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ALLOZYME VARIATION IN POPULATIONS OF *BOTHROPS ASPER* (SERPENTES: VIPERIDAE) IN COSTA RICA

MAHMOOD SASA[1,3] AND RAMIRO BARRANTES[2]

[1]*Instituto Clodomiro Picado and* [2]*Instituto de Investigaciones en Salud, Universidad de Costa Rica, San José, Costa Rica*

ABSTRACT: We investigated allozyme variation from Costa Rican populations of the fer-de-lance, *Bothrops asper*. Blood samples were taken from a total of 100 specimens from six localities representing three major physiographic regions: Atlantic versant, Central Pacific lowlands, and Southwestern Pacific lowlands. Five of 16 protein coding loci (phosphogluconate mutase, isocitrate dehydrogenase, glucose 6-phosphate isomerase, malate dehydrogenase, and carbonic anhydrase-1) were polymorphic. Average heterozygosity for these loci varied between 2.9% and 5.5%. Genetic distances among samples from localities from the same physiographic region were not significantly different from zero. Pairwise comparisons between regions also resulted in low estimated distances. Analysis of population structure suggests high levels of gene flow among populations. Rates of evolution of venom and morphology previously reported for populations of this species seem to be uncoupled from rates of allozyme variation.

Key words: Pitviper; *Bothrops asper*; Allozymes; Genetic variation; Population genetics; Costa Rica

THE lancehead, *Bothrops asper* (Garman), is a common pitviper of Central America and is considered the most important venomous snake in the region from the standpoint of human morbidity and mortality (Bolaños, 1984; Hardy, 1994). It has an extensive distribution ranging from the Atlantic versant of Tamaulipas (Mexico), throughout the Caribbean lowlands of Central America, to northeastern Colombia and Venezuela (Campbell and Lamar, 1989). Aside from a disjunct population occurring in western Chiapas and Guatemala, the distribution of this species is continuous from the south–central Pacific coast of Costa Rica to the Pacific versant of the Colombian and Ecuadorean Andes (Campbell and Lamar, 1989; Peters and Orejas-Miranda, 1970). In Central America, *B. asper* can be found from 0–1200 m altitude, although it is uncommon above 800 m. While the species is found in a great variety of habitats, it is particularly abundant in and well adapted to areas disturbed by humans (Greene and Campbell, 1992). At many localities, *B. asper* is the most common species of snake (Campbell and Lamar, 1989).

The geographic variation of morphological characters in this species is remarkable and undoubtly is the major factor responsible for the taxonomic chaos involving populations of *B. asper*, *B. atrox*, and other relatives from South America (Campbell and Lamar, 1992; Markezich and Taphorn, 1993). In Middle America, univariate and multivariate analyses of several morphological characters may distinguish among populations of *B. asper* (Sasa, 1996). In Costa Rica, specimens from the Atlantic slopes differ from those of the Pacific lowlands in body size, litter size, and some scutellation characters (Solórzano and Cerdas, 1989; Tejeira-Rodriguez, 1993). Toxicological variation also exists in populations of *B. asper*, with implications for snakebite therapy. Noticeable variation among populations in Costa Rica was reported in the expression patterns of myotoxin isoforms (Lomonte and Carmona, 1992; Moreno et al., 1988), the specific enzymatic activities of coagulant proteinases (Aragón and Gubensek, 1981), and the biological activities of the toxins (Gutiérrez et al., 1980). Thus, adult venoms from Atlantic populations are more hemorrhagic and myonecrotic, whereas those of the

[3] PRESENT ADDRESS: Department of Biology, University of Texas at Arlington, Arlington, TX 76019, USA. e-mail: SASA@UTA.edu

Central Pacific lowlands are more proteolytic. Venom of both regions have similar lethality, edema forming activity, and hemolytic effect (Gutiérrez et al., 1980).

Variation observed between snake populations might relate to overall genetic divergence (Arnold, 1988; Glenn et al., 1983; Murphy et al., 1987) or may be the result of the action of natural selection. For instance, venom variation probably resulted from natural selection for venom appropriate for feeding on local prey (Daltry et al., 1996*a*).

Aragón and Gubensek (1981) suggested that population isolation might account for the venom differences observed in *B. asper* from Costa Rica. In this country, a central axis of mountain ranges runs northwest–southeast and divides the Atlantic and Pacific lowlands, apparently precluding contact between populations located on each versant. In this study, we investigated whether the morphological and venom differences observed among populations of *B. asper* in Costa Rica are correlated with genetic divergence among them.

Materials and Methods

Specimens

A sample of 100 specimens of *Bothrops asper* were collected for venom extraction between July 1991 and November 1992 and brought to the Serpentarium of the Instituto Clodomiro Picado, Universidad de Costa Rica. We sampled only adult snakes (snout–vent length ≥85 cm, both sexes), all from six localities of Costa Rican lowlands (Fig. 1). These localities are located in three physiographic regions.

(1) Central Pacific Lowlands.—This area is located in the river basin of Grande de Tárcoles and Pirrís rivers, in the middle of the Pacific versant, and contains lands of moderate elevation (0–800 m) that are covered with Tropical Wet Forest and Tropical Moist Forest (Tosi, 1969). We sampled three localities in this area: Puriscal, Acosta, and Quebrada Ganado (Fig. 1). These localities are highly disturbed and all specimens collected came from grassland habitats.

(2) Southeastern Pacific Lowlands.—The life zone of this region is Tropical Wet Forest (Tosi, 1969). The specimens collected from this region came from two localities: Península de Osa and Golfito.

(3) Atlantic Versant.—Two life zones occur in this region: Premontane Wet Forest and Tropical Moist Forest (Tosi, 1969). Snakes were collected at Penshurt and Siquirres. Due to the proximity of these two localities, these specimens were considered as a single geographical sample.

Protein Electrophoresis

We extracted a 1 ml blood sample from each individual by tail clipping or by cardiac puncture using a heparinized syringe. We centrifuged the blood samples at 4 C at 5000 rpm and then separated plasma and cellular fractions. The erythrocytes were washed three times in saline solution and were hemolyzed with 0.1% sodium citrate. After centrifugation, we stored blood plasma and the hemolyzed erythrocytes at −70 C; these were used later without modification. We performed horizontal starch gel electrophoresis (Murphy et al., 1990) for each locus using 12% (w/v) starch gels at 5 C. Transferrin and lactate dehydrogenase (Ldh) products were assayed using a 7% polyacrylamide gel at 5 C. The Ldh reaction mixture followed Schwantes (1973). Other histochemical staining procedures followed Harris and Hopkinson (1976) and Murphy et al. (1990). Enzymes and buffer systems are listed in Table 1. We considered electromorphs homologous if they had the same mobility. Presumptive genotypes were assigned to each individual based on the enzyme banding pattern observed by eye and followed the criteria in Murphy and Crabtree (1985).

Data Analyses

Allelic frequency data were analyzed using BIOSYS-1 (Version 2.0: Swofford and Selander, 1981). The average locus heterozygosity (H) and percent of polymorphic loci (P) were used to measure genetic variability estimates for each locality. We calculated the coefficients of genetic distance of Nei (1978) and Rogers (1972). Genetic

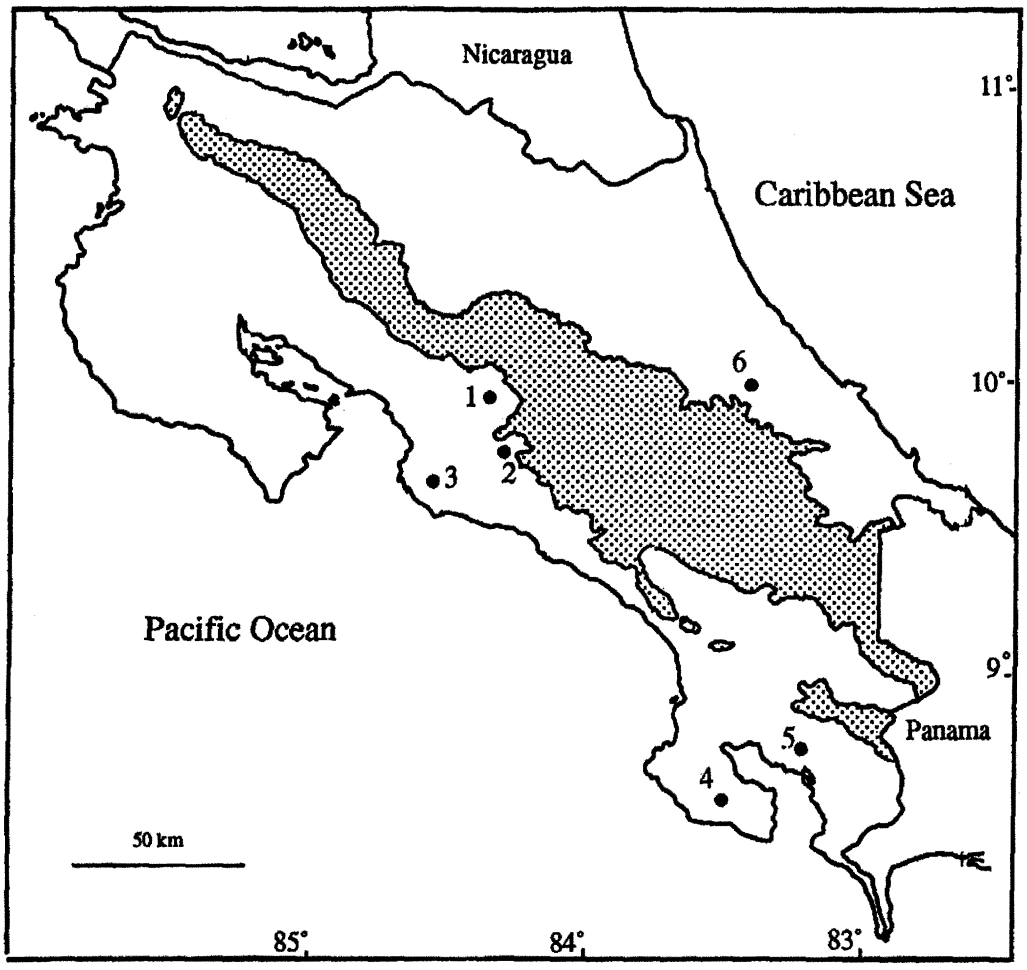

FIG. 1—Sampled localities of *Bothrops asper* in Costa Rica. Contour line represents elevations ≥1000 m. Locality numbers as follow: (1) Puriscal, (2) Acosta, (3) Quebrada Ganado, (4) Península de Osa, (5) Golfito, and (6) Penshurt-Siquirres.

diversity within and among populations was analyzed using Wright's (1965) *F*-statistics, according to the methods outlined by Weir and Cockerham (1984). We examined two levels of subdivision: physiographic region and localities within each region. Estimates of the variance of *F*-statistics were provided by a jackknife procedure (Reynolds et al., 1983). Gene flow was estimated from *F*-statistics and by Slatkin's (1981) private alleles procedure. This method is based on the strong dependence between the total level of gene flow among several populations and the average frequency of an allele conditioned on the number of populations in which this allele occurs (Slatkin and Barton, 1989).

Analyses of spatial correlation of genetic divergence among populations were performed using Mantel correlations (Mantel, 1967). We used Rogers (1972) distance for these analyses, because Nei distances depend upon mutation rates. The matrix of geographical distances was constructed from the linear distance following the altitudinal isocline between localities. In this way, the geographical barrier between the Atlantic and Pacific populations in lower Central America is taken into account. Comparisons with the random distribution of coefficients obtained after 5000 per-

TABLE 1.—Enzyme and electrophoretic conditions used in determining the allozyme characteristics. Prefixes "s" and "m" indicate supernatant and mitochondrial enzymes respectively. Enzyme nomenclature numbers are those of International Union of Biochemistry and Nomenclature Committee (1984). Locus assignments for enzymes follow Murphy and Crabtree (1985).

Enzyme system	Commission number	Locus	Assay* conditions
Acid phosphatase	3.1.3.2	Acp-1	A, B
Carbonic anhydrase	4.2.1.1	Ca-1	D
		Ca-2	D
Esterase**	3.1.1.1	Est-A	E, B
		Est-C	E, F
		Est-D	B, E, F
Glucose-6-phosphate isomerase	5.3.1.9	Gpi-A	B
Isocitrate dehydrogenase	1.1.1.42	slcdh-A	A, C
L-lactate dehydrogenase	1.1.1.27	Ldh-A	A, B, H
		Ldh-B	B, H
Malate dehydrogenase	1.1.1.37	sMdh-A	A, F
		mMdh-A	A, F
Phosphoglucomutase	2.7.5.1	Pgm-A	C, F
Tripeptidase***	3.4.11.13	Pep-5	E, G
Superoxide dismutase	1.15.1.1	sSod-A	C
Tranferrin		Tf	H

* Conditions: (A) 0.005 M DI-Histidine pH = 7 (gel), 0.41 M Sodium citrate (electrode) pH = 7.0, 5 v/cm, 17 h. (B) 0.1 M Tris, 0.028 M Citric acid, pH = 7.4, 5 v/cm, 12 h. (C) 0.25 M NaH_2PO_4, 0.15 M Citric acid pH = 5.9, 5 v/cm 12 h. (D) 0.9 M Tris, 0.5 M Boric acid, 0.02 M EDTA pH = 8.6, 5 v/cm, 18 h. (E) 0.1 M Tris, 0.1 M Maleic acid pH = 7.2, 5 v/cm 17 h. (F) 0.1 M Tris, 0.1 M Maleic acid, 0.01 M EDTA, 0.01 M $MgCl_2$, pH = 7.4, 18 h at 35 mA/gel. (G) 0.1 M Tris, 0.1 M NaH_2PO_4, pH = 7.4, 5 v/cm 18 h. (H) 0.04 M Tris, 0.18 M Glycine pH = 8.5, gel 7% acrylamide 4 h at 40 mA/gel.

** Substrate used: Naphthyl acetate (Est-A, Est-C) and Methyl umbelliferyl acetate (Est-D).

*** Substrate used: L-leucyl-glycyl-glycine.

mutations of the genetic distance matrices determined the significance of the observed correlation coefficients.

RESULTS

Of 16 loci assayed for 100 specimens (Table 1), 11 were monomorphic in all individuals studied: mMdh-A, Ldh-A, Ldh-B, Ca-2, Est-A, Est-C, Est-D, Pep-5, sSod-A, Acp-1, and Tf. Five loci were polymorphic, with the frequency of the common allele ≤0.99 (Table 2). One sample (Quebrada Ganado) had a rare allele (c) in s-Icdh-A (frequency of 0.026). The percentage of polymorphic loci varied between localities, being highest in snakes from Quebrada Ganado (P = 30.8%) and lowest in the Península de Osa sample (P = 7.7%) where the only variable locus was Gpi-A. Few alleles per locus (A ± SD = 1.21 ± 0.09) were found in these enzymatic systems in *B. asper*.

Low levels of geographic variation were observed in allelic frequencies of polymorphic loci. The Gpi-A common allele appeared fixed for the Acosta sample, perhaps due to the small sample size at that locality. Phosphoglucomutase (Pgm-A) was polymorphic only in samples from Golfito and Penshurt-Siquirres. In these localities, the isocitrate dehydrogenase fast electromorph was fixed. Allelic frequencies were in Hardy-Weinberg equilibrium at all loci, except the mMdh-A in the Atlantic sample (X^2 = 23, $P < 0.0001$) which exhibited a deficiency of heterozygotes. The mean locus heterozygosity (H) did not differ significantly between populations ($F_{[5, 72, 0.05]}$ = 0.19, P = 0.963), ranging from 0.029 in the Quebrada Ganado sample to 0.055 in snakes from Golfito (Table 2).

Genetic identity (Nei, 1978) ranged from 0.995–1.000 among sampled localities. Genetic distances within physiographic regions were not significantly different from zero; the pairwise comparisons between samples from those regions resulted in low estimated distances (Table 3). Nei's distance estimates the accumulated number of codon substitutions per locus since the time of divergence between the populations. Based on our sample of 16 loci, on average the population of *B. asper* had only about one codon substitution per 250 loci. These values suggested a recent di-

TABLE 2.—Summary of the allelic frequencies for five polymorphic loci in *Bothrops asper* for several localities of Costa Rica. Numerical designations for each population are as follow: 1 = Puriscal, 2 = Acosta, 3 = Quebrada Ganado, 4 = Peninsula de Osa, 5 = Golfito, 6 = Penshurt-Siquirres. Sample size for each locality is in parentheses.

Locus (allele)	Locality 1 (n = 15)	2 (n = 10)	3 (n = 20)	4 (n = 10)	5 (n = 22)	6 (n = 23)
SMdh-A						
(a)	1.000	1.000	1.000	1.000	0.972	0.957
(b)					0.028	0.043
Ca-1						
(a)	0.962	0.929	0.952	1.000	1.000	1.000
(b)	0.038	0.071	0.048			
Gpi-A						
(a)	0.955	1.000	0.944	0.688	0.800	0.900
(b)	0.045		0.056	0.313	0.200	0.100
sldh-A						
(a)	0.846	0.833	0.913	1.000	0.921	1.000
(b)	0.154	0.167	0.087		0.053	
(c)					0.026	
Pgm-1						
(a)	1.000	1.000	1.000	1.000	0.947	0.881
(b)					0.053	0.119
% loci polymorphic*	23.1	15.4	23.1	7.7	30.8	23.1
Alleles per locus	1.2	1.2	1.2	1.1	1.4	1.2
(H)**	0.037	0.037	0.029	0.048	0.055	0.034
(± SE)	(0.024)	(0.027)	(0.016)	(0.048)	(0.032)	(0.023)

* 0.99 criterion.
** Mean locus heterozygosity by direct count.

vergence of these populations and, or, a high degree of gene flow among them.

Low F-values and their variance estimators were found for the five polymorphic loci (Table 4). A slight negative value of the fixation index (F_{st}) was found for sMdh-A (Table 4), but F_{st} was so small that significant population subdivision could not be detected (Slatkin and Barton, 1989). The graphical analysis of rare alleles also showed a trend of high gene flow among the six sampled populations, as implied from the J-shaped relationship between conditional average allelic frequency and the occupancy number.

DISCUSSION

Measures of genetic variation presented here for populations of *Bothrops asper* are similar to those reported for other reptilian species based on allozyme data (Nevo, 1978). The results from the analysis of rare alleles and the low values for F-statistics (Table 4) were consistent with the hypoth-

TABLE 3.—Unbiased genetic distances of Nei (1978: above the diagonal) and Rogers (1972: below the diagonal) genetic distance coefficients for all pairwise combinations of populations of *Bothrops asper* from Costa Rica. Numerical designations for each population are as in Table 2.

Locality	1	2	3	4	5	6
Puriscal	—	0.000	0.000	0.006	0.001	0.002
Acosta	0.007	—	0.000	0.008	0.003	0.003
Quebrada Ganado	0.007	0.012	—	0.005	0.001	0.001
Peninsula de Osa	0.035	0.042	0.030	—	0.000	0.003
Golfito	0.028	0.035	0.023	0.020	—	0.000
Penshurt	0.031	0.039	0.026	0.029	0.019	—

TABLE 4.—Population *F*-statistics* for polymorphic loci in Costa Rican *B. asper*.

Loci	F_{st}	F_{it}	F_{is}	Nm
Ca-1	0.0007	−0.0181	−0.0188	342.2
cicdh-A	0.0308	−0.0529	−0.0865	8.0
Pgm-A	0.0446	−0.0251	−0.0738	5.3
sMdh-A	−0.0201	0.3213	0.3347	12.1
Gpi-A	0.0714	−0.0930	−0.1770	3.3
Jackknife mean	0.0240	0.0263	−0.0042	
Standard deviation	0.0160	0.0748	0.0884	

* Estimates of Wright (1965) *F*-statistics follow Weir and Cockerham (1984).

esis of high levels of gene flow among sampled geographic regions. These results are not totally unexpected when the extensive distribution of the species and its ability to adapt to human-disturbed areas are taken into consideration. The Continental Divide of Costa Rica is higher than the elevational limit of the species throughout most of the country, and, therefore, any region where substantial gene flow might be occurring between Atlantic and Pacific populations must be looked for extralimitally to Costa Rica. The dry-forest life zone on the Pacific versant of Nicaragua and northwestern Costa Rica is an effective barrier to the distribution of *B. asper* (Sasa, 1996). Thus, any gene flow between Atlantic and Pacific populations of this snake in Isthmian Central America must be occurring in Panamá. The Cordillera de Talamanca in Costa Rica ranges southeast into the Chiriquí Province of Panamá. This mountain range is connected with Cordillera Central by highlands above 1000 m. The Cordillera Central extends eastward as far as Cocle Province in central Panamá. Thus, contact between Atlantic and Pacific versant populations of *B. asper* in lower Central America is possible in central and eastern Panamá, where the lancehead is one of the most abundant snakes.

Despite the great variation previously recorded for *Bothrops asper* in venom components, morphological characters, and natural history traits (Sasa, 1996), our results suggest that there might be little genetic heterogeneity among populations of this species in Costa Rica. Mantel tests (Manly, 1991) were used to evaluate the association between the linear geographic distance and the estimates of genetic distance (Table 3). A significant correlation ($r = 0.56$, $P = 0.012$) was observed between Rogers' genetic distances and geographical distances. From these results, the slight genetic divergence among populations included in this study could be attributed to the effects of spatial correlation, or to an isolation by distance effect. In addition, morphological distances in samples of *B. asper* from different localities of Middle America are also correlated to spacial distances (Sasa, 1996).

Based upon the electrophoretic patterns and activities of L-aminoacid dehydrogenase and other venom components, Jiménez-Porras (1964) suggested that a distinct geographical pattern of venom variation occurs among samples from the Caribbean slope, Central, and South Pacific of Costa Rica, similar to those suggested by our allozyme data. The low values of genetic distances and the low number of loci sampled, however, make the concordance between those data sets suspect. Our interpretation is that allozyme and venom variation might not be correlated. The allozyme data do not reveal fixed differences among geographic localities, thus making it difficult to interpret the genetic basis for the observed phenotypic diversity.

The independent evolution of tissue allozymes from venom characters was reported previously for *Crotalus s. scutulatus*, where slight genetic differences, inferred from allozymes, existed between populations with different venom types (Wilkinson et al., 1991). In this species, the uncoupled rates of evolution of these characters may reflect geographically based selection on venom proteins, as opposed to the neutral evolution of allozyme characters (Kimura, 1982). In a recent study, Daltry et al. (1996*a*,*b*) showed that

venom variation among localities of the pitviper *Calloselasma rhodostoma* is associated with its diet and is unrelated to intraspecific phylogenetic relationships and, or, current gene flow. Consequently, geographical variation in venom composition might reflect directional selection for feeding in local prey (Daltry et al., 1996*a*).

If strong selection is acting on venom components of *Bothrops asper,* then these characters could be expected to change faster than allozymes, consistent with our results. Nevertheless, the local selection hypothesis should be invoked with caution, because it is still unclear if geographical variation in diet occurs in this species.

In summary, geographical variation in venom and in morphological characters previously reported for *Bothrops asper* in three regions of Costa Rica may suggest that restricted gene flow occurs among them, or that their populations were independently derived from a common source area. However, allozyme data showed high genetic similarity among populations of *Bothrops asper* in Costa Rica, and high gene flow apparently prevents further differentiation. Our results suggest that there is independent evolution of tissue protein characters from venom and morphological characters in this species that may be associated with local selective differences

Resumen

En Costa Rica, gran diversidad en caracteres morfológicos, toxicológicos y de historia natural han sido previamente reportados entre poblaciones de la serpiente *Bothrops asper.* Con el fin de investigar la relación genética de esas diferencias, se evaluó la variación isozímica entre poblaciones de *Bothrops asper* en ese país. Se tomaron muestras de sangre de un total de 100 individuos provenientes de seis localidades representando a tres regiones fisiográficas: Vertiente Caribe, Pacífico Central y Pacífico Sur. De los 16 loci estudiados, cinco (Pgm-A, Icdh-A, Gpi-A, sMdh-A) resultaron polimórficos. El porcentaje de heterozigosis promedio por población varió entre 2.9 y 5.5%. Las distancias genéticas estimadas entre poblaciones fueron muy bajas. El análisis de la estructura génica indica que poblaciones de *Bothrops asper* en Costa Rica poseen alto flujo génico y gran homogeneidad genética entre ellas. Nuestros análisis sugieren evolución independiente entre los caracteres genéticos y fenotípicos observados en estas poblaciones. Se sugiere que la gran diversidad en veneno y morfologia en esta especie pueda estar asociada con diferencias de selección a nivel local.

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