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Mitochondrial DNA Phylogeny of the *Bothrops atrox* Species Complex (Squamata: Serpentes: Viperidae)

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Zusammenfassung

Der *Bothrops atrox* Artenkomplex in Südamerika wird mit Hilfe einer phylogenetischen Analyse eines Sequenzabschnitts von 580 Basenpaaren des mitochondrialen Cytochrom b Gens untersucht. Die karibischen *Bothrops lanceolatus* und *B. caribbaeus* zeigen sich als Schwesterarten und bilden die Schwestergruppe zu allen südamerikanischen Populationen. Die mitochondriale Sequenzdivergenz der südamerikanischen Populationen ist gering, was einen rezenten Ursprung der Gruppe nahelegt. Mehrere Haplotypenlinien sind erkennbar, entsprechen jedoch nicht den konventionellen Arten, ebensowenig wie den geographischen Variationsmustern der Morphologie. Einige der Haplotypenlinien zeigen geographisch disjunkte Verbreitung. „Lineage sorting“ während pleistozäner „Flaschenhälse“ der Populationen könnte eine Genphylogenie erzeugt haben, die nicht die organismische Phylogenie widerspiegelt.

Abstract

We investigate the phylogeny of the *Bothrops atrox* species complex in South America and the Caribbean by means of phylogenetic analysis of a 580 base pair section of the mitochondrial cytochrome b gene. The Caribbean species *B. lanceolatus* and *B. caribbaeus* are sister species, and form the sister clade to all South American populations. The South American populations show low levels of mtDNA sequence divergence, suggesting a recent origin of the group. A number of haplotype clades are recognised. The conventional species do not correspond to these haplotype clades, and neither do patterns of geographic variation in morphology. Some of the haplotype clades represent geographically disjunct distributions. Lineage sorting during possible Pleistocene population bottlenecks may have resulted in a gene phylogeny which is not representative of organism phylogeny.

Introduction

The *Bothrops atrox* species complex comprises a number of populations of medium to large-sized pitvipers found throughout the tropical parts of Central and South America. Within this complex, there is considerable geographic variation in colour pattern, scalation, size and other characteristics. As a result, a considerable number of species or subspecies have been and are being described within this complex (e.g. *B. leucurus* WAGLER 1824; *B. colombiensis* HALLOWELL 1845; *B. pradoi* HOGE 1947; *B. marajoensis* and *B. moojeni* HOGE 1965; *B. isabellae* SANDNER MONTILLA 1979; *B. lanceolatus aida* SANDNER MONTILLA 1981; *B. lanceolatus nacaritae* SANDNER MONTILLA 1990). However, the status of many of these species remains controversial (CAMPBELL & LAMAR 1989), and supposedly diagnostic characters do not withstand comparisons of large samples (personal observation). An improved understanding of the population systematics of these snakes is of particular importance, as these snakes are the leading cause of snakebite mortality and morbidity in much of

their range (e.g. HAAD 1980/81, KOUYOUMDJIAN & POLIZELLI 1988, NISHIOKA & SILVEIRA 1992, OTERO et al. 1992, PIERINI et al. 1996). A poor understanding of the systematics of venomous snakes can lead to subsequent problems in the interpretation of toxicological data or in the production of effective antivenoms (WÜSTER & MCCARTHY 1996).

Most of the previous work on the group has relied on conventional systematic techniques. However, WÜSTER et al. (1996, 1997) used multivariate analysis of morphological characters and comparative mtDNA sequence analysis to investigate the systematics of this group, and came to the conclusion that the conventional species are weakly defined at best, or even indistinguishable. Furthermore, the conventional species did not correspond to clades of mtDNA haplotypes revealed by those studies. The aim of the present paper is to continue these investigations, based on an expanded database of mtDNA sequence information from a greater number of localities.

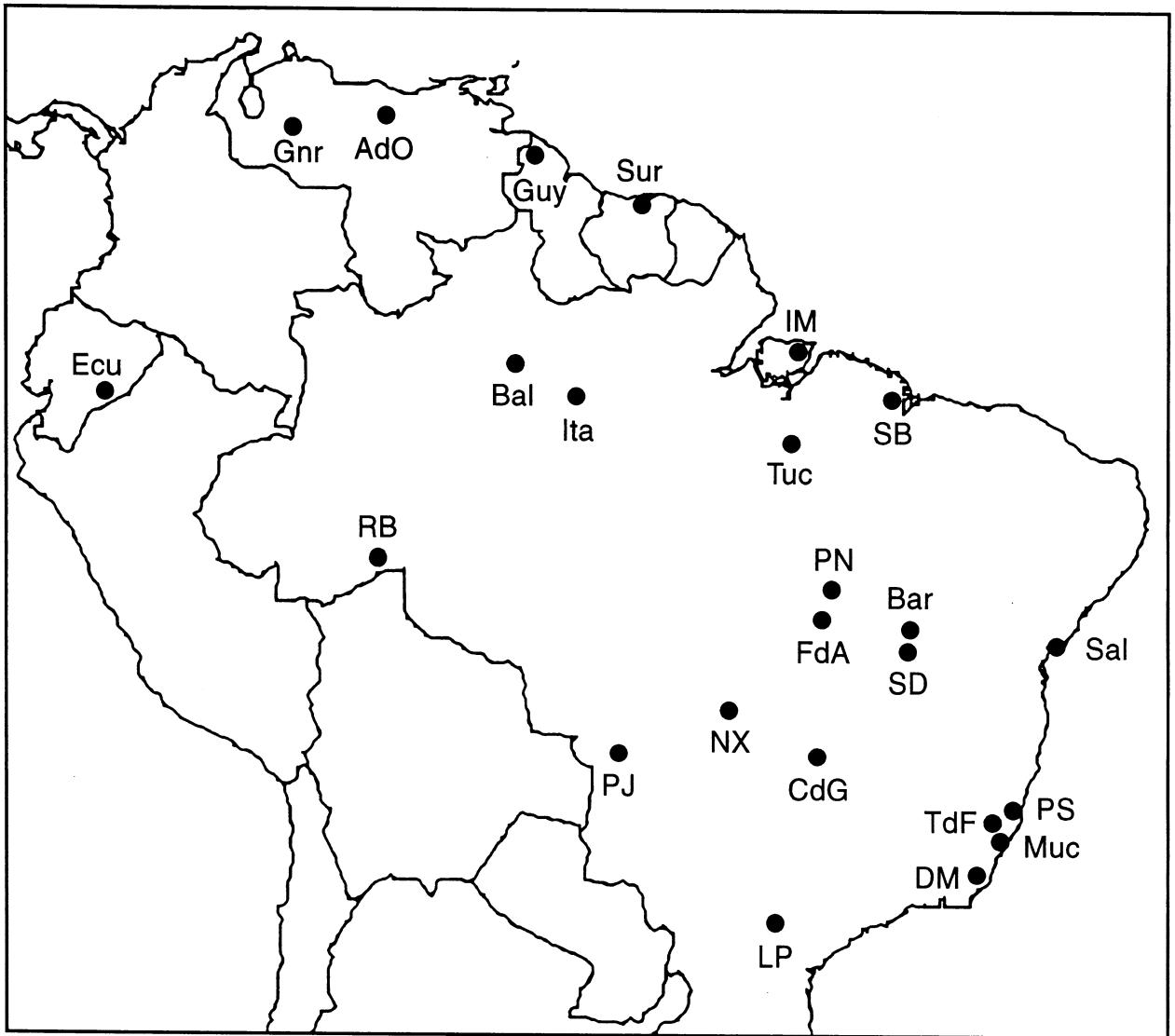


Fig. 1: Locality of samples of the South American *Bothrops atrox* group analysed in this study. For explanations of abbreviations see Tab. 1.

Materials and methods

Molecular methods

Tissue and/or blood samples were obtained from living or freshly road-killed specimens in the field or in captive collections. The localities from which samples were obtained are listed in Tab. 1 and illustrated in Fig. 1. DNA was purified from the samples by means of RNase and proteinase k digestion, phenol, phenol-chloroform and chloroform centrifugation and ethanol precipitation, or by the use of various commercial kits.

A 767 b.p. fragment of the cytochrome b gene was amplified by running the Polymerase Chain Reaction (PCR), using primers 5'-TCA AAC ATC TCA ACC TGA TGA AA-3' (L-strand, modified from KOCHER et al. 1989) and 5'-GGC AAA TAG GAA GTA TCA TTC TG-3' (H-strand, modified version of primer MVZ 16 of MORITZ et al. 1992). PCR was

carried out in 50 μ l of a solution of 20 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.52 μ M of each primer, 0.4 mM dNTP and 2.0 units Taq polymerase. Typical thermal cycle parameters were 4 min at 94 °C, then 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C, and finally 3 min at 72 °C and 10 s at 28 °C.

Sequencing was carried out manually, using standard laboratory protocols (HILLIS et al. 1996). The sequencing primers included the PCR primers as well as customised internal primers derived from the sequences obtained. Both strands were sequenced as far as possible, and many sequences were obtained several times from the products of several PCR reactions, thus minimising the likelihood of erroneous sequence data due to PCR copying errors. The sequences were aligned by eye against the published human mitochondrial DNA sequence (ANDERSON et al. 1981), relative to which there are no insertions or deletions.

Table 1: Samples used in this study, and their locality of origin. The abbreviations are those used in the cladograms and in Fig. 1, where they are illustrated. * = These populations were discussed under the designation *B. atrox* by HOGE et al. (1976/77), but were the populations referred to by HOGE & ROMANO (1972) and HOGE & ROMANO-HOGE (1978/79) in their statement that *B. marajoensis* occurs east to "the equatorial regions of Maranhão".

Locality, State/Province and Country	Conventional species	Abbreviation
Altagracia de Orituco, Guárico, Venezuela	<i>Bothrops colombiensis</i>	AdO
Balbina Hydroel. Dam, Amazonas, Brazil	<i>Bothrops atrox</i>	Bal
Barreiras, Bahia, Brazil	<i>Bothrops leucurus</i>	Bar
Baramita, Barina-Waini Dist., Guyana	<i>Bothrops atrox</i>	Guy
Corumbá de Goiás, Goiás, Brazil	<i>Bothrops moojeni</i>	CdG
Domingos Martins, Espírito Santo, Brazil	<i>Bothrops pradoi</i>	DM
Formoso do Araguaia, Tocantins, Brazil	<i>Bothrops moojeni</i>	FdA
Guanare region, Portuguesa, Venezuela	<i>Bothrops isabelae</i>	Gnr
Itacoatiara, Amazonas, Brazil	<i>Bothrops atrox</i>	Ita
Lençóis Paulista, São Paulo, Brazil	<i>Bothrops moojeni</i>	LP
Macuma, Morona-Santiago, Ecuador	<i>Bothrops atrox</i>	Ecu
Mucuri, Bahia, Brazil	<i>Bothrops leucurus</i>	Muc
Nova Xavantina, Mato Grosso, Brazil	<i>Bothrops moojeni</i>	NX
Porto Jofre, Mato Grosso, Brazil	<i>Bothrops moojeni</i>	PJ
Porto Nacional, Tocantins, Brazil	<i>Bothrops moojeni</i>	PN
Porto Seguro, Bahia, Brazil	<i>Bothrops leucurus</i>	PS
Rio Branco, Acre, Brazil	<i>Bothrops atrox</i>	RB
Salvador, Bahia, Brazil	<i>Bothrops leucurus</i>	Sal
Salvaterra, Marajó Island, Pará, Brazil	<i>Bothrops marajoensis</i>	IM
São Bento, Maranhão, Brazil	<i>Bothrops atrox/marajoensis</i> *	SB
São Desidério, Bahia, Brazil	<i>Bothrops leucurus</i>	SD
Teixeira de Freitas, Bahia, Brazil	<i>Bothrops leucurus</i>	TdF
Tijgerkreek, Saramacca Dist., Surinam	<i>Bothrops atrox</i>	Sur
Tucuruí, Pará, Brazil	<i>Bothrops atrox</i>	Tuc

Phylogenetic analysis

A 580 b.p. segment of the cytochrome b gene was selected for phylogenetic analysis on the basis of availability for all haplotypes of interest. A total of 27 haplotypes of the *Bothrops atrox* complex were included in the analysis. Sequences from *B. alternatus* and *B. jararacussu* were used as outgroups, as these species were found to constitute relatively far and near sister groups, respectively, by SALOMÃO et al. (1997). The species *B. caribbaeus* was previously found to be the sister group of the South American *B. atrox* group, and this species and the other Caribbean island species, *B. lanceolatus*, were also included in the analysis.

Maximum parsimony analysis, using the heuristic search option and tree bisection-reconnection branch swapping, was carried out using the package PAUP 3.1 (SWOFFORD 1993). Ten repetitions with randomised sample input order were carried out. Neighbour-joining analysis was carried out using the program MEGA 1.02 (KUMAR et al. 1993) and maximum likelihood and Fitch-Margoliash trees were obtained through the use of DNAML and FITCH programs of the PHYLIP 3.5 package (FELSENSTEIN 1993). Distance methods were based on Jukes-Cantor distances between haplotype sequences. In all cases, transversions and

transitions were not weighted differently, as there is no evidence of saturation of transition sites in this database.

Bootstrap support for the various nodes was assessed for the parsimony and neighbour-joining trees, but not for the maximum likelihood and Fitch-Margoliash tree, due to excessive time requirements and computational difficulties, respectively. For the maximum parsimony algorithm 100 replicates were performed, while 1000 replicates were carried out for neighbour-joining analysis.

Results

Mitochondrial DNA sequence information

Among the 580 base pair positions analysed, 135 were variable among all taxa included in the analysis, and 74 were informative under the parsimony criterion. Unadjusted pairwise divergences (obtained from PAUP) between haplotypes of the ingroup (South American *B. atrox* group) and the outgroup taxa *B. alternatus* and *B. jararacussu* ranged from 9.7 to 11.9% and 7.4 to 9.6%, respectively. Within the ingroup, pairwise divergences among haplotypes ranged from 0.4 to 4.8%. Thirty different ingroup haplotype sequences, from 24 lo-

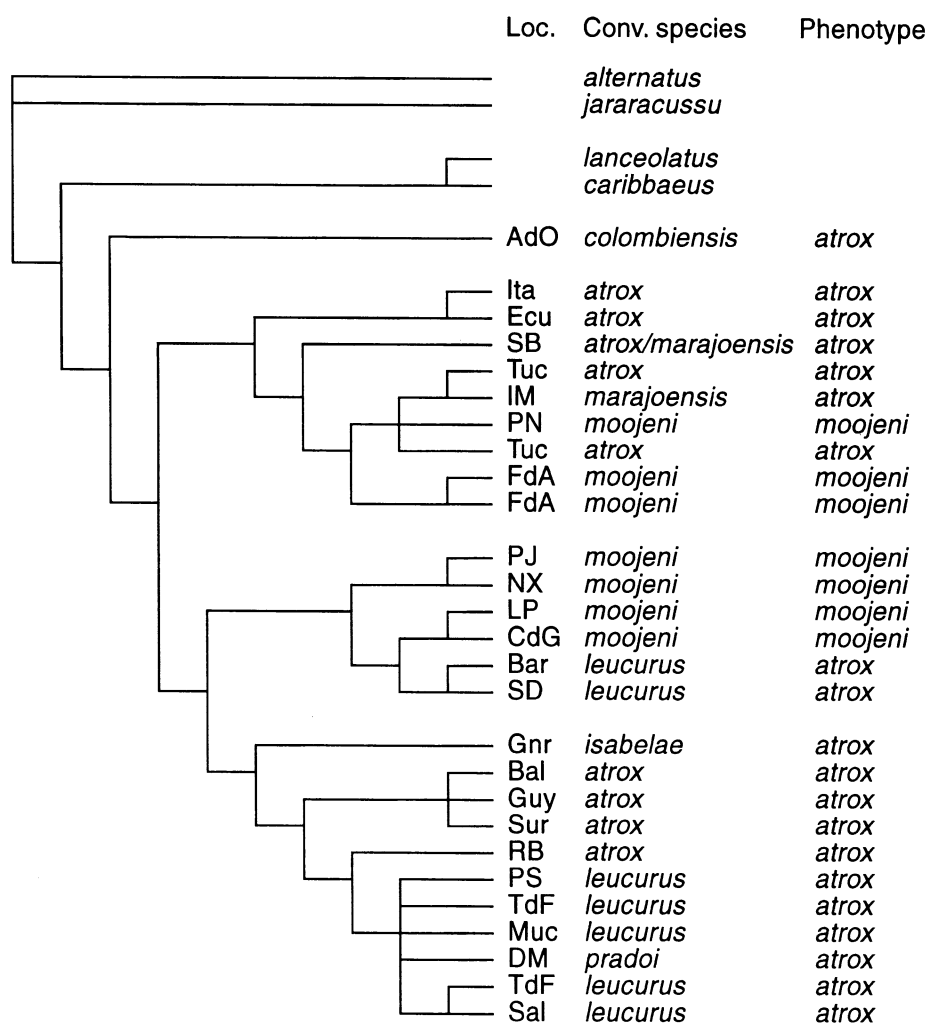


Fig. 2: Strict consensus of six equally parsimonious trees generated by the heuristic search algorithm of PAUP 3.1. Column headings: Loc. = location of samples; abbreviations correspond to those used in Tab. 1; for geographic locations see Fig. 1. Conv. species = the conventional species to which these populations have been assigned in most recent texts; no recognition of these species as valid is implied. The phenotype refers to the *B. atrox* and *B. moojeni* phenotypes identified by WÜSTER et al. (1996, 1997).

calities, were identified. Sequences from several specimens, without substitutions, were obtained for most populations. Where sequences from different specimens from one locality displayed substitutions but formed a monophyletic group in preliminary analyses, only one of the haplotypes was included in the final analysis which included 27 ingroup haplotypes. The sequences will be submitted to GenBank upon completion of the study.

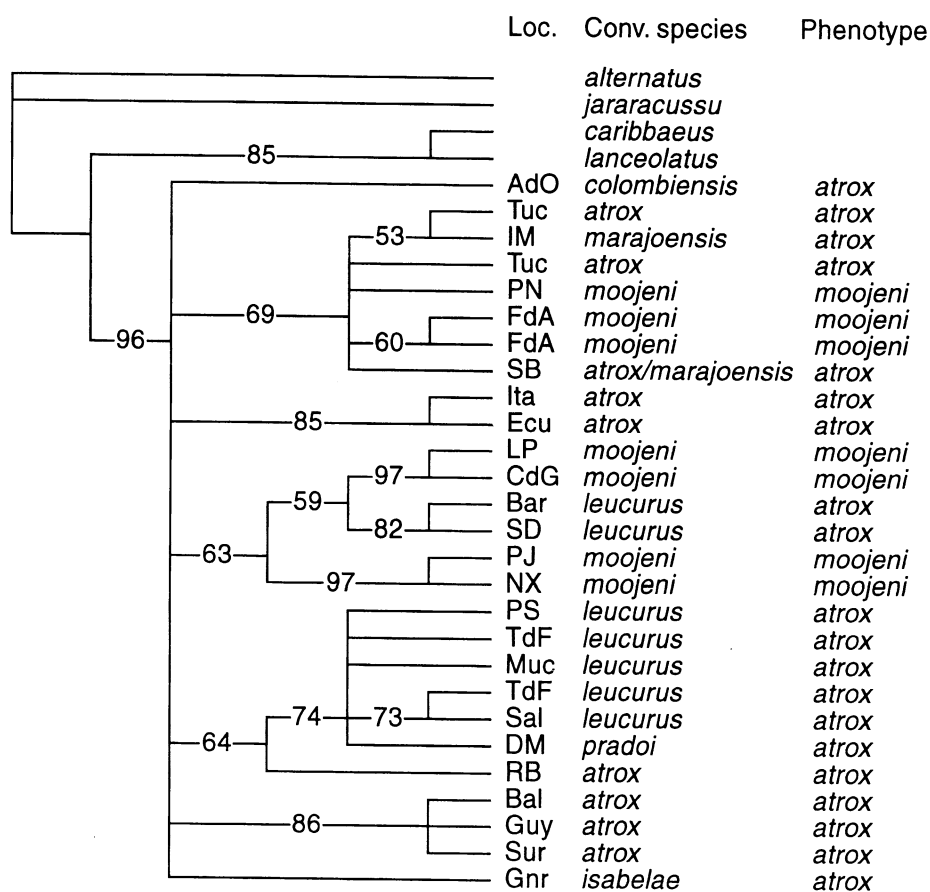
Phylogenetic analysis of mtDNA sequences

Parsimony analysis of the sequence data found six equally most parsimonious trees, with a length of 219 steps (consistency index 0.67, homoplasy index 0.33, retention index 0.74, rescaled consistency index 0.50). A strict consensus tree of these six trees is shown in Fig. 2 and the corresponding bootstrap tree in Fig. 3. The trees resulting from the maximum likelihood (ML), Fitch-Margoliash (FM) and neighbour-joining (NJ) algorithms are shown in Figs. 4, 5 and 6, respectively.

There is considerable agreement between the trees obtained using the different methods. The following phylogenetic relationships are represented in the trees generated by all methods and receive high bootstrap support (see Fig. 7):

- the monophyly of the South American *Bothrops atrox* complex east of the Andes
- a sister group relationship between the Antillean island species *B. caribbaeus* and *B. lanceolatus* and the position of this clade as a sister clade to the South American mainland populations
- a south-central Brazilian clade containing populations from the states of Goiás, São Paulo and Mato Grosso (conventionally assigned to *B. moojeni*) and two populations from western Bahia (conventionally assigned to *B. leucurus*); three pairs of populations are consistently recognised as forming monophyletic groups within this clade
- populations from the Atlantic coast of the Brazilian states of Bahia and Espírito Santo (conventionally assigned to *B. leucurus* and *B. pradoi*) are monophyletic, with a haplotype from Rio Branco, Acre State, Brazil (south-western Amazonia) as a sister group
- a sister group relationship between a population from Itacoatiara (eastern Amazonas State, Brazil) and Amazonian Ecuador
- a clade consisting of haplotypes from Guyana, Surinam and a region north of Manaus (Bal)

Fig. 3: 50% majority rule bootstrap tree from parsimony analysis.



- a clade representing populations from the south-eastern periphery of the Amazonian forests (eastern Pará State, Marajó Island, northern Maranhão and Tocantins)

Several other nodes are supported by all analyses, but with low or negligible bootstrap support:

- the northern Venezuelan haplotype (AdO) is consistently placed as the sister group to all other South American mainland haplotypes
- all trees place the Itacoatiara-Ecuador clade as sister clade to the south-eastern Amazon periphery clade, forming what might be termed an Amazon valley clade
- all trees group the Guyanan clade, the western Venezuelan haplotype and the Acre-Atlantic coast group into what could be termed an Amazon periphery clade
- all trees place the south-central Brazilian clade as the sister clade of the Amazon periphery clade

Discussion

The haplotype phylogenies determined in this study are far from being the final state of knowledge on the phylogeography of the *Bothrops atrox* complex; nevertheless, they do provide a basis for discussing some interesting features of the pattern of mtDNA variation among the populations of this complex.

All trees confirm the monophyly of the two Antillean species, *B. caribbaeus* and *B. lanceolatus*. This is not surprising, as the two forms occur on neighbouring islands; nevertheless, this phylogeny must be interpreted with care, as no sequence information from Central and South American populations usually assigned to *B. asper* was available at the time of writing. The Antillean species have been regarded as conspecific with '*B. asper*' by a few workers (e.g. SANDNER MONTILLA 1990). Although it is unclear how this classification was derived, the interrelations between '*B. asper*' and the Antillean species, and the phylogenetic position of the latter, require further investigation. However, it is clear that the Antillean forms do not originate from a particularly recent overwater dispersal event from the South American mainland, as appears to be the case in *Corallus enydris* (HENDERSON & HEDGES 1995).

The monophyly of the South American *B. atrox* group is strongly supported by results from all methods and by high bootstrap values. Within the group, levels of sequence divergence are low; average levels of pairwise divergence between haplotypes of the more basal clades range mostly between 2.6 and 3.6%, suggesting a relatively recent divergence of the basal lineages. For comparison, levels of pairwise sequence divergence in the cytochrome b gene of Asiatic cobra species range from

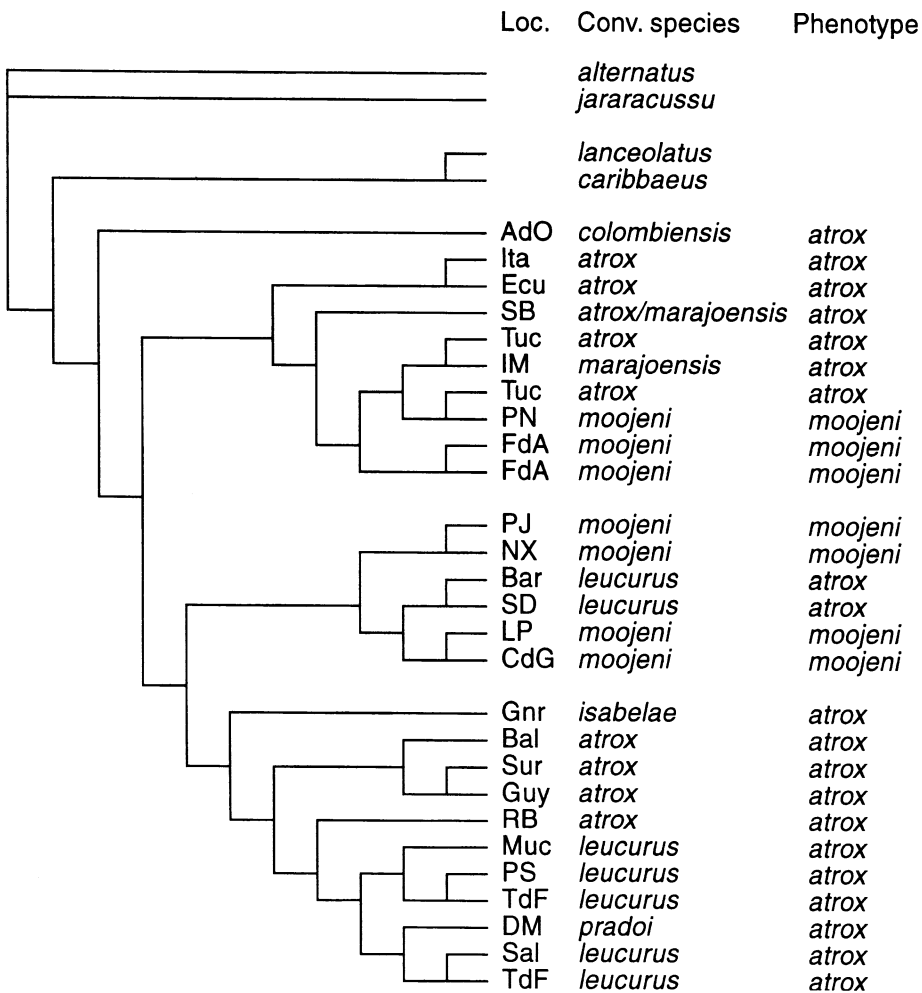


Fig. 4: Cladogram of the maximum likelihood tree of the 31 haplotypes included in this study. See Fig. 2 for explanations.

around 5% among allopatric sister species (e.g. *Naja siamensis* and *N. sputatrix*) to nearly 10% among less closely related species (e.g. *N. naja* and *N. sputatrix*) (W. WÜSTER, unpublished data). This complex was formerly believed to be monospecific, but more recent work has shown it to consist of at least ten full species (WÜSTER & THORPE 1991, WÜSTER et al. 1995).

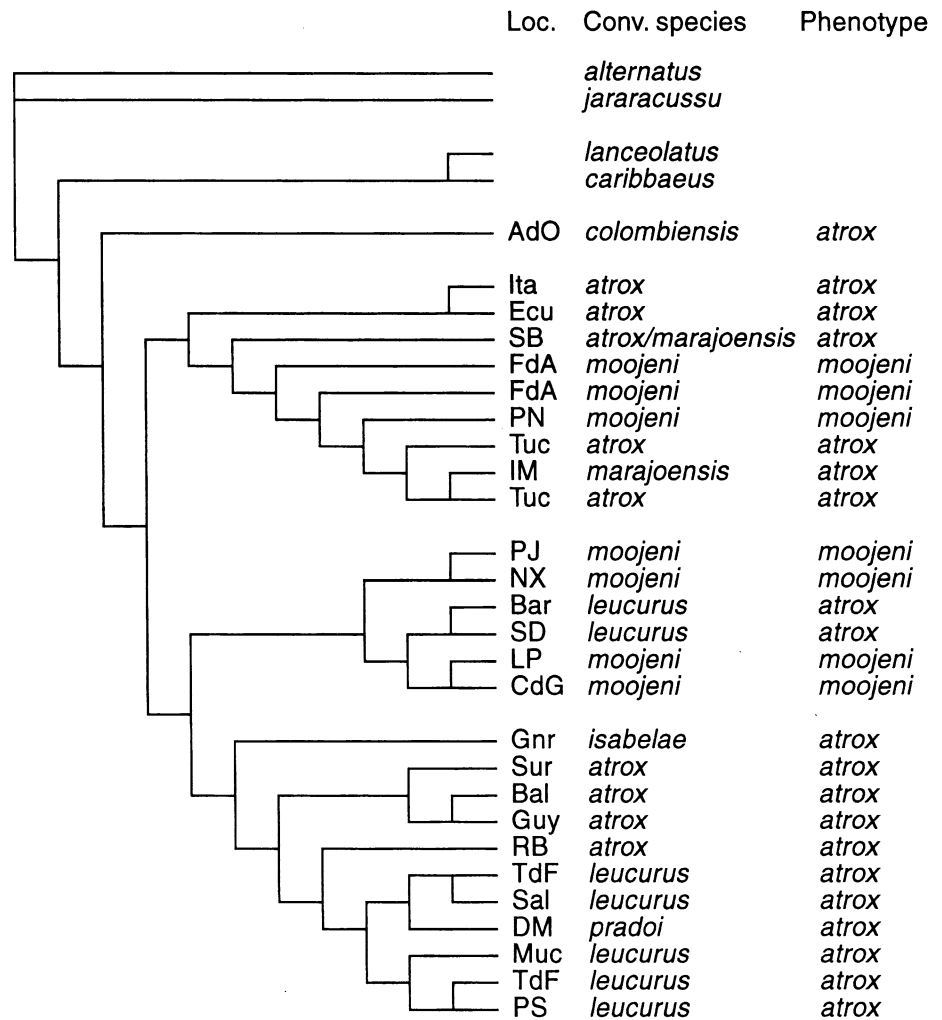
The present study further confirms the work of WÜSTER et al. (1997), who noted that the conventional species recognised within the South American populations of the *B. atrox* complex do not correspond to the mtDNA haplotype clades revealed by the analysis of our sequence data. Incongruence between mtDNA clades and conventional species is found at all levels of the phylogeny, even within the well-supported distal clades: the south-eastern Amazon clade contains populations conventionally assigned to *B. atrox*, *B. marajoensis* and *B. moojeni*, the south-central Brazilian clade contains populations variously assigned to *B. moojeni* and *B. leucurus*, and the Acre-Atlantic coast clade contains populations conventionally assigned to *B. atrox*, *B. leucurus* and *B. pradoi*.

Similarly, incongruence between mtDNA haplotype lineages and morphological variation, already

noted by WÜSTER et al. (1997), is further emphasised and found to occur even within the well-supported distal clades. WÜSTER et al. (1996, 1997) identified two main phenotypes within the *B. atrox* complex: the '*moojeni*' phenotype, which is found in populations normally assigned to *B. moojeni*, from central-southern Brazil (states of Paraná and São Paulo north to Tocantins and Mato Grosso), and the '*atrox*' phenotype, which is found in all other populations. These phenotypes were shown not to correspond entirely to the mtDNA haplotype clades identified in the study. This study provides evidence of further incongruence between morphology and phylogeography: specimens from western Bahia, exhibiting the '*atrox*' phenotype, are found within the clade comprising the southern '*B. moojeni*' populations, and populations exhibiting the '*atrox*' and the '*moojeni*' phenotype are rooted within the south-eastern Amazon clade.

The relationship between geographic distribution and the mtDNA clades revealed here also shows some interesting patterns. Most of the more distal clades within the various trees represent geographically coherent groups; the main exception is the sister group relationship between the

Fig. 5: Cladogram of the Fitch-Margoliash tree of the 31 haplotypes included in this study. See Fig. 2 for explanations.



populations from the Brazilian Atlantic coast and that from Acre which are separated by several thousand kilometres and various intervening populations representing the south-central Brazilian clade. Among the more basal clades, the "Amazonian periphery clade" includes widely scattered populations from north and south of the Amazon as well as the Brazilian Atlantic coast.

It is difficult to imagine a simple dispersal or vicariance scenario that could have given rise to the pattern of mtDNA phylogeography seen in this complex. It is possible that repeated multiple simultaneous population bottlenecks, perhaps due to Pleistocene climatic and vegetation fluctuations, may have resulted in lineage sorting among the more ancestral haplotypes, thus resulting in a situation where the mtDNA gene phylogeography does not reflect the phylogeography of the whole organism.

The results presented here do not yet allow a new classification of the *B. atrox* complex or the determination of species limits within the group. In order to achieve this, it will be necessary to determine to what extent the two main morphological phenotypes, or the clades revealed here

based on mtDNA sequence data, represent evolutionarily independent lineages, and thus different species. However, it should be noted that the evidence currently available, showing low levels of mtDNA differentiation among populations, incongruence between morphological and mtDNA variation even within terminal clades, the lack of morphologically discrete groups of populations, and an association between phenotype and habitat (WÜSTER et al. 1997) is more consistent with a hypothesis of a single, highly variable species than with the recognition of multiple species in the South American *B. atrox* complex. More work on these problems is currently in progress.

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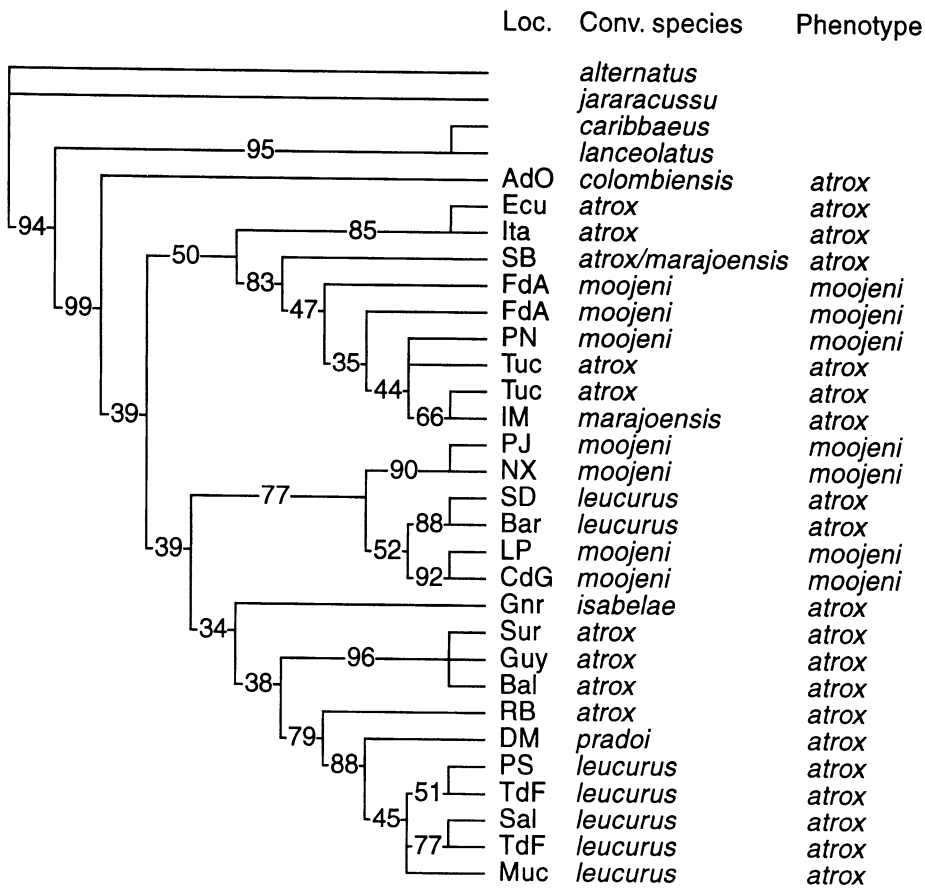


Fig. 6: Cladogram of the neighbour-joining tree of the 31 haplotypes included in this study. See Fig. 2 for explanations.

obtaining live *Bothrops* specimens or tissue samples for our DNA analyses. We also thank INEFAN (Quito, Ecuador), and STINASU (Paramaribo, Surinam) for permission to export tissue samples from their respective countries. The program PHYLIP was run using the Seqnet Facility of the Central Laboratory of the Research Councils (Daresbury Laboratory), Daresbury, U.K. This study was funded by the Wellcome Trust (Research Career Development Fellowship to W.W.), the Science and Technology for Development Programme of the European Union (contracts TS3-CT91-0024 and IC18-CT96-0032), Fundação Banco do Brasil and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP – grants 95/9056-9 and 97/2445-5) and the British Council.

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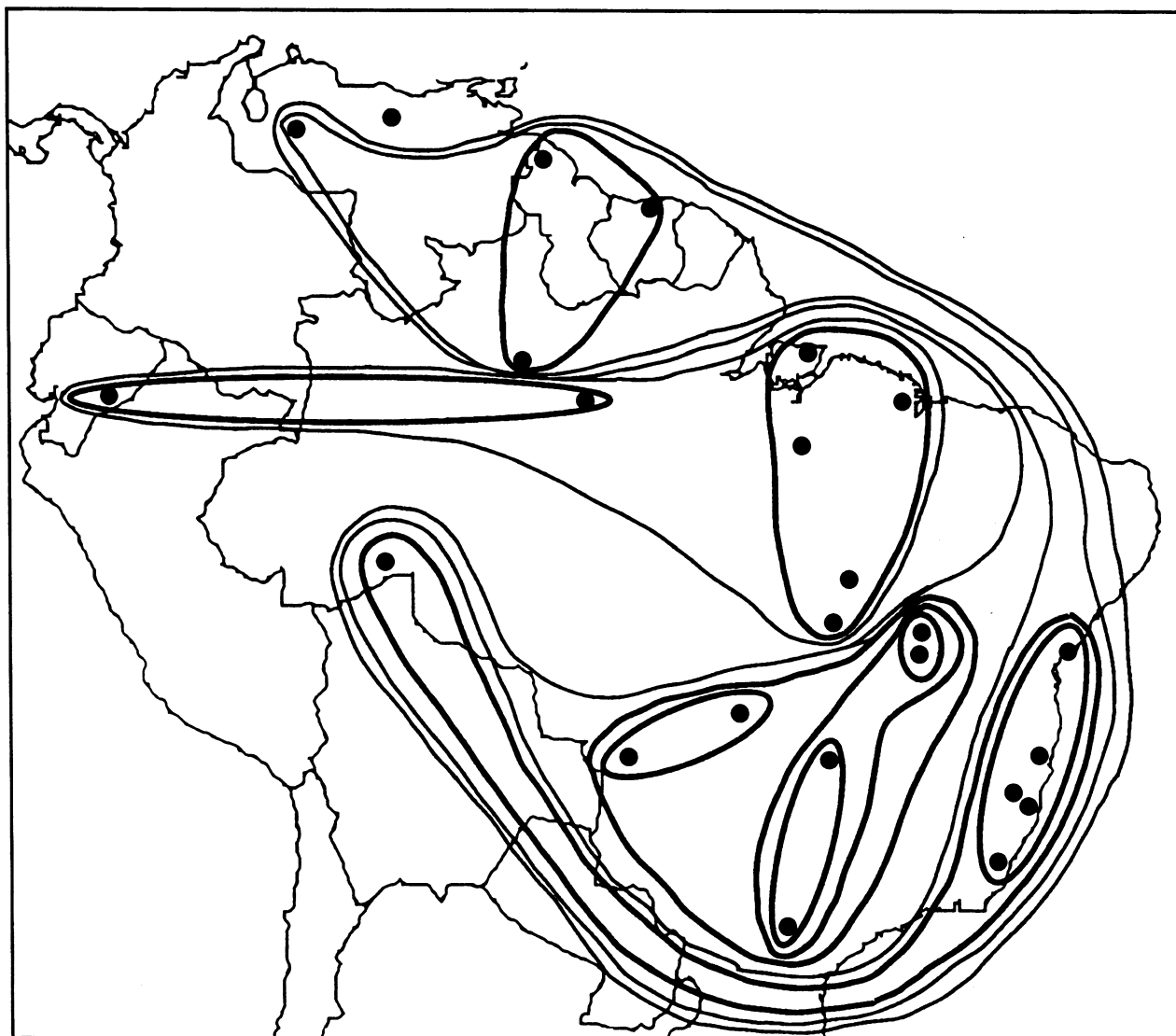


Fig. 7: Distribution of the mtDNA haplotype clades identified by the various algorithms. Clades outlined in bold are strongly supported by bootstrap analysis, those outlined with fine lines were recognised by all algorithms, but without strong bootstrap support.

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