

Random Amplified Polymorphisms Between Two South American Subspecies of Rattlesnakes (*Crotalus durissus collilineatus* and *Crotalus durissus terrificus*)

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ABSTRACT

The present work was made to determine the suitability of RAPD analysis for the identification of specimens of two subspecies of South American rattlesnakes (*Crotalus durissus terrificus* and *Crotalus durissus collilineatus*). The 11 arbitrary primer sequence tested amplified a total of 161 bands, of which 31% were polymorphics when compared with *Crotalus* specimens. The combining results from different primers allowed the identification of all the specimens under analysis. Several characteristic bands of each subspecies were identified. Dendrogram, based on RAPD markers, show three groups that corresponded to the subspecies of *Crotalus*, and to *Bothrops jararaca* specimen.

Key words: Rattlesnake, RAPD, genetic variability, molecular markers

INTRODUCTION

The snakes of the genus *Crotalus* originated in North America migrated to Central and South America. Central and South American rattlesnakes are classified as a single species, *Crotalus durissus*, which is separated by several authors into various subspecies (Campbell and Lamar, 1989). Based on the geographical distribution and several morphological traits such as the pattern of longitudinal bands on the neck, the presence of more or less contrasting rhomboid blotches, the shape and intensity of paravertebral nuchal stripes and the presence of loreal scales, Peter and Oreja-Miranda (1970) separated *Crotalus durissus* in the following subspecies: *C.d. durissus*, *C.d. terrificus*, *C.d. unicolor*, *C.d. collilineatus*, *C.d. cascavela*,

C.d. ruraima, *C.d. dryinus*, *C.d. tzabcan*, *C.d. marajoensis* and *C.d. cumanensis*.

The separation of *Crotalus durissus* into different subspecies raises a conceptual argument, since it is difficult to guarantee the existence of these subspecies based on a few morphological characters, most of which exhibit a large variation within each group (Santos and Laporta Ferreira, 1992). Nevertheless, the comparative analysis of venom composition has confirmed the existence of at least two subspecies, *C.d. durissus* and *C.d. terrificus*, and several groups within the last one (Barrio, 1960).

Four of the subspecies of *C. durissus* are found in Brazil: *C.d. marajoensis*, restricted to the Marajo island, *C.d. carcavela*, found in the dry region of the northeast of Brazil, *C.d. collilineatus*, distributed from central Brazil to north Argentina,

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and *C.d.terrificus*, spread over the south of Brazil, Argentina and Uruguay (Hoge, 1965; Hoge and Romano-Hoge, 1979; Campbell and Lamar, 1989). The last two subspecies have a sympatric region distribution on southeast Brazil.

The molecular approaches utilizing genomic DNA have revolutionized the studies of ecology, evolution, population genetics and systematics. Identification of restriction fragment length polymorphisms (RFLP), DNA sequence disparities, DNA fingerprintings, and random amplified polymorphic DNA (RAPD) have been used to distinguish among individuals, populations, species and higher taxa, ant to evaluate phylogenetic relationships in plants, animals and microorganisms (Burke and Bruford, 1987, Parker and Whiteman, 1993, Master, 1995). In an attempt to better characterized two subspecies of *Crotalus durissus*: *C. d. terrificus* and *C. d. collilineatus*, we investigated molecular differences between individuals of both subspecies, based on random amplified polymorphic DNA markers.

MATERIALS AND METHODS

Eight adult snakes were used in the present study. Three were representatives of *C. d. collilineatus* from central Brazil (Goiás and Mato Grosso states), four were representatives of *C. d. terrificus* from south Brazil (Rio Grande do Sul state), and one representative of *Bothrops jararaca*, used as a pattern of intergeneric variation. All the animals were collected in different locations. All this individuals are part of the collection of the Museum of Natural Sciences of the University of Caxias do Sul.

Blood samples from each individual, collected by the cardiac puncture method described by Branch (1973), were stored at -20°C prior to DNA purification. Total DNA was extracted according to procedures adapted from Hillel *et al.* (1989). A 0.1ml blood aliquot was mixed with 0.4ml of 1xSSC and homogenated with an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2). The suspension was centrifuged at 13000xg for five minutes at 4°C . The layer was transferred to a clean tube and subjected to a second extraction by the addition of an equal volume of

chloroform:isoamyl alcohol (24:1) followed by mixing and centrifugation as described above.

The DNA present in the aqueous layer was precipitated by the addition of two volume of ethanol (-20°C), followed by at least one hour incubation at -20°C . The DNA was pelleted by centrifugation at 13000xg for five minutes at 4°C , air dried at room temperature and dissolved in 400 μl of TE (pH8.0). The DNA was precipitated again by the addition of 4 μl of %M sodium acetate and 1ml of ethanol (-20°C), pelleted by centrifugation at 13000xg for five minutes at 4°C , air dried and dissolved in 50 μl of TE. DNA concentration and purity was measured by spectrophotometry at A_{260} and A_{280} . The extracted DNAs were stored at -20°C until used for DNA amplification.

DNA amplifications were carried out in an MJ Research, Inc. MiniCycler using the method described by Williams *et al.* (1993). The amount of template DNA was of 100ng per reaction. Taq DNA polymerase was obtained from Pharmacia Co. Primers were obtained from Operon Technologies, Inc. In the course of the study, 60 primers of the kits OPB, OPX and OPY were evaluated. Each RAPD amplification was prepared at least three times to determine reproducibility of the patterns. Amplification products were electrophoresed through 1.5% agarose gels in 1xTBE, stained with ethidium bromide and visualized under UV light.

Amplification products were listed as discrete character states per strain (present/absent). These data were analyzed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) package, version 1.5 (Rohlf, 1989) to determine the similarities among the individuals. Similarities were computed using the Jaccard's coefficient. The individuals were clustered by the unweighted pair-group method using arithmetic averages (UPGMA) in order to present the results in the form of a phenogram.

RESULTS AND DISCUSSION

From the 60 primers evaluated for the generation of amplified DNA products of specific size from rattlesnakes, 18 primers provided more than three scorable bands and were selected for the studies. A

fragment was judged to be scorable if it was intense enough and different enough in size from neighboring bands to be scored with confidence, as well as being reproducible from experiment to experiment. From these 18 primers, 11 generated reproducible markers with some degree of variability between the individuals. The other seven primers generated markers that were highly conserved among the *Crotalus* specimens examined.

The selected primers (Table 1) generated a total of 161 scorable amplification products that were analyzed and used as variables in our analysis. The variation between *Crotalus* and *Bothrops* was higher than the variation between the two subspecies of *Crotalus durissus* (Fig.1 and Fig.2). Of the selected bands, 83.9% were polymorphics between *Crotalus* and *Bothrops*, what was expected when comparing different genera.

Table 1 - Primers identification and corresponding number of scorable, polymorphic and specific bands, amplified from rattlesnake DNA. (Cdt – *C. d. terrificus*, Cdc- *C. d. collilineatus*)

Primer ^a	Primer sequence 5' > 3'	Nº scorable bands	Polymorphic bands	Specifics of Cdt	Specifics of Cdc
OPB-04	GGACTGGAGT	04	01	0	1
OPB-08	GTCCACACGG	21	02	0	1
OPB-10	CTGCTGGGAC	30	14	0	2
OPB-13	TTCCCCGCT	05	02	0	0
OPX-01	CTGGGCACGA	16	06	1	1
OPX-03	TGGCGCAGTG	10	03	2	0
OPX-06	ACGCCAGAGG	24	05	2	0
OPX-11	GGAGCCTCAG	12	05	5	0
OPX-13	ACGGGAGCAA	16	06	0	0
OPY-01	GTGGCATCTC	08	01	1	0
OPY-02	CATCGCCGCA	15	04	0	3

^aPrimer designation refers to the kit (last letter) and the specific primer (number) purchase from Operon Techn.

Conversely, only 50 bands (31%) were polymorphics when comparing the seven specimens of *Crotalus*. From these, 31 bands showed a random polymorphism distribution among the individuals of both subspecies allowing for the identification of all the specimens. These band patterns could be useful in population studies, but were not able to discriminate between the subspecies. The remaining 19 bands (38%) of the polymorphic bands between *Crotalus* specimens were specific of each subspecies. Eight bands were present only in the four specimens of *C. d. collilineatus*, and 11 were specific of *C. d. terrificus*. One of these specific bands can be observed in Figure 1.

The 161 RAPD fragments were used to calculate similarity values and to estimate the relatedness among the specimens. As can be observed in Table 2, the similarity between individuals of the same subspecies ranged from 0.98 and 0.87. These values were very high when compared with the interspecific variation obtained in other organisms

(Ferreira and Grattapaglia, 1996). This results supported their separation in sub-species.

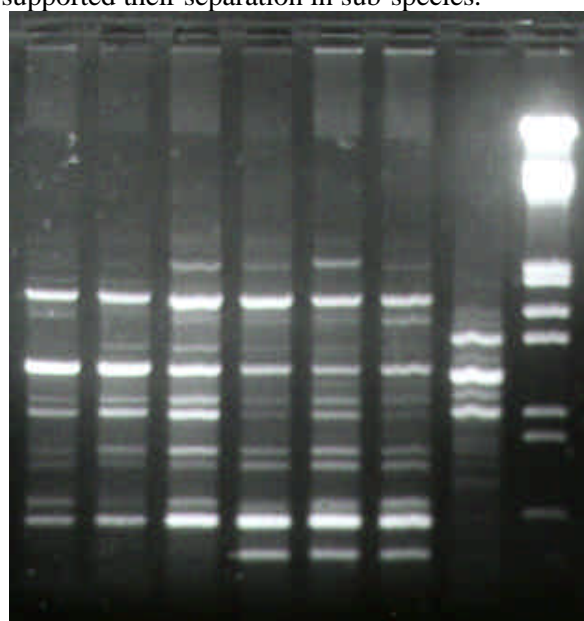


Figure 1 - RAPD products generated using primers OPB-08. Lanes 1-3, specimens of *C.d. terrificus*, lanes

4-6, specimens of *C.d. collilineatus*, lane 7, a specimen of *B. jararaca*, and lane 8, DNA size standard (λ /EcoRI-HindIII fragments).

Table 2 - Distance matrix between each of eight specimens of snakes, based on their RAPD profiles. Cdt - *C. d. terrificus*, Cdc- *C. d. collilineatus*, Bj- *Bothrops jararaca*.

Cdt	Cdt	Cdt	Cdt	Cdc	Cdc	Cdc	Bj
1	2	3	4	1	2	3	
1.0							
0	1.00						
0.9	0.93	1.00					
4			1.00				
0.9	0.93	1.00					
1				1.00			
0.8	0.92	0.93	1.00				
8					1.00		
0.7	0.83	0.82	0.85	1.00			
9						1.00	
0.7	0.82	0.79	0.83	0.96	1.00		
8							1.00
0.8	0.83	0.80	0.84	0.96	0.98	1.00	
0							
0.3	0.34	0.32	0.33	0.34	0.34	0.35	1.00
3							

The similarity between individuals of *C.d. terrificus* and *C.d. collilineatus* ranged from 0.78 and 0.85. These values were slightly lower than those obtained between individuals of the same subspecies, allowing separating them in two populations. The similarity values between *Crotalus* and *Bothrops* were of the order of 0.33.

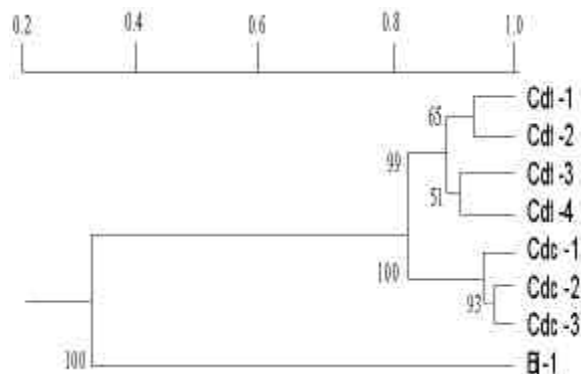


Figure 2- Similarity tree based on the shared bands in pairwise comparisons using UPGMA and Jaccard's

distances. Cdt - *C. d. terrificus*, Cdc- *C. d. collilineatus*, Bj- *Bothrops jararaca*.

The similarity matrix was used to generate a phenogram (Figure 2). Expectedly, *Crotalus* and *Bothrops* were clustered in different groups with low relation between them. The phylogenetic tree showed that the *Crotalus* specimens clearly form two separate groups that corresponded to the two subspecies studied in this work. The lowest similarity among individuals of each group was 92%, while the similarity between groups was 81%.

Considering that each specimen represented a different genetic pool and, as a whole, they gave a good estimative of the genetic variability of each subspecies, the present data supported the separation of *C. durissus*, into *C.d. terrificus* and *C.d. collilineatus*. Moreover, this molecular markers would be useful to confirm the crosses between the two subspecies in the sympatric region (São Paulo state), reported by Hoge (1965) and Santos and Laporta Ferreira (1992), and to evaluate the gene flow between them.

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RESUMO

O presente trabalho foi desenvolvido visando determinar a aplicabilidade da análise de RAPD na identificação de indivíduos pertencentes a duas subespécies de cascavéis sul-americanas (*Crotalus durissus terrificus* e *Crotalus durissus collilineatus*). As 11 sequências arbitrárias inicializadoras testadas nestes experimentos, amplificaram um total de 161 bandas, das quais 31% foram polimórficas na comparação entre espécimes de *Crotalus*. Os resultados combinados

obtidos com as distintas sequências inicializadoras, permitiram a identificação de todos os indivíduos avaliados. Várias bandas características de cada sub-espécie foram identificadas. O dendrograma, baseado em marcaodres de RAPD, mostra tres grupos que correspondem às duas sub-espécies de *Crotalus* e ao espécimen de *Bothrops jararaca*.

REFERENCES

- Barrio, A. (1960), Consideraciones en torno a *Crotalus durissus terrificus crotaminicus* Moura Gonçalves. *Physis* (Buenos Aires) **22**, 141-147
- Branch, B. (1973), The collection of blood by cardiac puncture from surgically anesthetized snakes. *J. Herp. Assoc. Africa*. **11**, 5-6
- Burke, T. and M. W. Bruford (1987), DNA fingerprinting of birds. *Nature*. **327**, 149-152
- Campbell, J. A and W. W. Lamar (1989). The venomous reptiles of Latin America. Cornell Univ. Press, Ithaca, N.Y., 425
- Duellmann, W. E. (1979), The South American herpetofauna: its origin, evolution and dispersal. W.E. Wuellmann, Ed., Monogr. Mus. Nat. Hist., Univ. Kansas, nº 7.
- Ferreira, M. E., and D. Grattapaglia. (1996), Introdução ao uso de marcadores moleculares em análise genética. 2 ed. EMBRAPA-CENARGEM,
- Hillel, J., T. Schaap, A Haberfield, A J Jeffrey, Y. Plotzky, A Cahaner and U. Lavi. (1989), DNA fingerprinting of poultry. *Animal Genetics* **20**, 25-35
- Hoge, A. R. (1965), Preliminary account on Neotropical Crotalinae (Serpentes, Viperidae). Mem. Inst. Butantan. **32**, 109-184
- Hoge, A. R. and S. A. R. W. D. L. Romano-Hoge (1979), Sinopse das serpentes peçonhentas do Brasil. Mem. Inst. Butantan. **42/43**, 373-496
- Master, B. S. (1995), The use of RAPD markers for species identification in demognathine salamanders. *Herpetol. Ver.* **26**, 92-95
- Peters, J. A and B. Oreja-Miranda (1970), Catalogue of the Neotropical Squamata: Part I. Snakes. *Bull. U.S. Natl. Mus.* **297**, 1-347
- Rohlf, F. J. (1989), NTSYS-pc numerical taxonomy and multivariate analysis system, version 1.70. Exeter Software, Setauket, N.Y.
- Santos, S. and I. L. Laporta Ferreira. (1992), Discriminação geográfica de *Crotalus durissus terrificus* and *Crotalus durissus collilineatus* no Estado de São Paulo. Res. Congr. Latino-Americano de Zoologia, Belém, PA, Brasil, 123.
- Williams, J. G. K.; J. A. Rafalski and S. V. Tingey. 1993. Genetic analysis using RAPD markers. *Methods Enzymol.* **218**, 704-740

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