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Protein polymorphism in populations of *Boa constrictor occidentalis* (Boidae) from Córdoba province, Argentina

Paula C. Rivera[1], Margarita Chiaraviglio[1], Gabriela Pérez[2], Cristina N. Gardenal[3]

Abstract. *Boa constrictor occidentalis*, the only subspecies of the genus *Boa* present in Argentina, is endangered because of the strong hunting pressure due to the skin and pet-shop trade and the destruction and fragmentation of its habitat. We estimated levels of protein polymorphism and determined the degree of genetic differentiation between two populations of *B. c. occidentalis* in Córdoba province, Argentina. We obtained blood samples from 93 specimens in two sampling sites 200 km apart. A total of 17 proteins affording information on 25 presumptive loci were studied by gel electrophoresis techniques. Only four loci were polymorphic: *6-Pgdh-1*, *Cat-1*, *Ldh* and *Hp*. The mean heterozygosity per locus was low (0.06 and 0.07). Life history and ecological traits of this snake may explain the low levels of polymorphism found. No evidence of inbreeding was detected. The average genetic differentiation between the two sampled areas was not significant ($\theta = 0.004$). These results would indicate a relatively recent fragmentation of an original gene pool.

Introduction

Boa constrictor, the only species included in the genus *Boa*, is distributed throughout America from 30°N to 36°S. Taxonomists currently recognize eight to ten subspecies, which are poorly differentiated on the basis of external characters (Forcart, 1951; Stimson, 1969; Peters and Orejas-Miranda, 1970; Langhammer, 1983; Kluge, 1991; Price and Russo, 1991). *B. c. constrictor*, *B. c. imperator*, *B. c. amarali*, *B. c. occidentalis*, *B. c. ortonii*, *B. c. melanogaster* and *B. c. mexicana* are cited for the mainland of South and Central America, the last three are now considered by some authors to be undeserving of subspecific status. The remaining subspecies, *B. c. nebulosus*, *B. c. sabogae*, *B. c. orophias*, and *B. c. sigma*, have very restricted geographic ranges. *Boa constrictor occidentalis* is the southernmost subspecies and it is the only member of the genus *Boa* present in Argentina. It is found in the semiarid plains of the phytogeographic regions of Chaco, Monte and Espinal of Argentina. It is also distributed in Paraguay from the 21°S up to the borderline with Argentina (Peters and Orejas-Miranda, 1970). This snake is subject to strong hunting pressure due to the skin and pet-shop trade (Gruss, 1991; Avila and Acosta, 1996), and to the destruction and fragmentation of its habitat by the farming and cattle raising activities in the area. It is considered an endangered species and has been included in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (1997). Despite the current legal protection, hunting pressure is an ongoing problem for this snake. Studies to increase our understanding of the biology of *B. c. occidentalis* in its natural habitat are critical for its conservation (Chiaraviglio et al., 1998b). Since specimens cannot be captured with funnel traps because of their large size (1.5 to 4 m) field studies are particularly difficult. Moreover, its cryptic color patterns make it almost impossible to perform visual surveys.

In recent years, conservation biology has been particularly concerned with the genetic structure of threatened species as data on the degree of intrapopulation polymorphism, genetic

1 - Biología del Comportamiento, Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Av. Velez Sarsfield 299, CP 5000, Córdoba, Argentina
e-mail: riverapauc@yahoo.com.ar
2 - Química Biológica, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Av. Valparaiso s/n, CP 5000, Córdoba, Argentina
3 - Genética de Poblaciones y Evolución, Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Av. Velez Sarsfield 299, CP 5000, Córdoba, Argentina

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isolation among demes and breeding structure contribute to the design of management plans (e.g. Loeschcke et al., 1994; Prior et al., 1996). Among the genetic markers used to study the degree and distribution of genetic variability in time and space, protein polymorphism revealed by gel electrophoresis and specific staining techniques is one of the most widely used. As they are co-dominant genetic markers, they also provide some evidence on possible departures from random mating.

Although knowledge on the degree of genetic similarity among populations is highly relevant in conservation and evolutionary studies, no more than a few studies on genetic structure of populations have been reported in Serpentes (Prior et al., 1996; Sasa and Barrantes, 1998; King and Lawson, 2001).

Detection of inbreeding is another key issue in population biology since increased mating among relatives can reduce individual and population fitness (Charlesworth and Charlesworth, 1987). Madsen et al. (1995) reported inbreeding depression (reduced litter size relative to maternal body size and a higher proportion of deformed and stillborn offspring) in a geographically isolated population of a *Vipera* species.

The purpose of this study is to estimate levels of protein polymorphism in two populations of *B. c. occidentalis* from the Argentinian Chaco and to determine the degree of genetic differentiation between them.

Materials and methods

Samples. *B. c. occidentalis* is distributed in Córdoba province (Argentina) in the Northern, Western and Northwestern areas of the province (Di Fonzo de Ábalos and Bucher, 1981, 1983; Chiaraviglio et al., 1998a). Animals were captured in two districts: Pocho (31°50′S; 65°50′W) in the west, and Sobremonte (29°55′S, 64°20′W) in the northwest. Locations of capture were determined with a Global Positional System (Garmin III). Each sampling site covers an area de 400 km^2 and they are separated by 200 km. Pocho belongs to the phytogeographic region of Chaco Arido, and Sobremonte to Chaco Serrano (Bridarolli and di Tada, 1996). Although the vegetation structure shows distinctive characteristics in each area, underbrush and secondary xerophytic wood resulting from deforestation are common in both regions (Cabido and Zak, 1999); farming and cattle raising activities also produce a patchy distribution of natural habitats. There are not physical barriers separating the two sampling areas.

A total of 93 individuals, adults and sub-adults, were captured by hand during trips carried out each month between 1998 and 2001: 48 specimens were found in Pocho (27 males and 21 females) and 45, in Sobremonte (26 males and 19 females). Blood samples (about 3 ml) were obtained from the caudal vein using a syringe, placed in tubes with sodium citrate, refrigerated and carried to the laboratory. Before releasing the individuals to their original places, they were marked by ventral scale clipping and by subcutaneous implantation of microchips (Passive Integrated Transponder System, ID-100, Trovan) to avoid possible recaptures.

Electrophoresis. Red cells were separated by centrifugation, washed in saline solution and lysed with distilled water. Red cells lysate and plasma were submitted to gel electrophoresis (table 1). Starch gels were prepared in a concentration of 10% (W/V) and poliacrilamide gels, in a concentration of 7.5%. The buffer Tris-borate-EDTA pH 8.6, 0.1 M (Markert and Faulhaber, 1965) was used with a voltage gradient of 8 V/cm during 16 hrs. at +4°C and the buffer Tris-citric, pH 6.3 (gel) and pH 6.7 (electrodes) (Gardenal and Blanco, 1985), with a voltage gradient of 8 V/cm during 4 hrs. at +4°C. Poliacrilamide gels were run during 1:30 hrs. at 150 V, in buffer Tris-HCl pH 8.6, 1.5 M (gel) and Trisglycine pH 8.3 (electrodes) (Hames and Rickwood, 1981).

Specific staining procedures to reveal the activity of different enzymes were performed as described by Murphy et al. (1996). Plasma proteins were stained with Coomasie Blue R 250. For descriptive purposes, alleles detected at polymorphic loci were designated with letters in order of decreasing anodal mobility.

Statistical analysis. Allelic frequencies, mean heterozygosity per locus (H), percentage of polymorphic loci (P99% criteria), and mean number of alleles per locus (A) were calculated for each locality. Agreement between observed and expected genotypic frequencies was analyzed by a chi-square test. All the estimates were obtained using the BIOSYS-2 program (Black IV, 1997). Genetic distance between populations was calculated using Rogers' index (Wright, 1978).

Genetic differentiation among populations was examined using the corrected method of Weir and Cockerham (1984), where θ is an estimator of the parameter F_{ST}, a measure of the amount of differentiation among populations. We used f as an estimator of F_{IS}, the inbreeding coefficient within subpopulations, which represents reduction or increase of heterozygosity due to non-random mating. Significance of θ for individual loci was obtained by permutation of alleles between samples, and that of f for individual loci was obtained by permutation of alleles within samples. The FSTAT program was used for these calculations (Goudet, 2000).

Table 1. Enzymes, loci and assays conditions used in the study of *Boa constrictor occidentalis*. H: hemolizate. P: plasma.

Protein	EC number	Locus	Tissue sample	Support	Buffer system
6-phosphogluconate dehydrogenase	1.1.1.44	*6-Pgdh-1*, *6-Pgdh-2*	H	SGE[a]	I
Acid phosphatase	3.1.3.2	*Acp-1*, *Acp-2*	H	PAGE[b]	III
Alkaline phosphatase	3.1.3.1	*Alp*	P	PAGE	III
Catalase	1.11.1.6	*Cat-1*, *Cat-2*	H	SGE	I
Esterase	3.1.1.1	*Est-1*, *Est-2*	P	SGE	I
Fumarate hydratase	4.2.1.2	*Fumh*	H	SGE	I
Glucose phosphate isomerase	5.3.1.9	*Gpi-1*, *Gpi-2*	H	SGE	I
Hemoglobin	–	*Hb*	P	SGE	I
Isocitrate dehydrogenase	1.1.1.42	*Idh*	H	SGE	II
Lactate dehydrogenase	1.1.1.27	*Ldh*	H	SGE	I
Malate dehydrogenase	1.1.1.37	*Mdh*	H	SGE	II
Phosphoglucomutase	5.4.2.2	*Pgm-1*, *Pgm-2*	H	SGE	I
Plasma Protein 1	–	*Pp-1*	P	PAGE	III
Plasma Protein 2	–	*Pp-2*	P	PAGE	III
Plasma Protein 3	–	*Pp-3*	P	PAGE	III
Plasma Protein 4	–	*Pp-4*	P	PAGE	III
Superoxide dismutase	1.15.1.1	*Sod-1*, *Sod-2*, *Sod-3*	H	SGE	I

[a] SGE: Starch gel electrophoresis.
[b] PAGE: Polyacrylamide gel electrophoresis. I: Tris-borate-EDTA, pH 8.6, 0.1 M. II: Tris-citric, pH 6.3 (gel) and pH 6.7 (electrodes). III: Tris-glycine pH 8.3 (electrodes), Tris-HCl pH 8.6, 1.5 M (gel).

Results

A total of 17 proteins affording information on 25 presumptive loci were studied. The enzymes fumarate hydratase, alkaline phosphatase, isocitrate, malate and lactate dehydrogenases showed one anodic band. 6-phosphogluconate dehydrogenase, phosphoglucomutase, acid phosphatase and esterases presented two anodic zones of activity, each one representing the product of two different loci. Hemoglobin, glucose phosphate isomerase, and superoxide dismutase exhibited one, two and three cathodic bands respectively. The catalase showed two zones of activity, one cathodic (locus *Cat-2*) and one anodic (locus *Cat-1*).

Four main anodic fractions of plasma proteins were revealed (*Pp-1* to *Pp-4*). On the basis of patterns reported for many vertebrate species, the three more anodical would correspond to albumin, transferrin, and haptoglobin, in that order. The loci *Mdh*, *Idh*, *Hb*, *Alp*, *Acp-1*, *Acp-2*, *Est-1*, *Est-2*, *Gpi-1*, *Gpi-2*, *Fumh*, *Pgm-1*, *Pgm-2*, *6-Pgdh-2*, *Pp-1*, *Pp-2*, *Pp-4*, *Cat-2*, *Sod-1*, *Sod-2* and *Sod-3* were monomorphic for the same allele in both populations.

Only four loci were polymorphic: *Pp-3*, *Cat-1*, *6-Pgdh-1*, and *Ldh-1*. In *Pp-3*, we observed the three possible phenotypes determined by two codominat alleles; the heterozygote presented two bands (fig. 1a). For *Cat-1*, a tetrameric enzyme, two phenotypes were found (fig. 1b) which can be explained by the presence of two codominant alleles, a and b, the latter being predominant in both populations. Seven different phenotypes were observed in *6-Pgdh-1* (fig. 1c), which would be the product of four different alleles in the locus; three of them presented one band (homozygous individuals); the other four showed two bands. A dimeric structure has been reported for this enzyme in a variety of organisms, including snakes, which would determine a three banded pattern in heterozygous individuals. If a mutation in one allele produces a structural change that precludes the formation of the heterodimer, only two bands are detected in heterozygotes

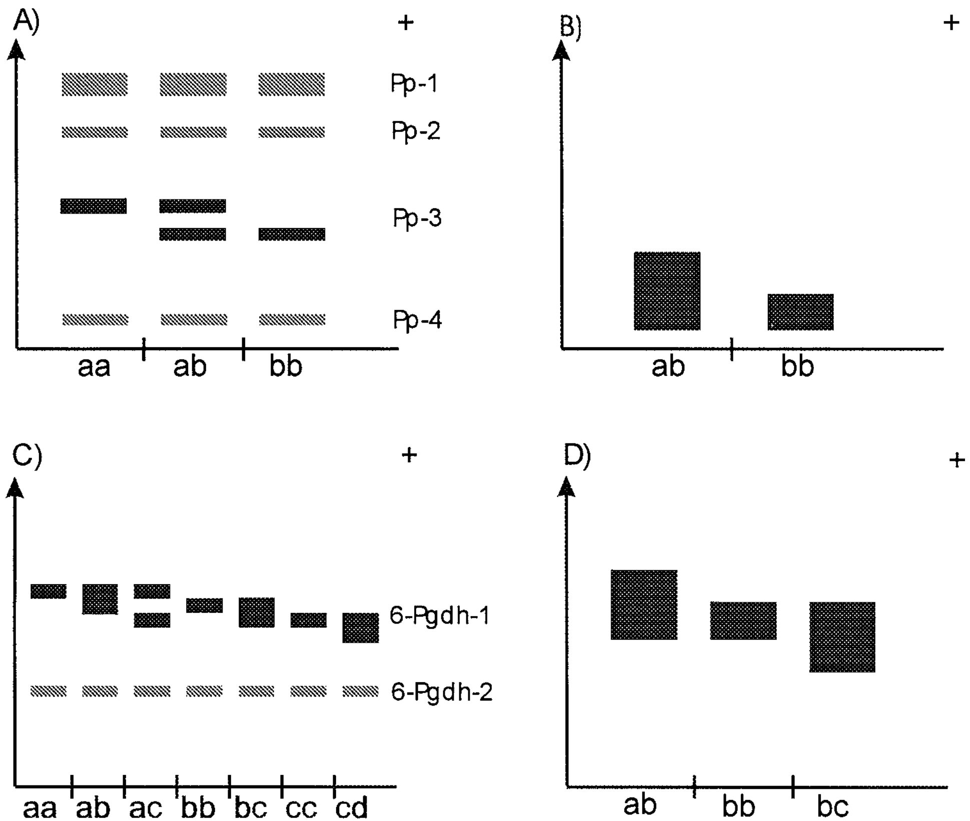

Figure 1. Schematic representation of electrophoretic patterns of Plasma Proteins (A), *Cat-1* (B), *6-Pgdh* (C) and *Ldh* (D) in *Boa constrictor occidentalis*.

(Ward et al., 1992). This would be the case for *B. c. occidentalis*.

Only one locus of *Ldh* (a tetrameric enzyme) was expressed in red cells of *B. c. occidentalis*, the *Ldh-1* or B4. The pattern for homozygous individuals is similar than the one reported by Schwantes (1973) in heart of *B. c. amarali*. In *B. c. occidentalis*, the heterocigotes show a wide zone of activity, which would include the five tetramers expected; it is possible to infer from these patterns the existence of three alleles at the locus *Ldh-1*.

Table 2 presents the allele frequencies and the measures of genetic variation (P, A, H_o and H_e) of the polymorphic loci in each population. In all the polymorphic loci, the observed genotype frequencies were not significantly different from those expected from Hardy-Weinberg equilibrium. Average θ between the two areas was 0.004. Neither this value nor those per locus were significant. Rogers' genetic distance between the studied populations was 0.030.

Discussion

This is the first report on genetic variability in the species *B. constrictor* and on variance in allele frequencies between two populations of *B. c. occidentalis*. Measures of genetic variation (P, A, H_o and H_e) showed low levels of polymorphism in the two populations studied. H_e (0,058 and 0,067) is lower than that cited for Reptilia by Ward et al. (1992) ($H_e = 0.078 \pm 0.007$) although it is close to that referred by Nevo et al. (1984) ($H_e = 0,055 \pm 0.006$) for this taxon.

Low heterozygosity has also been reported in other snake species: 0.029-0.055 in *Bothrops asper* (Sasa and Barrantes, 1998), 0.038-0.045

Table 2. Allelic frequencies and levels of allozymic polymorphism of *Boa constrictor occidentalis* in Pocho and Sobremonte departments.

Locus alleles	Pocho ($n = 48$)	Sobremonte ($n = 45$)
Cat-1		
A	0.074	0.178
B	0.926	0.822
6-Pgdh-1		
a	0.156	0.244
b	0.490	0.511
c	0.333	0.233
d	0.021	0.011
Pp-3		
A	0.563	0.535
B	0.438	0.465
Ldh		
A	0.053	0.033
B	0.926	0.900
C	0.021	0.067
Percentage of polymorphic loci (P)	16.7	16.7
Mean number of alleles per locus (A)	1.3	1.3
Mean heterozygosity per locus observed (H_o)	0.058	0.067
Mean heterozygosity per locus expected (H_e)	0.056	0.065

in *Crotalus scutulatus scutulatus* (Wilkinson et al., 1991), 0.041-0.08 in *Thamnophis sirtalis* (Sattler and Guttman, 1976), and 0-0.005 in the genus *Nerodia* (Thompson and Crother, 1998). In the case of *Cerrophidion golmani*, absence of allozymic polymorphism was reported, on the basis of 15 loci analysed (Sasa, 1997).

Different explanations can be proposed for the low levels of protein polymorphism here detected. Individuals of *B. c. occidentalis* reach very large body sizes (4 m). It is considered a long-lived species (Cei, 1993), with a generation span longer than a year (Chiaraviglio, pers. observ.). These traits have been consistently correlated with low levels of mean heterozygosity (Nevo et al., 1984). These authors also demonstrated that, in general, specialists display significantly lower genetic variability than generalists. The few available data on the ecology of *B. c. occidentalis* suggest that it has quite specific habitat requirements as those related with use of refuges and thermal regulation mechanisms (Attademo et al., 2004).

Table 3. Values of f, F and θ in *Boa constrictor occidentalis*

Locus	f	θ
6-Pgdh-1	0.046	0.003
Hp	−0.055	−0.009
Ldh	−0.064	−0.001
Cat-1	−0.161	0.039
Total	−0.029	0.004

Population bottlenecks reducing mean number of alleles per locus and proportion of polymorphic loci could also explain our findings. Although at present no data on population densities are available for *B. c. occidentalis*, the existence of drastic reductions in population size as a consequence of hunting pressure and destruction of woody habitat in the area cannot be discarded.

No evidence of inbreeding was found. In the four polymorphic loci detected, observed genotype frequencies were in agreement with those expected under Hardy-Weinberg equilibrium; f values were also non-significant (table 3). These results would indicate that the populations have not been forced to inbreeding as a consequence of a severe reduction in effective population size, at least in a degree detectable by the methodology used in this study. However, we must be cautious in the interpretation of these results until data of more populations are available, because the natural habitat of this boid is rapidly and severe deteriorating.

Genetic differentiation between the two populations analysed, measured as Rogers' genetic distance and mean θ values, was non-significant. The high genetic similarity between populations from Pocho and Sobremonte could indicate that at present they maintain an important degree of gene flow. A more likely explanation is that the two populations became isolated quite recently from the same original gene pool and had not enough time to accumulate allelic differences at structural loci. The drastic environmental changes that determined a patchy distribution of favorable habitat for the species

in the area, begun in the last decades of the nineteenth century (Bucher and Huszar, 1999), which is a short period for a long-lived species with a large generation time. New studies in other populations covering a larger geographic range using highly polymorphic genetic markers would be needed to get a deeper knowledge of processes determining the genetic structure of this snake.

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