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8 Systematics of the *Bothrops atrox* complex: new insights from multivariate analysis and mitochondrial DNA sequence information

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Synopsis

We use multivariate analysis of morphological characters and comparative sequencing of a 520 bp fragment of the cytochrome *b* gene (mitochondrial DNA) to investigate patterns of geographic variation in the *Bothrops atrox* species complex, and the population phylogeny of the group in parts of South America. Populations conventionally assigned to *B. atrox* and *B. moojeni* constitute morphologically distinct groupings, with a zone of phenetically intermediate specimens where their ranges meet. *Bothrops marajoensis*, *B. isabelae*, *B. leucurus* and *B. pradoi* are poorly differentiated or undifferentiated from *B. atrox* and each other. Phylogenetic analysis of the cytochrome *b* sequence information reveals that *B. caribbaeus* constitutes an outgroup to the South American populations of the *B. atrox* complex. Within the South American radiation, the conventional species, as generally understood, largely lack historical reality. Two well-corroborated basal clades are revealed: one includes the populations from the lower Amazon and Tocantins–Araguaia drainages, as well as Marajó Island. The other comprises two further basal clades within it, the first consisting of the populations conventionally assigned to *B. moojeni*, the other a number of populations from the eastern coast of Brazil, various places in western and central Amazonia and western Venezuela. Mantel matrix correlation tests show morphological variation among the populations of the complex to be most strongly associated with vegetation type. This suggests that morphological variation represents primarily an adaptation to current selection pressures, probably for crypsis, and not a reflection of population phylogeny.

Introduction

The *Bothrops atrox* species group constitutes a complex group of venomous snakes with a long history of taxonomic confusion. The group displays a complex pattern of among- and within-population variation. Over the last 50 years, this has led to the description as separate species of several groups of populations subjectively perceived as distinct (*B. pradoi* — Hoge 1947; *B. marajoensis* and *B. moojeni* — Hoge 1965; *B. isabelae* — Sandner Montilla 1979; *B. atrox aidae* — Sandner Montilla 1981). However, the status of these forms, as well as that of the populations from the eastern coast of Brazil assigned to *B. leucurus*, remains uncertain (Campbell & Lamar 1989; Cunha & Nascimento 1993). Additional problems concern the relationship between these Amazonian and Brazilian taxa with populations from northern South America, Central America and Caribbean islands, which are variously assigned to *B. asper*, *B. xanthogrammus*, *B. caribbaeus* and *B. lanceolatus*. The species of this group are the principal cause of snakebite mortality and morbidity in much of their range (Haad 1980/81; Bolaños 1984; Kouyoumdjian & Polizelli 1988; Cardoso 1992; Nishioka & Silveira 1992; Otero *et al.* 1992). Understanding the population systematics of this group is therefore of potential importance for the treatment of their bites.

Previous attempts to resolve the systematics of this complex used standard taxonomic methods (i.e. the use of single characters to attempt to diagnose taxa defined *a priori* on the basis of subjective perceptions of differences). However, the *B. atrox* complex displays an intricate pattern of geographic, sexual and ontogenetic variation. In such complex groups, the conventional approach to systematics almost inevitably results in seriously flawed systematic conclusions. Recent analyses of the systematics and phylogeny of the genus *Bothrops* and related taxa, based on hemipenis morphology and plasma protein electrophoresis (Pesantes 1989), and a combination of morphology, anatomy, isozymes and allozymes (Werman 1992), have clarified many problems posed by the genus. In both these studies, the nominal species in the *B. atrox* group were considered as units. However, it has never been tested whether the populations making up these units do in fact constitute natural groups. These previous studies therefore cannot resolve the problem of species definitions within this complex.

Three different approaches have been found useful in investigating the systematics of such complex groups. Multivariate morphometrics allows the simultaneous analysis of patterns of variation in large numbers of characters; comparative mitochondrial DNA sequencing can reveal cryptic species as well as population phylogeny without the confounding effects of natural selection on morphological traits; finally, partial Mantel tests can reveal significant associations between observed variation in the phenotype of the animals and hypothesized causes of this variation, such as phylogeny (inferred from DNA sequence information, or hypotheses derived from other sources of information, such as geological, climatic or vegetational history) and current natural selection for present-day ecological conditions (e.g., vegetation, climate), and can thus further the understanding of the underlying phenomena under investigation. These three approaches have been useful in elucidating the population systematics of other complex groups of venomous

snakes, such as Asiatic cobras (Wüster & Thorpe 1989, 1990, 1991, 1992, 1994; Wüster, Thorpe, Cox *et al.* 1995) and Russell's viper (Wüster, Otsuka, Malhotra & Thorpe 1992; Wüster, Otsuka, Thorpe & Malhotra 1992), and for investigating the phenomenon of geographic variation in general (Thorpe, Brown *et al.* 1994; Thorpe, Malhotra *et al.* 1995).

Here, we use a combination of multivariate morphometrics, comparative mitochondrial DNA sequencing and Mantel tests of association between morphological variation and hypothesized causes to analyse the population systematics of the populations normally assigned to the species *B. atrox*, *B. moojeni*, *B. marajoensis*, *B. isabelae*, *B. leucurus* and *B. pradoi*, and to attempt to gain an insight into the causes of the pattern of morphological variation in this complex. Comparative sequencing of the same region of cytochrome *b* in a variety of *Bothrops* species and related genera (Salomão *et al.*, this volume pp. 89–98) has shown that the populations assigned to these nominal species constitute a monophyletic group, with *B. caribbaeus* as the sister group.

Materials and methods

Multivariate morphometrics

Approximately 260 preserved specimens were examined by the first author, and 87 morphological characters relating to the scalation, colour pattern, head and body proportions and visceral topography were recorded for each specimen. Details of character selection and recording can be found in Wüster, Thorpe, Puerto & BBBSP (in press).

Specimens were grouped into operational taxonomic units (OTUs) on the basis of collecting gaps and patterns of variation in the generalized phenotype as revealed by principal components analysis (PCA) of individual specimens. The OTUs used in this study are tabulated in Table 1. All characters were tested for significant among-OTU variation by means of analysis of variance (ANOVA) and those characters showing significant variation were selected for further analysis. Canonical variate analysis (CVA) was used to investigate patterns of geographic variation in the populations of the complex. In order to negate the effect of sexual dimorphism, male and female specimens were analysed separately.

Molecular methods

Tissue and blood samples for DNA analysis were collected from field-caught, roadkilled or captive specimens of *Bothrops* and stored in 75% ethanol. The localities of origin of the specimens are shown in Fig. 1. DNA extraction was carried out using the Puregene™ DNA isolation kit (Gentra Systems Inc.). A 767 bp fragment of the cytochrome *b* gene was amplified by means of the polymerase chain reaction (PCR). The amplification was carried out using modified versions of the cytochrome *b* primers of Kocher *et al.* (1989) and primer MVZ 16 of Moritz, Schneider & Wake (1992). The PCR product was purified with the SpinBind® DNA Extraction Unit (FMC BioProducts). Sequencing was carried out by the chain termination method (Sanger *et*

al. 1977). Both strands were sequenced wherever possible. Sequences were aligned against the published human mitochondrial DNA sequence (Anderson *et al.* 1981).

Table 1. OTUs used in multivariate analysis and sample sizes for each sex

OTU Locality	Sample size	
	Males	Females
110. Guyana	2	2
120. Northern Peru and neighbouring Colombia	7	6
121. Departamento de Cuzco, Perú	1	0
122. Manú National Park, Departamento Madre de Dios, Perú	3	2
123. Panguana, Rio Lullapichis, Rio Pachitea, Huánuco, Perú	2	1
130. Pastaza Province, Ecuador	5	12
150. Balbina Dam, Pres. Figueiredo, Amazonas, Brazil	8	8
151. Itacoatiara, Amazonas, Brazil	5	7
152. Manaus area, Amazonas, Brazil	4	6
153. Iauarête, Amazonas, Brazil	0	1
154. Porto Velho Region, Rondônia, Brazil	1	3
155. Acre State, Brazil	1	2
156. Belém area, Pará, Brazil	6	10
157. Tome-Açu, Pará, Brazil	2	0
158. Left side of Rio Tocantins, Tucuçu, Pará, Brazil	8	4
159. Right side of Rio Tocantins, Tucuçu, Pará, Brazil	0	8
160. Marabá, Pará, Brazil	0	1
161. Itaituba, Pará, Brazil	0	3
162. Curral Grande, Monte Alegre, Santarém, Pará, Brazil	11	7
163. Mouth of Rio Trombetas, Pará, Brazil	0	1
164. Barra do Corda region, Maranhão, Brazil	3	0
165. Oiapoque, Amapá, Brazil	1	0
166. Serra do Navio, Amapá, Brazil	0	1
167. Guaraí, Tocantins, Brazil	0	1
200. Southern Goiás and Brasília region, Brazil	7	6
201. Presidente Prudente region, São Paulo, Brazil	4	5
202. Itaipú Dam, Foz do Iguaçu, Paraná, Brazil	6	5
203. Ilha Solteira Dam, Mato Grosso do Sul, Brazil	8	7
204. Eastern and north-eastern Mato Grosso, Brazil	4	2
205. Ilha do Bananal, Tocantins, Brazil	0	1
300. São Bento, coastal Maranhão, Brazil	8	8
301. Eastern part of Marajó Island, Pará, Brazil	3	3
302. Breves, western part of Marajó, Pará, Brazil	2	3
303. Macapá area, Amapá, Brazil	1	0
400. Conceição do Araguaia, Pará, Brazil	3	1
401. Cachimbo, Pará, Brazil	1	0
402. Almas, Natividade, Tocantins	2	1
403. Uruçuí, Piauí, Brazil	1	1
404. Avelino Lopes, Piauí, Brazil	0	1
405. Formoso do Araguaia, Tocantins, Brazil	0	1
501. Southern Bahia and northern Espirito Santo, Brazil	2	1
601. Ibiracçu (formerly Pau Gigante), Espirito Santo, Brazil	2	0
701. Portuguesa State, Venezuela	2	0

Species designations follow mostly Campbell & Lamar (1989), with modifications according to phenotype: OTUs 100–199 would be classified as *B. atrox*, OTUs 200–299 as *B. moojeni*, OTUs 300–399 as *B. marajoensis*; OTUs 400–499 are populations phenotypically intermediate between *B. moojeni* and *B. atrox*, OTU 501 represents *B. leucurus*, OTU 601 *B. pradoi* and OTU 701 *B. isabellae*.

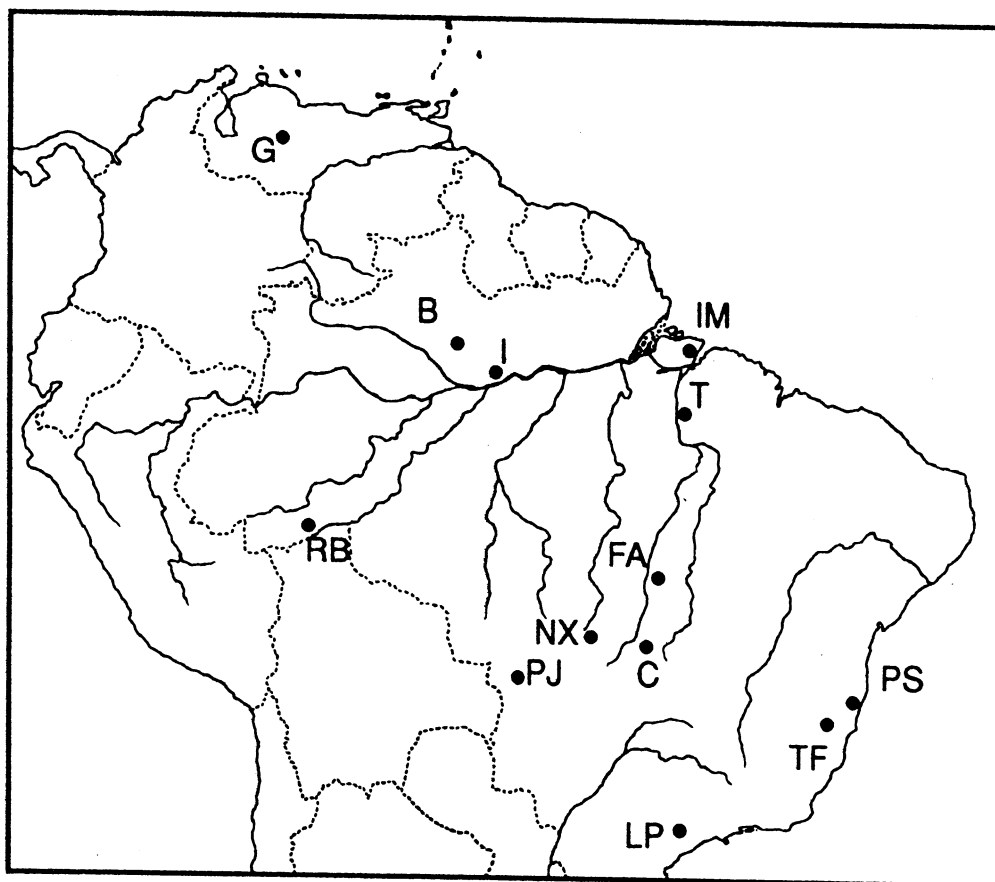


Fig. 1. Localities from which tissue samples were obtained, and nominal species to which these populations were assigned. The codes for the localities are as follows: B: UHE Balbina, Presidente Figueiredo, Amazonas, Brazil (*atrox*); C: Corumbá, Goiás, Brazil (*moojeni* — near type locality); FA: Formoso do Araguaia, Tocantins, Brazil (*atrox* × *moojeni* phenotypic intergrade); G: Guanare, Portuguesa, Venezuela (*isabelae* — type locality); I: Itacoatiara, Amazonas, Brazil (*atrox*); IM: Ilha de Marajó, Pará, Brazil (*marajoensis* — type locality); LP: Lençóis Paulista, São Paulo, Brazil (*moojeni*); NX: Nova Xavantina, Mato Grosso, Brazil (*moojeni*); PJ: Porto Jofre, Mato Grosso, Brazil (*moojeni*); PS: Porto Seguro, Bahia, Brazil (*leucurus*); RB: Rio Branco, Acre, Brazil (*atrox*); T: UHE Tucuruí, Pará, Brazil (*atrox*); TF: Teixeira de Freitas, Bahia, Brazil (*leucurus*).

Phylogenetic analysis

A homologous region of 520 base-pair positions was subjected to phylogenetic analysis. Four different algorithms were used in order to assess the robustness of the phylogenetic hypotheses generated here: maximum parsimony analysis (heuristic search method — PAUP 3.0 — Swofford 1989), Fitch–Margoliash with and without the assumption of a molecular clock (FITCH and KITSCH programs on PHYLIP 3.5 — Felsenstein 1993), and the Maximum Likelihood method (DNAML — PHYLIP 3.5 — Felsenstein 1993). The outgroup sequences for tree rooting were from *Bothrops alternatus* and *B. jararacussu*, both of which were shown to be suitable outgroups for the *B. atrox* complex by Salomão *et al.* (this volume pp. 89–98). All analyses were run both without character weighting and with transversions weighted twice as heavily as transitions.

Testing hypotheses with Mantel tests

Partial Mantel tests were used to test for significant associations between observed phenotypic variation in the *Bothrops atrox* group and potential causes of geographic

variation. Five potential causes were investigated in this study: rainfall regime, temperature regime, vegetation type, geographic distance and hypothesized Pleistocene refugia.

Rainfall and temperature regimes and vegetation types are known to be associated with geographic variation in various reptiles (Thorpe & Brown 1989; Castellano, Malhotra & Thorpe 1994). Geographic proximity provides a measure of the opportunities for gene flow between localities, as well as accounting for other effects related to geography.

Pleistocene changes in vegetation cover in South America may have led to the fragmentation of the range of the populations of *Bothrops* and hence to differentiation. In particular, the fragmentation of the Amazonian forest into small refugia during the Pleistocene has been hypothesized to be a major cause of speciation or population differentiation among Amazonian faunal elements (Haffer 1969; Vanzolini & Williams 1970). The refuge scenario used in this paper is based on a synthesis of the ideas of Ab'Sáber (1977), Brown (1979) and Brown & Ab'Sáber (1979), as presented in Haffer (1987).

For each specimen, temperature regimes (represented as a combined score derived from the number of months with a mean temperature under 25 °C and the number of months with mean temperature under 20 °C), rainfall regimes (represented as a combined score derived from total annual rainfall, the number of months with under 100 mm and the number of months with under 25 mm of precipitation), and vegetation type of the locality of collection were converted into a between-specimen distance matrix by means of principal co-ordinates analysis. Geographic distance was calculated on the basis of latitude and longitude of the locality of each specimen. In the case of the hypothesized Pleistocene refugia, each specimen was assigned to the refugium closest to its collecting locality. The assumption is that specimens descended from the same refugium population should be morphologically more similar to each other than specimens descended from different refugium populations.

Partial Mantel tests were run on individual specimens using 1000 randomizations. One test was run on the generalized phenotype of all specimens (expressed as a taxonomic distance matrix obtained by a principal co-ordinates analysis), and on nine individual characters or constellations of functionally and statistically related characters (such as number of dorsal scale rows at various points along the body).

Results

Multivariate morphometrics

The CVA of male specimens (Fig. 2a) indicates a clear separation along the first canonical variate between populations conventionally assigned to *Bothrops moojeni* and the remaining populations. However, there are several phenotypically intermediate specimens from regions where the ranges of *B. atrox* and *B. moojeni* meet, in the Brazilian states of Tocantins, Piauí and south-western Pará. The populations normally assigned to *B. marajoensis* group with *B. atrox*, as do those assigned to *B. leucurus*, *B. pradoi* and *B. isabellae*. In the females (Fig. 2b), the pattern is similar, except that some of the populations assigned to *B. marajoensis* are more phenotypically similar to *B. moojeni*.

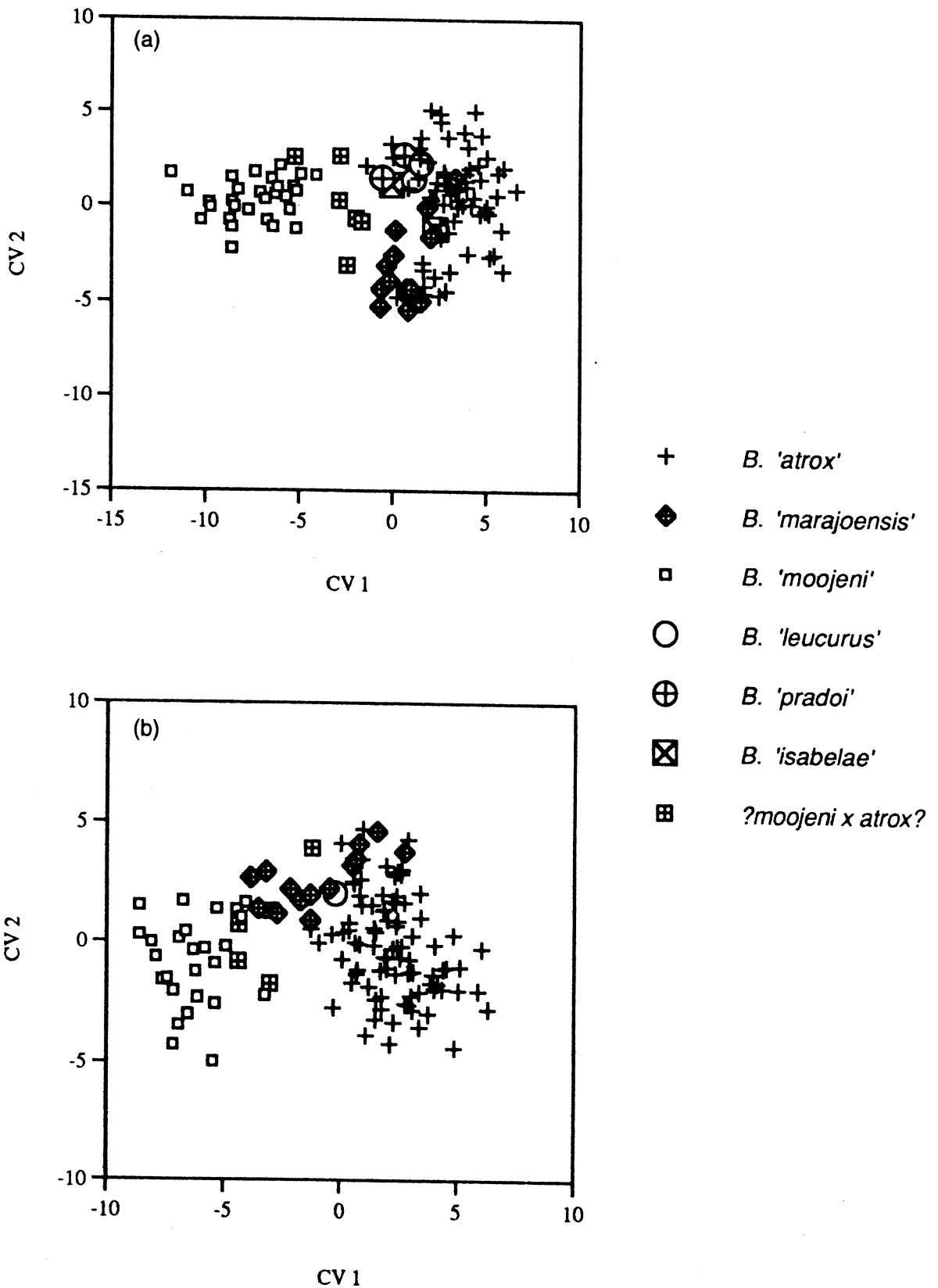


Fig. 2. Ordination of the male (a) and female (b) specimens of the *Bothrops atrox* complex along the first two canonical variates. Species names represent the nominal species to which these specimens would conventionally be assigned.

Cytochrome *b* sequence analysis

A total of 19 ingroup haplotypes were identified and used in the phylogenetic analyses. Of the 520 base-pair positions analysed, 111 (21.3%) were variable overall, and 69 (13.3%) within the ingroup. The pairwise sequence divergence among the ingroup haplotypes ranged from 0.2% to 6.6%, mean 2.87% (0.2–4.5%, mean 2.56%, if *caribbaeus* is excluded) of all positions. The transversion-to-transition ratio among ingroup haplotypes ranged from 0 to 0.77. Owing to the low level of pairwise divergences, saturation of transition sites does not appear to be a problem.

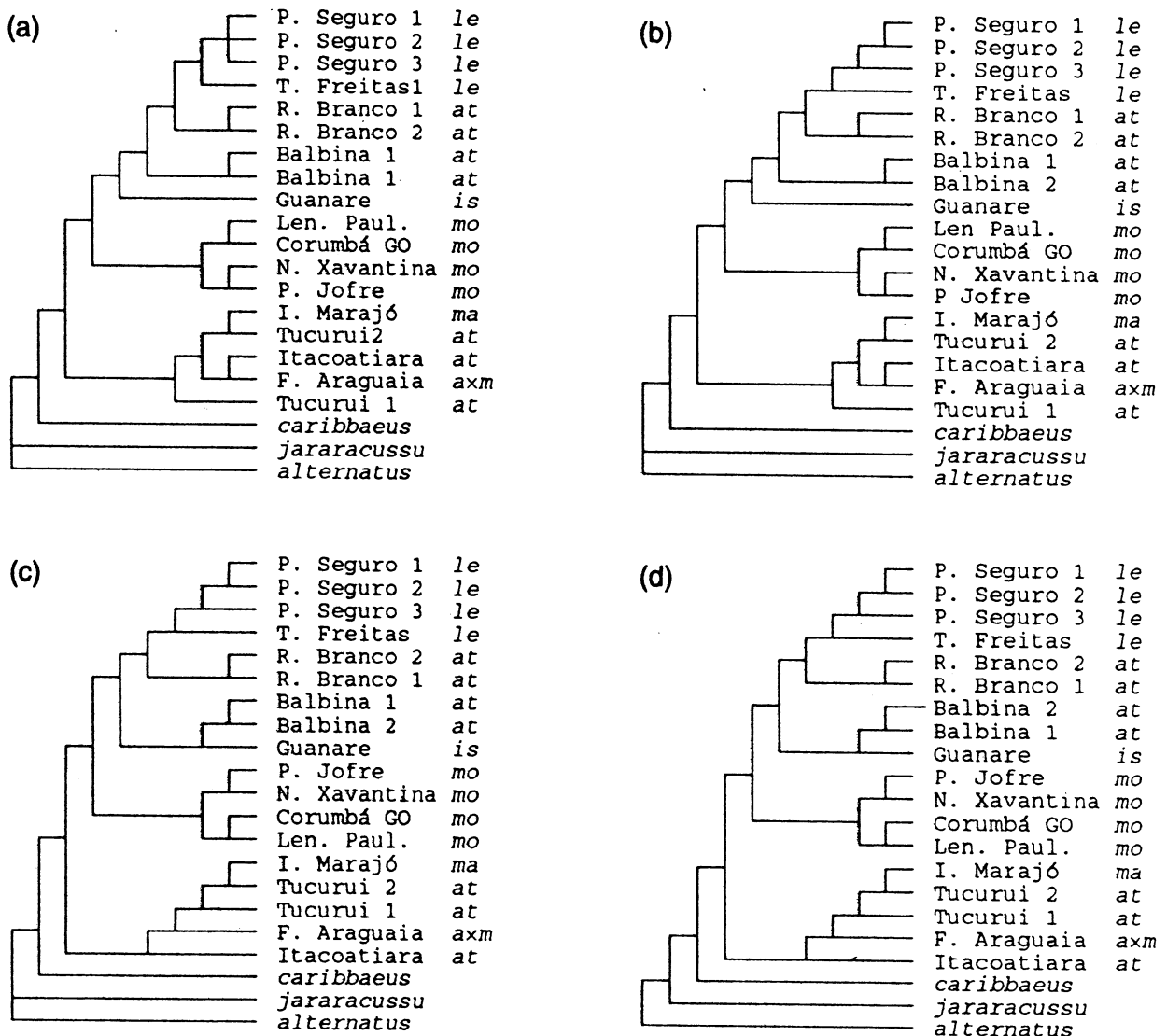


Fig. 3. Phylogenetic trees obtained through the use of four algorithms: (a) maximum parsimony analysis; (b) maximum likelihood analysis; (c) Fitch–Margoliash algorithm; (d) Fitch–Margoliash algorithm with molecular clock assumption. These trees show the results of an analysis using equal weighting for all base-pair substitutions. Double weighting of transversions results in identical trees, except for the branching order of the three Porto Seguro haplotypes. Abbreviations in italics denote the nominal species to which these populations are normally assigned. (*at* = *B. atrox*; *is* = *B. isabelae*; *le* = *B. leucurus*; *ma* = *B. marajoensis*; *mo* = *B. moojeni*; *a × m*: specimen from zone of morphological intergradation between *B. 'atrox'* and *B. 'moojeni'*)

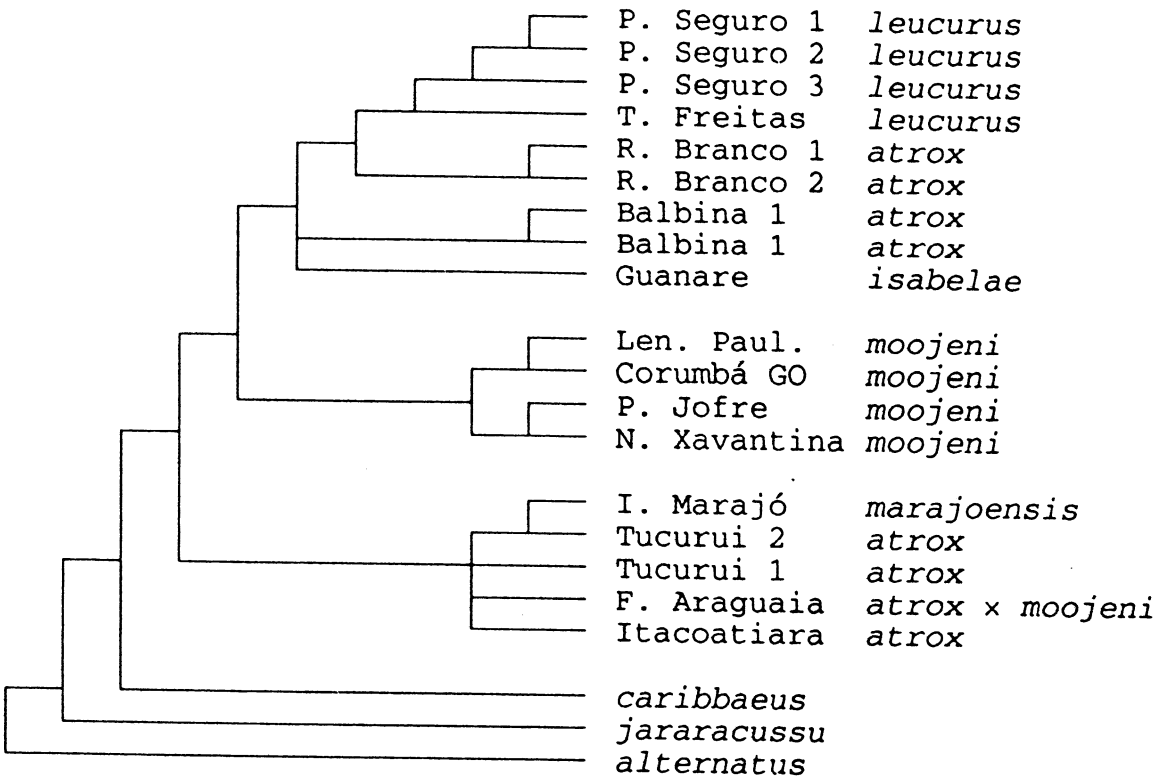


Fig. 4. Semistrict consensus of the trees generated by the four algorithms used in this study.

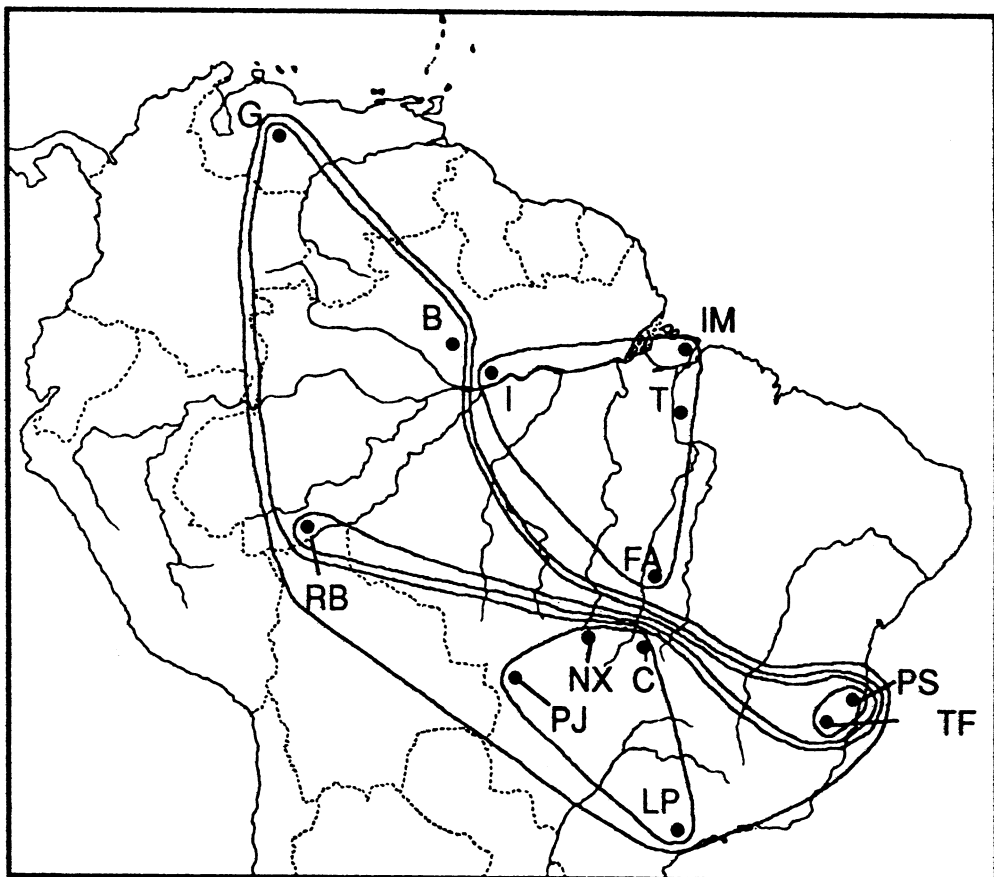


Fig. 5. Geographic distribution of the clades revealed by this study. Locality abbreviations are as in Fig. 1.

The trees resulting from analyses of unweighted substitutions are essentially identical to those resulting from analyses with transversions weighted twice as heavily as transitions (the only differences concern the branching order of the three haplotypes from Porto Seguro). The trees resulting from the analysis of unweighted data are shown in Fig. 3, and a semistrict consensus tree of the trees obtained with the four methods is shown in Fig. 4. In all trees, the South American populations of the *B. atrox* complex constitute a monophyletic group with *B. caribbaeus* as a sister taxon.

Within the *B. atrox* group, the basal dichotomy separates a group of populations from the lower Amazon Basin and the Tocantins–Araguaia drainage from the remaining South American populations. This remainder is again split into two further clades: the first of these comprises the populations conventionally referred to as *B. moojeni*; the second comprises a set of populations from the east coast of Brazil, and several populations from the upper Amazon Basin and the Orinoco drainage. The haplotypes from the eastern coast of Brazil form a monophyletic group. The clades revealed by these analyses are mapped in Fig. 5.

Mantel tests

The Mantel tests of association between geographic variation in the generalized phenotype of the South American *B. atrox* group clade show that the generalized phenotypes of both sexes are most strongly associated with the vegetational zone inhabited by the relevant population (Table 2). In the case of the females, vegetation is the only hypothesized cause significantly associated with the observed pattern of variation. In the males, generalized phenotype is also associated with Pleistocene refugia and geographic distance. Similarly, in both sexes, vegetation was associated with the highest number of single characters or character suites.

Discussion

In this analysis of the systematics of the *Bothrops atrox* complex, the pattern of geographic variation revealed by multivariate morphometrics and the population phylogeny revealed by mtDNA sequence analysis are largely incongruent, and both are incongruent with the conventional taxonomic arrangement of this group.

The multivariate analysis revealed that only the southern populations currently assigned to the nominal species *B. moojeni* are clearly morphologically distinct from the remaining populations. These populations occupy a zone of relatively open cerrado vegetation, with a highly seasonal climate, whereas most of the remaining populations originate from moister, less seasonal regions, usually covered by more or less permanently humid forest. The remaining populations, which mostly originate from rainforest habitats, are all relatively similar to each other.

The phylogenetic analysis of the cytochrome *b* sequence data reveals a population phylogeny that is largely incongruent with both the pattern of morphological variation discovered through multivariate morphometrics, and with the conventional taxonomy of these forms. The only populations that are morphologically distinct from all others, the populations commonly called *B. moojeni*, from the seasonally dry areas of central-western Brazil, do constitute a monophyletic group.

However, the remaining populations, with the 'atrox' phenotype, fall into two different clades, among which the 'moojeni' clade is nested. It would appear that the phenotype of the 'moojeni' populations is a derived adaptation to the different vegetation, whereas the 'atrox' phenotype is a plesiomorphy of the *B. atrox* group.

Table 2. Probabilities for the null hypothesis of no association between generalized phenotype and individual morphological characters and hypothesized potential causes of the observed pattern of variation

	Geographic distance	Rainfall	Temperature	Refugia	Vegetation
General phenotype	♂♂ 0.002	0.017	0.707	0.002	≤ 0.001
	♀♀0.032	0.976	0.084	0.017	≤ 0.001
Ventral number	♂♂0.652	0.009	0.014	≤ 0.001	≤ 0.001
	♀♀0.829	0.018	0.717	0.009	≤ 0.001
Subcaudal number	♂♂ ≤ 0.001	0.005	0.003	0.134	0.090
	♀♀ ≤ 0.001	0.931	0.033	0.333	0.011
Dorsal scale rows	♂♂0.035	0.006	0.872	0.194	0.319
	♀♀ 0.003	0.125	0.237	0.588	0.983
Caudal scale reductions	♂♂ ≤ 0.001	0.018	0.789	0.466	0.003
	♀♀ 0.003	0.125	0.237	0.588	0.983
Supralabials in postocular stripe	♂♂0.992	0.143	0.008	0.167	≤ 0.001
	♀♀0.186	≤ 0.001	≤ 0.001	0.055	≤ 0.001
Percentage of last supralabial in postocular stripe	♂♂0.327	0.022	≤ 0.001	0.083	≤ 0.001
	♀♀0.457	0.915	0.090	0.006	0.106
Ventral pigmentation	♂♂0.994	≤ 0.001	0.042	≤ 0.001	0.008
	♀♀0.045	0.800	0.01	0.037	0.013
Separation of upper & lower patterns	♂♂ 0.002	0.522	0.012	0.087	≤ 0.001
	♀♀0.035	0.919	0.012	0.871	≤ 0.001
Intercanthal	♂♂0.487	0.411	0.025	0.566	0.076
	♀♀0.939	0.471	≤ 0.001	0.931	0.009

P values in bold type are significant at the 0.05 level after Bonferroni correction for the number of hypotheses tested for each character.

With respect to formal classification, the results presented here do not allow any definitive conclusions, except to note that the conventional species as currently defined (Campbell & Lamar 1989) do not generally correspond either to phenetically distinct and homogeneous units, or to evolutionary lineages. *Bothrops atrox*, as conventionally understood, is polyphyletic according to the models of Farris (1974) and Oosterbroek (1987). The haplotypes of the two '*B. leucurus*' populations included in this study form a clade, but are nested within a clade containing several other populations conventionally assigned to *B. atrox*. The population of '*B. marajoensis*' is nested within the clade from lower Amazonia, and the population

of *B. isabellae* is nested within a clade containing populations commonly assigned to *B. atrox* and *B. leucurus*. It is clear from this that studies which take the nominal species of the *B. atrox* complex as given units cannot provide any useful information about the sister-group relationships within the *B. atrox* complex. More information on the distribution of these haplotypes is needed, and material from the type localities of all species will be required before a revision of the nomenclature of these forms can begin. We urge other researchers working with this complex, and especially toxinological and medical researchers, to obtain and publish a maximum of information on the locality of origin of their material, to enable their results to be reconciled with future developments of the classification of the group.

The Mantel tests showed a particularly significant association between vegetation formation and external morphology of the complex as a whole, reflecting largely the different vegetational formations occupied by the morphologically differentiated '*B. moojeni*' populations. Although relatively little published information on the natural behaviour of these snakes is available, most pitvipers, including large *Bothrops*, are thought to be primarily 'sit-and-wait' predators, relying on their camouflage to remain unnoticed by approaching prey animals (Greene 1992; Sazima 1992). We therefore hypothesize that the morphological differences of the divergent '*B. moojeni*' populations represent adaptations to the vegetational differences resulting from a drier, more seasonal, climate within its range. The fact that the morphology of this complex appears to represent primarily an adaptation to ecological conditions rather than a reflection of phylogeny in this group is likely to be one of the principal causes of the general failure to arrive at a stable and robust classification for these animals (see also Markezich & Taphorn 1993).

The data presented here are insufficient to indicate whether the clades revealed in this analysis represent separate, independently evolving species, or whether the mitochondrial DNA gene trees presented here are contained within a single, highly variable species. The resolution of this problem requires more sequence information from a much greater number of localities than is currently available, in order to identify the distribution of different mtDNA haplotype lineages among the populations of this complex. Furthermore, a close investigation of contact zones between the clades identified here by means of nuclear as well as mitochondrial markers is likely to be necessary in order to understand the patterns of genetic exchange between these lineages.

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