TECHNIQUES

Herpetological Review, 2007, 38(2), 158–162.

Detection of Crotamine and Crotoxin Gene Sequences in Genomic DNA from Formaldehyde-fixed Rattlesnakes

POLIANA G. CORRÊA
Laboratório de Herpetologia – Instituto Butantan
Av. Vital Brazil, 1500, São Paulo 05503-900, SP, Brazil

VALDIR J. GERMANO
Laboratório de Herpetologia – Instituto Butantan
Av. Vital Brazil, 1500, São Paulo 05503-900, SP, Brazil

GANDHI RÁDIS-BAPTISTA
Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami - Universidade Federal de Pernambuco
Av. Professor Moraes Rego, S/N, Recife 50670-420, PE, Brazil

and
NANCY OGUIURA*
Laboratório de Herpetologia – Instituto Butantan
Av. Vital Brazil, 1500, São Paulo 05503-900, SP, Brazil
e-mail: nancyoguiura@butantan.gov.br

** Corresponding author*

Museum collections are an important source of material for studies of phylogeny, systematics, and phylogeography. These collections can represent rare or extinct species and provide historical samples and may be very useful for genetic studies of reptile populations. The material is usually formalin–fixed and stored in ethanol. Formalin (an aqueous solution of formaldehyde) is the fixative agent most widely used to preserve tissues and organisms, but it causes cross-links in DNA-DNA, DNA-protein and protein-protein (Schander and Halanych 2003; Srinivasan et al. 2002). Several chemical reactions between formaldehyde and nucleic acids have been demonstrated, such as the addition of a hydroxymethyl group (-CH_2OH) in any nucleotide, formation of a methylene bridge between two amino groups, generation of apurinic and apyrimidic sites, and hydrolysis of phosphodiester bonds (Masuda et al. 1999; Srinivasan et al. 2002), causing the break of DNA, mutations and the inhibition of *in vitro* polymerization reactions of nucleic acids. The polymerase chain reaction (PCR) enzymatically amplifies specific sequences of nucleic acids, using very small amounts of DNA as template. In addition to innumerable current applications, PCR also allows nucleic acids from fixed tissues to be used in molecular analyses (Chatigny 2000; France and Kocher 1996; Gioia et al. 1998; Karlsen et al. 1994).

Given that the Herpetological Collection Alphonse Richard Hoge at Instituto Butantan in São Paulo, Brazil has the largest Neotropical collection of snakes with a majority of Brazilian species, we decided to investigate if these formalin-fixed samples are useful for molecular analysis of venom variation. Thus, we studied the crotamine polymorphism of some Brazilian rattlesnakes.

The rattlesnake *Crotalus durissus* occurs throughout most of South America except in Ecuador and Chile. Seven subspecies occur in Brazil: *C. d. cascavella*, *C. d. collilineatus*, *C. d. dryinas*, *C. d. marajoensis*, *C. d. ruruima*, *C. d. terrificus,* and *C. d. trigonicus* (Campbell and Lamar 1989). The venom of *C. d. terrificus* contains four main toxins: crotoxin, convulxin, gyroxin and crotamine (Bercovici et al. 1987). Crotoxin, the most toxic component in *C. durissus* venom, is a neurotoxin with a basic and weakly toxic phospholipase A_2 subunit (Crotoxin B or CB), and an acidic, non-toxic subunit (Crotoxin A, crotapotin or CA) (Hendon and Fraenkel-Conrat 1971). The cDNA precursors of both subunits have been cloned and sequenced. The 5'-untranslated tracts of cDNAs encoding CA and CB are nearly identical and the 3'-untranslated tracts are very similar (Bouchier et al. 1991).

The other major toxic component in *C. durissus* venom is crotamine. Crotamine—a cationic peptide (4.9 kDa, pI 10.8)—translocates into cells *in vitro*, and causes hind limb paralysis and myonecrosis *in vivo* (reviewed in Oguiura et al. 2005). Gonçalves (1956) described a crotamine polymorphism in the South American rattlesnake venoms: the crotamine-positive venoms have the toxin while the crotamine-negative venoms do not. Later, Schenberg (1959) defined two regions in São Paulo state: in the northeastern region he found only crotamine-positive venoms, but in the southwestern region he found predominantly crotamine-negative venoms. Collares et al. (2006) observed that snakes in the northwestern part of Paraná had the highest proportion of crotamine-positive venoms, with the majority of the venoms (71%) having crotamine content more than 10%. Although the individual venom sample sizes varied among the regions, Collares et al. (2006) observed crotamine-positive venoms less frequently in the northern regions including the states of Maranhão and Bahia.

Crotamine-positive rattlesnakes possess the crotamine gene, which is organized in three exons and is located in the extremity of the long arm of chromosome 2 (Rádis-Baptista et al. 2003). The crotamine cDNA of 340-360 base pairs encompasses an open reading frame of 198 nucleotides with 5' and 3' untranslated regions of variable size, signal peptide sequences, and can produce two crotamine isoforms (crotamine and crotamine-Ile19) (Rádis-Baptista et al. 1999). Furthermore, it was demonstrated immunologically that crotamine-negative rattlesnakes do not have crotamine in their venom using (Oguiura et al. 2000) or crotamine mRNA in their venom glands (Rádis-Baptista et al. 1999). Although the crotamine gene is absent in crotamine-negative snake genome, a paralogous crotamine-like gene is present (Rádis-Baptista et al. 2004).

In this work, we present data concerning to the crotamine gene polymorphism in the genome of fixed *C. durissus* subspecies, which are maintained for up to 22 years in the Alphonse Hoge Collection at Instituto Butantan (São Paulo, Brazil), by using PCR and genomic DNA from preserved samples.

Material and Methods

Tissues.—Table 1 indicates the sites where the snakes were collected, their voucher numbers, and their storage time.

DNA.—The DNA was purified using the method described by Ausubel et al. (2000). One hundred milligrams of scraped tissues were washed in TE solution (pH 8.0) for 24 hours at 4°C and then treated at 37°C for 18 h with 0.5% SDS, 10 mM Tris.HCl, 0.1 mM EDTA, 0.1 M NaCl, 0.16 mg/ml Proteinase K and 40 ng/ml RNAse A. The DNAs were purified by phenol/chloroform extraction and

TABLE 1. *Crotalus durissus* tissue samples and sources used in this study.

Sample	Tissue	Taxon	Locality	State	Voucher number	Storage time
1	Blood	*C. d. terrificus*	—	—	—	non-fixed
2	Heart	*C. d. terrificus*	Cianorte	Paraná	61.808	two years
3	Liver	*C. d. terrificus*	Cianorte	Paraná	61.808	two years
4	Gut	*C. d. terrificus*	Tapejara	Paraná	61.805	two years
5	Lung	*C. d. terrificus*	Tapejara	Paraná	61.805	two years
6	Kidney	*C. d. terrificus*	Cianorte	Paraná	61.807	two years
7	Kidney	*C. d. terrificus*	Ponta Grossa	Paraná	61.811	two years
8	Heart	*C. durissus* ssp.	Itapagipe	Minas Gerais	60.130	five years
9	Liver	*C. durissus* ssp.	Pouso Alegre	Minas Gerais	60.288	five years
10	Skin	*C. durissus* ssp.	Ponta Grossa	Paraná	61.812	two years
11	Trachea	*C. durissus* ssp.	Ponta Grossa	Paraná	61.813	two years
12	Skin	*C. durissus* ssp.	Ponta Grossa	Paraná	61.813	two years
13	Liver	*C. durissus* ssp.	Pouso Alegre	Minas Gerais	60.287	five years
14	Liver	*C. durissus* ssp.	Itapagipe	Minas Gerais	60.118	five years
15	Liver	*C. d. marajoensis*	São Luís	Maranhão	44.779	22 years
16	Liver	*C. d. cascavella*	São Luís	Maranhão	45.305	21 years
17	Liver	*C . d. cascavella*	São Luís	Maranhão	45.310	21 years
18	Liver	*C. d. cascavella*	São Luís	Maranhão	46.968	20 years
19	Liver	*C. d. cascavella*	Brumado	Bahia	51.791	19 years
20	Liver	*C. d. cascavella*	Juazeiro	Bahia	51.794	19 years
21	Liver	*C. d. cascavella*	Ibiquera	Bahia	54.952	12 years
22	Liver	*C. d. cascavella*	Grajaú	Bahia	57.306	seven years
C+	Liver	*C. d. terrificus*	—	—	—	non-fixed

acetate (pH 7)/ethanol precipitation. The DNAs were analyzed by 1% agarose gel electrophoresis in TBE buffer (89 mM Tris; 89 mM boric acid; 25 mM EDTA) and stained with 1 µg/ml ethidium bromide.

Primers.—The crotoxin primers, F crotox (forward): 5'- CCC CTG CCT GGC TTC TCC TTC- 3' and R crotox (reverse): 5'- CCT CAA TCC AGA CCT GGG AA- 3', were synthesized based on cDNA sequences described by Bouchier et al. (1991). The crotamine primers, 5 crot (forward): 5'- CAG TGT CAT AAG AAA GGA GG- 3' and 3 crot (reverse): 5'- CAT CTC CAT CGA CAG TCC AT- 3', were designed based on cDNA sequences described by Rádis-Baptista et al. (1999).

PCR conditions.—The PCR of crotoxin gene was done in 30 µl, using 600 ng of genomic DNA, 6 mM dNTPs, 60 mM $MgCl_2$, 4 pmol of each primer and 1 U of Biotools DNA polymerase and temperature profiles of: 94ºC for 4 min; 40 cycles of 94ºC for 45 sec, 49ºC for 45 sec, and 72ºC for 30 sec. The PCR of crotamine gene was performed in two rounds using 30 µl reaction, and a temperature profile of: 94ºC for 4 min; 25 cycles of 94ºC for 20 sec, 45ºC for 20 sec, 72ºC for 20 sec; and 72ºC for 2 min. In the first round, we used 100 ng of genomic DNA as template and two micro-liters from this first step were added to the second reaction round.

PCR reaction analysis.—Five micro-liters of each amplification product was analyzed in 7.5% polyacrylamide gel electrophoresis (PAGE) in TBE buffer and was stained with 1 µg/ml ethidium bromide.

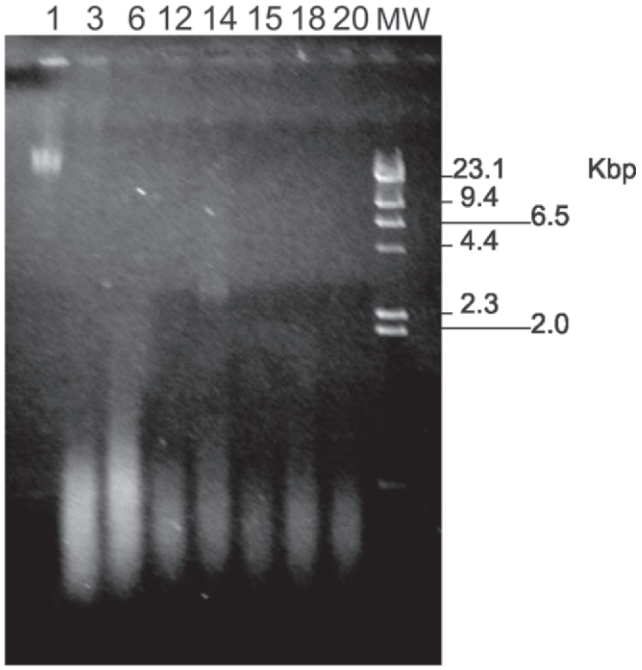

FIG. 1. 1% agarose gel of DNA purified from fresh and fixed tissues. The molecular weight standard (MW) was lambda DNA digested with *Hind* III purchased from Life Technologies.

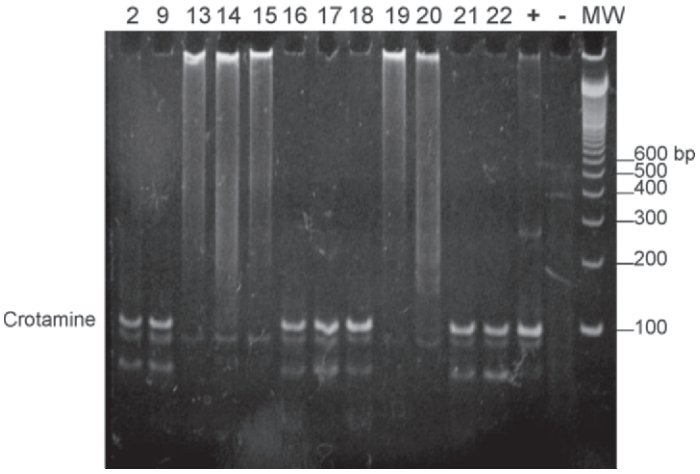

FIG. 2. 7.5% PAGE of PCR of fixed samples, a fresh sample (+), and no DNA (-). The arrow indicates the 100 bp fragment amplified using 5 crot and 3 crot primers. MW is the 100bp DNA ladder purchased by New England Biolabs.

RESULTS

The purification of DNA from fixed tissues took longer to be prepared by proteinase K/SDS method. More amount of proteinase K and longer time of digestion were necessary to disrupt the snake fixed tissue, in comparison to fresh tissues. Even so, a smaller quantity of DNA was obtained from fixed tissues than from non-fixed ones, starting from the same initial amount of material. In addition, the DNA purified from fresh tissue was longer than 23.1 kbp, as indicated in lane 1, Fig. 1, for DNA isolated from blood. Samples 3, 6, 12, 14, 15, 18, and 20 that represent genomic DNA from fixed tissues were markedly degraded, showing DNA lengths of less than 500 bp.

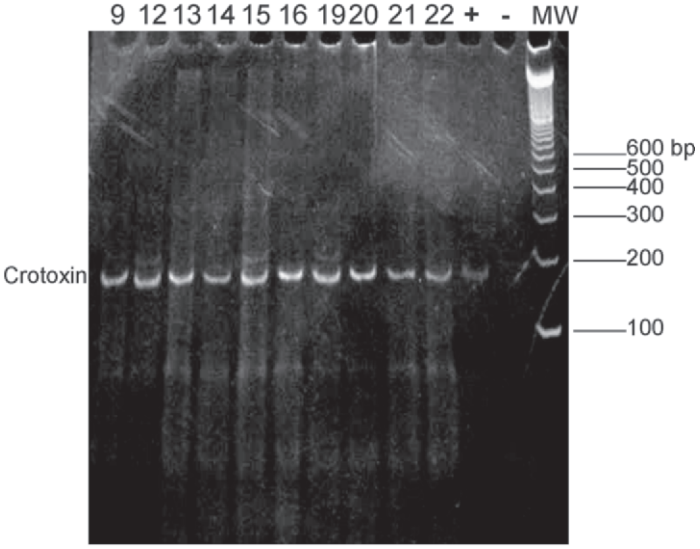

FIG. 3. 7.5% PAGE of PCR of fixed samples, one fresh sample (+) and no DNA (-). The arrow indicates the 200 bp fragment amplified using F-crotox and R-crotox primers. MW is the 100bp DNA Ladder purchased from New England Biolabs.

The PCR product (amplicon) of the crotamine gene was 100 bp long (Fig. 2) while that of the crotoxin gene was 200 bp (Fig. 3). Table 2 shows the PCR results for all the samples. Samples 2, 4, 6, 7, and 10 from the state of Paraná; 8 and 9 from Minas Gerais; 16, 17, and 18 from Maranhão; and 21 and 22 from Bahia were crotamine-positive. On the other hand, samples 5, 11, and 12 from the state of Paraná; 13 and 14 from Minas Gerais; 15 from Maranhão; and 19 and 20 from Bahia were crotamine-negative. As expected, all the samples have the crotoxin gene amplified, since all *C. durissus* in Brazil contain this neurotoxin in their venom. We observed some smear and faint bands in crotamine gel that are non-specific amplifications probably appeared in function of the high number of cycles used, more than 50 in polymerase chain reaction (double PCR reaction).

DISCUSSION

As it may be widely known, formalin fixation imposes a harsh condition of tissue preservation that damages nucleic acids inside the cells, despite its powerful ability to keep the intact structure of any preserved tissue. Huge collections of animals and plants that are potentially useful for studies of the molecular history of such organisms along space and time are kept in museums. For example, the Alphonse Richard Hoge Collection at Instituto Butantan (São Paulo, Brazil), contains over 70,000 catalogued Neotropical snake specimens, some of which are up to 100 years old.

Here, using PCR and specific primers, two genes encoding toxic components of rattlesnake venom (crotoxin and crotamine) were successfully amplified from genomic DNA extracted from formalin-fixed snake specimens. We were able to amplify the majority of DNA tested (approx. 90% of all samples), even in conditions of snake fixation that are not ideal for use in molecular biology protocols.

The low yield of DNA purified from formalized tissues may have resulted from the loss of low molecular weight (LMW) nucleic acid sequences that bind to proteins. During phenol treatments, it is possible to diminish the loss of DNA linked to protein by using DTE (dithioerythritol) or DTT (dithiothreitol) in extraction buffer, because these reducing agents break the cross-links between DNA and protein (Chatigny 2000; Schander and Halanych 2003). It is also possible to improve the recovery of LMW DNA during nucleic acid precipitation using carriers such as tRNA or glycogen (De Giorgi et al. 1994). The links between DNA-protein and DNA-DNA can also be broken by heating the DNA from fixed tissue in TE at 70°C, which improves the DNA yield (Masuda et al. 1999).

Even though the sizes of recovered genomic DNA samples were around 500 bp, they were useful for the molecular analysis of crotamine gene polymorphism and for accessing the presence of crotoxin gene. This is because the amplicons' size we were investigating lie between 100 and 200 bp. In fact, the lengths of amplicons in the PCR of genomic DNAs from fixed and fresh tissues were identical (that is, 100 bp) when using primers for crotamine gene (5 crot and 3 crot) and 200 bp when using crotoxin-specific primers (F crotox and R crotox). Both gene fragments were amplified from a single exon: exon II, in the case of crotamine, and the 5'-untranslated region of CA which is identical in CB, in the case of crotoxin. The crotamine gene polymorphism is well

TABLE 2. PCR of **CRO** – crotamine sequence and **CTX** – crotoxin sequence that resulted in amplification (+), no amplification (-) and not tested (**NT**).

Sample	CRO	CTX	Sample	CRO	CTX
1	NT	NT	13	-	+
2	+	+	14	-	+
3	NT	+	15	-	+
4	+	+	16	+	+
5	-	+	17	+	+
6	+	+	18	+	+
7	+	+	19	-	+
8	+	+	20	-	+
9	+	+	21	+	+
10	+	+	22	+	+
11	-	+	C+	+	+
12	-	+			

known among herpetologists: crotamine-negative rattlesnake have neither crotamine transcripts in their venom gland nor crotamine in their venom.

Our molecular results on fixed samples are consistent with the expected pattern of crotamine distribution. For example, Itapagipe and Pouso Alegre cities are in a region described as a mixed one, where both varieties are found, in the south-central region of Minas Gerais state (Bicalho et al. 1990). In the northwestern region of Paraná, including Cianorte and Tapejara, the rate of crotamine-positive venoms is high (Schenberg 1959), Crotamine was also detected in some venoms from Bahia it was using immunological method in a venom from Ibiquera (Collares et al. 2006). On the other hand, crotoxin is an ubiquitous toxin that is expressed in all *C. durissus* venom, independent of the local where snake are found. So, as expected all genomic DNA samples have the crotoxin gene fragment amplified, what worked as a positive control of PCR reaction. Lung tissue proved not to be useful because of the false negative result of sample 5.

In a parallel experiment, the PCR results of samples 2 to 14 were confirmed using DNA taken from the frozen livers from the same animals (data not shown). The amplification of gene sequences from genomic DNA isolated from fixed tissues posed greater difficulties than from fresh ones, since a larger amount of template was required to amplify the crotoxin sequence. Moreover, this was particularly true in the amplification of crotamine gene, for which an extra PCR round was carried out when compared with the PCR conditions routinely used in our laboratory, i.e., 100 ng of genomic DNA and 30 cycles. However, even in these circumstances, the formalin-fixed snakes constitute a valuable source to investigate the natural history of these animals and the correlation with their respective toxic traits.

Crotalus durissus terrificus is the predominant rattlesnake subspecies found in Brazil. In addition to it, we tested two subspecies, *C. d. marajoensis,* and *C. d. cascavella*, whose crotamine polymorphism has been little studied. *C. d. marajoensis* occurs in northern Brazil (Ilha de Marajó, Pará) and *C. d. cascavella* in northeastern Brazil (states of Maranhão, Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, and Bahia). Although the venoms of rattlesnakes from the states of Bahia, Pernambuco, Piauí, and Maranhão are believed to be crotamine-negative (Santoro et al. 1999, Toyama et al. 2005), we detected the crotamine gene in *C. d. cascavella* from São Luiz (Maranhão) and from Ibiquera and Grajaú (Bahia). Dr I. Biondi also observed the effect of some crotamine-positive venoms from Bahia in mice which showed myonecrosis in hystological preparations from muscles (personal communication). Our findings therefore indicate not only that these specimens tested positive for crotamine gene but also that these populations of *C. d. cascavella* contain crotamine-positive individuals. The only *C. d. marajoensis* snake we tested was crotamine-negative. Interestingly, this is the first time that crotamine has been detected in the rattlesnake *C. d. cascavella* inhabiting the northeastern regions of Brazil, encompassing the states of Bahia and Maranhão. Thus, in addition to molecular analysis by northern and southern blot and immunological approaches like ELISA, PCR of fixed tissue appears to be a useful method to study venom variation in preserved rattlesnakes obtained from museum collections.

In conclusion, this study demonstrated that PCR is a reliable technique for detecting crotamine and other toxin genes, and to analyze their distribution among population of snakes without using the snake venom itself. This is valid for the investigation of genomic DNA isolated from fresh tissues, as well as from fixed specimens.

Acknowledgments.—To Fundação de Amparo à Pesquisa de Estado de São Paulo (FAPESP) for its financial support of this work, and to FUNDAP for the fellowship granted to Poliana G. Corrêa. We are also indebted to K. F. Grego for his help in handling and caring for the snakes and to H. Suzuki for providing the frozen tissues and suggesting improvements to the manuscript.

LITERATURE CITED

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN, J. A. SMITH, AND K. STRUHL (eds.). 2000. Current Protocols in Molecular Biology. John Wiley & Sons Inc., New York.

BICALHO, A. F. X.; U. S. SILVEIRA, H. B. MAGALHÃES, AND M. R. V. DINIZ. 1990. Distribuição geográfica da serpente *Crotalus durissus*, variedade crotamino positivo e *Crotalus durissus*, variedade crotamino negativo no Estado de Minas Gerais *In* I Simpósio da Sociedade Brasileira de Toxinologia. São Paulo.

BERCOVICI, D., A. M. CHUDZINSKI, W. O. DIAS, M. I. ESTEVES, E. HIRAICHI, N. Y. OISHI, Z. P. PICARELLI, M. C. DA ROCHA, C. M. P. UEDA, N. YAMANOYIE, AND I. RAW. 1987. A systematic fractionation of *Crotalus durissus terrificus* venom. Mem. Inst. Butantan 49:69–78.

BOUCHIER, C., J.–C. BOULAIN, C. BON, AND A. MÉNEZ. 1991. Analysis of cDNAs encoding the two subunits of crotoxin, a phospholipase A2 neurotoxin from rattlesnake venom: the acidic non-enzymatic subunit derives from a phospholipase A2-like precursor. Biochim. Biophys. Acta 1088:401–408.

CAMPBELL, J. A., AND W. W. LAMAR. 1989. The Venomous Reptiles of Latin America. Comstock Publishing Associates, Ithaca, New York.

CHATIGNY, M. E. 2000. The extraction of DNA from formalin-fixed, ethanol-preserved reptile and amphibian tissues. Herpetol. Rev. 31:86–87.

COLLARES, M. DE A.; P. G. CORRÊA, H. SUZUKI, M. F. D. FURTADO, AND N. OGUIURA. 2006. The gene family of crotamine and variations of its content in venoms of South American rattlesnake *Crotalus durissus*. *In* 15th World Congress on Animals, Plants, and Microbial Toxins. Glasgow.

De Giorgi, C., M. F. Sialer, and F. Lamberti. 1994. Formalin-induced infidelity in PCR-amplified DNA fragments. Mol. Cell. Probes 8:459–462.

France, S. C. and T. D. Kocher. 1996. DNA sequencing of formalin-fixed crustaceans from archival research collections. Mol. Marine Biol. Biotechnol. 5:303–313.

Gioia, L., L. J. Vogt, W. M. Freeman, A. Flood, B. A. Vogt, and K. E. Vrana. 1998. PCR-based apolipoprotein E genotype analysis from archival fixed brain. J. Neurosci. Methods 80:209–214.

Gonçalves, J. M. 1956. Estudos sobre venenos de serpentes brasileiras. II – *Crotalus terrificus crotaminicus*, subespécie biológica. An. Acad. Bras. Ciênc. 28:365–367.

Hendon, R. A., and H. Fraenkel-Conrat. 1971. Biological roles of the two components of the crotoxin. Proc. Natl. Acad. Sci. USA 68:1560–1563.

Karlsen, F., M. Kalantari, M. Chitemerere, B. Johansson, and B. Hagmar. 1994. Modifications of human and viral deoxyribonucleic acid by formaldehyde fixation. Lab. Invest. 71:604–611.

Masuda, N., T. Ohnishi, S. Kawamoto, M. Monden, and K. Okubo. 1999. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res. 27:4436–4443.

Oguiura, N., M. Boni-Mitake, and G. Rádis-Baptista. 2005. New view on crotamine, a small basic polypeptide myotoxin from South American rattlesnake venom. Toxicon 46:363–370.

———, M. E. Camargo, A. R. P. Da Silva, and D. S. P. Q. Horton. 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.

Rádis-Baptista, G., T. Kubo, N. Oguiura, A. R. B. P. Da Silva, M. A. F. Hayashi, E. B. Oliveira, and T. Yamane. 2004. Identification of crotasin, a crotamine-related gene of *Crotalus durissus terrificus*. Toxicon 43:751–759.

———, ———, ———, M. Svartman, T. M. B. Almeida, R. F. Batistic, E. B. Oliveira, A. M. Vianna-Morgante, and T. Yamane. 2003. Structure and chromosomal localization of the gene for crotamine, a toxin from the South American rattlesnake, *Crotalus durissus terrificus*. Toxicon 42:747–752.

———, N. Oguiura, M. A. F. Hayashi, M. E. Camargo, K. F. Grego, E. B. Oliveira, and T. Yamane. 1999. Nucleotide sequence of crotamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*). Toxicon 37:973–984.

Santoro, M. L., M. C. C. Sousa-e-Silva, L. R. C. Gonçalves, S. M. Almeida-Santos, D.F. Cardoso, I. L. Laporta-Ferreira, M. Saiki, C. A. Peres, and I. S. Sano-Martins. 1999. Comparison of the biological activities in venoms from three subspecies of the South American rattlesnake (*Crotalus durissus terrificus*, *C. durissus cascavella* and *C. durissus collilineatus*). Comp. Biochem. Physiol. C 122:61–73.

Schander, C. and K. M. Halanych. 2003. DNA, PCR, and formalized animal tissue—a short review and protocols. Org. Divers. Evol. 3:195–205.

Schenberg, S. 1959. Geographical pattern of crotamine distribution in the same rattlesnake subspecies. Science 129:1361–1363.

Srinivasan, M., D. Sedmak, and S. Jewell. 2002. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am. J. Pathol. 161:1961–1971.

Toyama, D. O., A. C. Boschero, M. A. Martins, M. C. Fonteles, H. S. Monteiro, and M. H. Toyama. 2005. Structure-function relationship of new crotamine isoform from the *Crotalus durissus cascavella*. Protein J. 24:9–19.