-Huntington and Majewski (2007) analyzed the dynamics of intron loss/gain in 17,000 genes in mammals, and observed 122 cases of intron loss and no evidence of intron gain in rodents. The majority of lost introns had less than 150 bp. We speculate that initially snakes would have β-defensin genes with three exons, and depending on the animal, the small intron 2 could be lost due to its size or by mutation that introduces a stop codon just after the pair of cysteines maintained the 3D structure. Another possibility is the presence of the two forms of genes, with two or three exons, in the ancestral snake, where there would be duplication of one form in some lineages and that of the other form in other lineages. Although the majority of β-defensin genes of the lizard *Anolis carolinensis* have a three exons structure, Dalla Valle et al. (2012) also

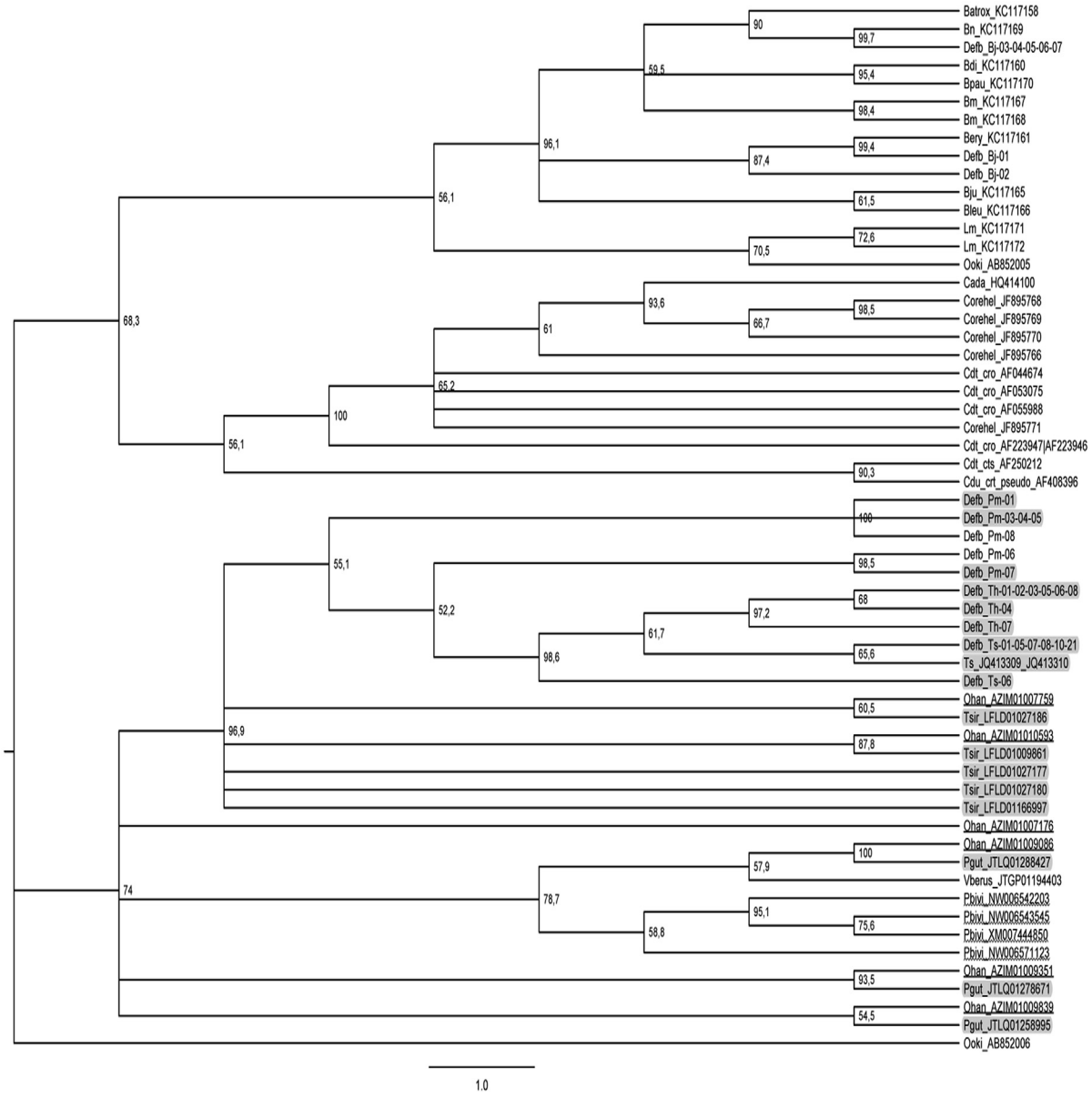

Fig. 2. Maximum likelihood cladogram of β-defensin-coding nucleotide sequences of snakes. Sequences were analyzed using TreeFinder (Jobb et al., 2004) based on substitution model TN [Optimum, Empirical]: G [Optimum]: 5; the bootstrap values are shown at each node (1000 replicates). The graphic view of cladogram was produced using FigTree (Andrew Rabaut, Institute of Evolutionary Biology, University of Edinburgh). Scale bar below the tree measures evolutionary distances in substitutions per site. Different marks illustrate the snake families (no mark = Viperidae; gray highlight = Colubridae; straight underline = Elapidae; wavy underline = Pythonidae). In Viperidae sequences, only Vberus is of subfamily Viperinae, the others are Crotalinae (pit vipers). Sequences extracted from GeneBank are indicated with their accession numbers. Abbreviations indicate the snake species: Batrox (*Bothrops atrox*), Bn (*B. neuwiedi*), Bdi (*B. diporus*), Bpau (*B. pauloensis*), Bery (*B. erythromelas*), Bj (*B. jararaca*), Bju (*B. jararacussu*), Bleu (*B. leucurus*), Bm (*B. matogrossensis*), Lm (*Lachesis muta*), Ooki (*Ovophis okinavensis*), Corehel (*Crotalus oreganus helleri*), Cdt-cro (*Crotalus durissus terrificus* – crotamine), Cdt-cts (*C. d. terrificus* – crotasin), Cdu-crt-pseudo (*C. durissus* – crotamine pseudogene), Ts (*Thamnodynastes strigatus* cDNA), Ohan (*Ophiophagus hannah*), Tsir (*Thamnophis sirtalis*), Pgut (*Panterophis guttatus*), Pbivi (*Python bivitatus*). The sequences described in this manuscript are Defb_Bj (β-defensin of *B. jararaca*), Defb-Pm (β-defensin of *Phalotris mertensi*), Defb-Th (*Thamnodynastes hypoconia*), Defb-Ts (*T. strigatus*).

described some two exons genes. A third possibility is an intron loss after the speciation of snakes followed by an intron insertion after the separation of Viperidae family. The first two hypotheses seem to be more parsimonious.

What are the consequences of having or not the second intron for the defensins? Other example of intron loss in snakes is related to the minimization of the protein structure of metalloproteinase toxins to disintegrins (Bazaa et al., 2007). Which is not the case of defensins, the intron lack does not interfere with the protein structure, however the presence of third exon in rattlesnakes (Cdt, Corehel) and other pit vipers (Bj, Bdi, Bpau) increases the tail length. Besides, it is reported that short introns are frequently found in regions of high recombination in genomes of *Drosophila* and vertebrates (Lynch, 2002) what could be an indication of a mechanism of increasing sequence variability. Also, introns can be considered as an enhancer of meiotic crossing over within coding sequences (Fedorova and Fedorov, 2003). We saw here that in the colubrid snake genes there is a high similarity among the cloned coding sequences, differently from the pit viper sequences with three exons.

The cladogram using β-defensin coding sequences (Fig. 2) shows two main branches that of three exons (β-defensin of genera *Bothrops* and *Lachesis*; the crotamine-like sequences of rattlesnakes of South and North America) and other with two exons (colubrid, pythonide and elapide snake sequences). The β-defensins of rattlesnakes are grouped and related to crotasin, it is known that the crotamine-like are venom toxins with positive net charge while the function of crotasin is unknown and it has a predicted net charge of −1. The β-defensin of Japanese pit viper *Ophiophagus okinavensis* is related to Brazilian pit viper *Lachesis muta* sequences. The colubrid and pythonide snake β-defensin sequences are grouped as well. The topology of the gene tree did not reflect the snake phylogeny but grouped the genes by structure and also by peptide function, because the family of small basic myotoxins of rattlesnake venom was assembled in a monophyletic group.

Our data indicate differences in evolution of β-defensin genes among the snakes, and the presence of an additional intron could drive the evolution of coding sequences as the tree constructed with, grouped the branches according to number of exons. An increase on the number of β-defensin genes with known structure is necessary to confirm our hypothesis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2018.02.048.

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