
7 DNA evolution of South American pitvipers of the genus *Bothrops* (Reptilia: Serpentes: Viperidae)

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Synopsis

The Neotropical pitvipers currently and formerly included in the genus *Bothrops* comprise a large number (> 60) of species. The phylogenetic relationships among the taxa included in this group remain incompletely understood. Here, we analyse the relationships among various species of *Bothrops* (*B. alternatus*, *atrox*, *caribbaeus*, *cotiara*, *insularis*, *isabelae*, *jararaca*, *jararacussu*, *leucurus*, *marajoensis* and *moojeni*) and *Bothriopsis* (*B. taeniata*) by means of comparative mitochondrial DNA sequencing. Portions of the cytochrome *b* gene were amplified by means of the polymerase chain reaction (PCR), and sequenced. A 565 bp region of the cytochrome *b* gene was subjected to phylogenetic analysis, using maximum parsimony, Fitch–Margoliash and maximum likelihood algorithms. All trees confirmed the monophyly of the *Bothrops atrox* group exclusive of *Bothrops jararacussu*, the rooting of *Bothriopsis taeniata* within the genus *Bothrops*, the basal dichotomy between species with and without a lacunolabial scale, and the sister-species relationships between *B. jararaca* and *B. insularis*. On the basis of these results, we synonymize the genus *Bothriopsis* with *Bothrops*. Our results are discussed with reference to previous analyses of the phylogeny of Neotropical pitvipers, and points of consensus and remaining problem areas are identified.

Introduction

The snakes of the genus *Bothrops* are the most important cause of snakebite accidents in Latin America (Bolaños 1984; Campbell & Lamar 1989; Ministério da Saúde 1990; Cardoso 1992; Otero *et al.* 1992). In spite of this, the systematics and the relationships among members of this group remain controversial and unclear. Problematic areas include the status of some of the genera resurrected by Burger (1971) and the population systematics of the different species of the *B. atrox* group.

Morphology has been widely used to infer the affinities among the species of *Bothrops* (Amaral 1921; Brattstrom 1964; Hoge & Romano-Hoge 1978/79), and to divide the genus into the genera *Bothriechis*, *Bothriopsis*, *Bothrops* and *Porthidium* (Burger 1971; Schätti, Kramer & Touzet 1990; Schätti & Kramer 1993). However, these studies lacked rigorous phylogenetic analysis of the data obtained.

Recently other approaches have been used in an attempt to resolve different levels of the branching hierarchy in these groups. These include analysis of hemipenis morphology and electrophoresis of plasma proteins (Pesantes 1989), and combined allozyme, isozyme and morphological analysis (Werman 1992; this volume pp. 79–88). However, despite these studies, a number of problems remain unresolved.

The use of molecular systematic techniques has contributed considerably to the understanding of the systematics of many groups where morphological data have proved inconclusive (Patterson, Williams & Humphries 1993). The use of mitochondrial DNA sequence analysis has recently increased enormously through the development of the polymerase chain reaction (PCR) (Mullis *et al.* 1986; Saiki *et al.* 1988) and of direct sequencing of PCR-amplified DNA fragments using conserved primers (Kocher *et al.* 1989). Among venomous snakes, mtDNA sequence information has been used to formulate phylogenetic hypotheses at various taxonomic levels (Knight & Mindell 1993, 1994; Knight *et al.* 1993; Malhotra & Thorpe, this volume pp. 114–128). MtDNA sequence information has also been used for the identification of cryptic species in morphologically complex groups, such as the Asiatic cobras (Wüster & Thorpe 1994; Wüster *et al.* 1995).

Among the mitochondrial genes, cytochrome *b* has been used extensively in vertebrates not only because of the availability of conserved primer sites, but also because the rate of base-pair substitution makes it a useful source of information about relationships among closely related taxa (Irwin, Kocher & Wilson 1991; Simon *et al.* 1994).

This study aims to use mitochondrial DNA sequence information to provide additional evidence towards a robust phylogenetic hypothesis for the genus *Bothrops* and related South American pitvipers. Particular attention has been paid to the following problems: (1) Is there a basal dichotomy in the genus *Bothrops sensu* Burger (1971) between species with and without a lacunolabial scale? (2) Is the genus *Bothrops* paraphyletic with respect to *Bothriopsis*, as suggested by the results of Werman (1992)? (3) Is the *B. atrox* species complex monophyletic, especially with respect to *B. jararacussu* (see Werman 1992)?

Materials and methods

Tissue and/or blood samples were obtained from road-killed specimens or live, anaesthetized wild-caught or captive animals. The specimens used in this study and their localities are shown in Table 1. Tissue samples were collected and stored in 70–100% ethanol.

Table 1. List of specimens examined in this study and their localities

Taxon	Locality	Sample size
<i>B. taeniata</i>	Shell Macuma, Centro Pañyai, Morona Santiago, Ecuador	1
<i>B. alternatus</i>	Santa Rita do Passaquatro, São Paulo, Brazil	2
<i>B. atrox</i> ^a	UHE Balbina, Presidente Figueiredo, Amazonas, Brazil	2
<i>B. caribbaeus</i>	Saint Lucia	1
<i>B. cotiara</i>	Herval D'Oeste, Santa Catarina, Brazil	1
<i>B. insularis</i>	Ilha da Queimada Grande, São Paulo, Brazil	2
<i>B. isabelae</i> ^a	Guanare, Portuguesa, Venezuela	1
<i>B. jararaca</i>	São Bento do Sul, Santa Catarina, Brazil	2
<i>B. jararacussu</i>	Miracatu, São Paulo, Brazil	3
<i>B. leucurus</i> ^a	Porto Seguro, Bahia, Brazil	1
<i>B. marajoensis</i> ^a	Marajó Island, Pará, Brazil	1
<i>B. moojeni</i> ^a	Lençóis Paulista, São Paulo, Brazil	2
<i>C. durissus</i>	Pindamonhangaba, São Paulo, Brazil	1
<i>T. albolabris</i>	Pantar Island, Indonesia	1

^a The samples of the 'species' *atrox*, *isabelae*, *leucurus*, *marajoensis* and *moojeni* are simply of representative populations normally assigned to these taxa, and should not be taken to imply recognition of these taxa as valid species.

Genomic DNA was obtained through digestion with proteinase K (20 mg/ml). RNase solution (10 mg/ml) was added to the lysate and protein precipitation was carried out by adding 5 M ammonium acetate solution to the RNase-treated cell lysate. A DNA pellet was obtained by ethanol precipitation which was eluted in TE buffer (Puregene DNA isolation kit — Gentra Systems Inc.).

A 767 bp region of the cytochrome *b* gene was amplified using *Thermus aquaticus* DNA polymerase (GIBCO). The primers were MVZ 16 (Moritz, Schneider & Wake 1992), and modified versions of the universal cytochrome *b* primers of Kocher *et al.* (1989). PCR product was purified from the reaction solution through the use of SpinBind DNA Extraction Unit (FMC BioProducts).

Sequencing was carried out by the double-stranded dideoxy chain termination method (Sanger, Nicklen & Coulson 1977). Sequencing reaction products for both short and long fragments of cytochrome *b* gene were separated at 2000 V in 6% polyacrylamide, 7 M urea, 50 cm × 30 cm × 0.4 mm gels and were visualized by autoradiography. The sequences were aligned against the published human cytochrome *b* sequence (Anderson *et al.* 1981). Both strands were sequenced for most parts of the sequence analysed here.

A homologous region of 565 base-pair positions was subjected to phylogenetic analysis. In order to assess the robustness of the phylogenetic hypotheses generated here, we used four different algorithms: 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 *Trimeresurus albolabris* (A. Malhotra pers. comm.) and *Crotalus durissus terrificus*.

Results

Sequence data

A 565 bp section of the cytochrome *b* gene was available for the taxa of interest in this study, and was subjected to phylogenetic analysis. Of these 565 sites, 142 (25.13%) were variable among the ingroup taxa. The level of base-pair divergence between *Crotalus durissus* and the ingroup taxa varied from 15.1% to 17.1%, between *Trimeresurus albolabris* and the ingroup taxa from 13.8% to 16.1%, and among the ingroup taxa from 2.0% to 13.3%. The transition: transversion (TS:TV) ratio between *C. durissus* and the ingroup taxa varied from 1.22 to 1.71, between *T. albolabris* and the ingroup taxa from 1.66 to 2.71, and among the ingroup taxa from 1.73 to 14.50. Among the latter, there was no indication of a negative correlation between total base-pair divergence and the TS:TV ratio, which suggested that saturation of transitions is not a problem with this database. However, the uniformly low TS:TV between the outgroups and the ingroup taxa suggests considerable saturation of transition sites. As a compromise measure, taking into account both the saturation of transition sites in the outgroup comparisons and the fact that the transitions are likely to carry a useful phylogenetic signal within the ingroup, transversions were weighted twice as heavily as transitions in the phylogenetic analyses.

Phylogenetic analysis

The cladograms obtained by the four algorithms used in this study are shown in Fig. 1, and a semistrict consensus tree of the trees obtained by the four algorithms is shown in Fig. 2. All trees support the placement of the clade containing the two

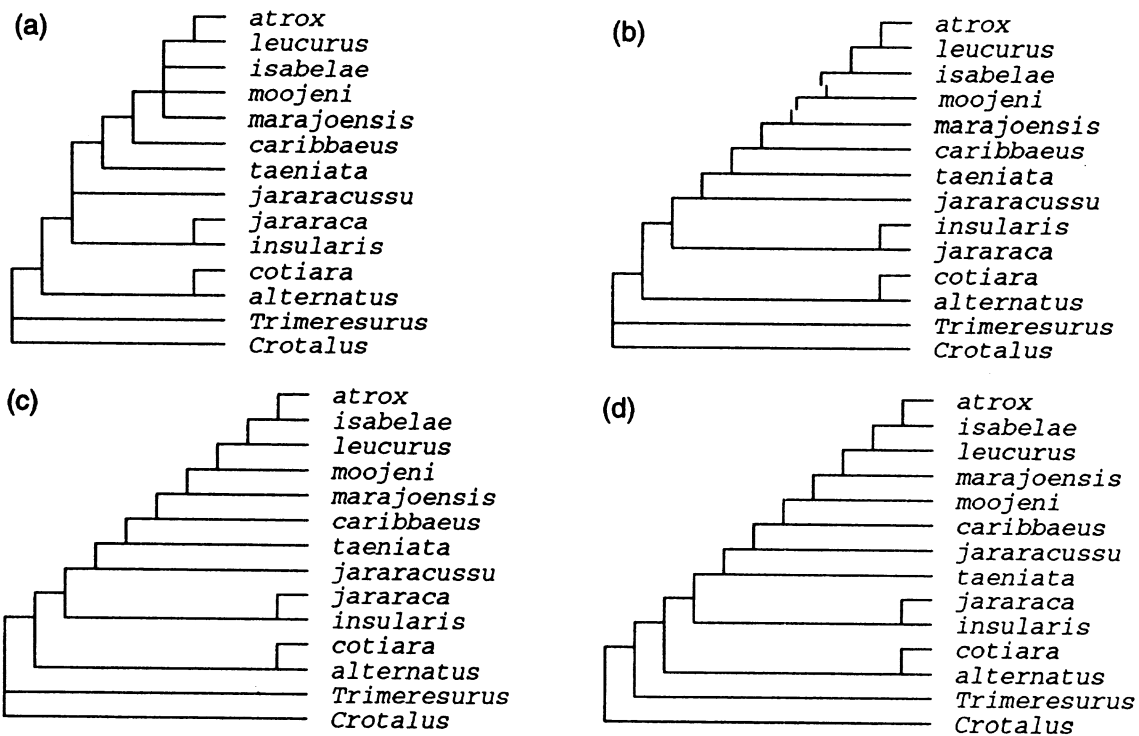


Fig. 1. Phylogenetic trees for the species of *Bothrops* and *Bothriopsis* obtained through the use of four different algorithms. (a) Maximum parsimony algorithm. This is a 75% majority rule consensus tree resulting from 11 equally parsimonious trees revealed by the analysis. (b) Maximum likelihood method. (c) Maximum parsimony algorithm. (d) Maximum likelihood method.

species with a divided lacunolabial scale (*Bothrops alternatus* and *B. cotiara*) as a sister clade to the remaining taxa, which have an undivided lacunolabial scale. All trees support the monophyly of the South American representatives of the *B. atrox* group, to the exclusion of *B. jararacussu*, and all support the status of *B. caribbaeus* as a sister taxon to this clade. The nesting of *B. jararacussu* and *Bothriopsis taeniata* varies depending on the algorithm used. *Bothriopsis taeniata* is consistently nested within the genus *Bothrops*, usually as a sister taxon to the *B. atrox* group (including *B. caribbaeus*), except in the KITSCH tree. *Bothrops jararaca* and *B. insularis* consistently form a monophyletic group, and represent the sister clade to all other species with an undivided lacunolabial scale in all but the parsimony tree, where the nesting of this clade, *B. jararacussu* and *B. taeniata*, are unresolved. *Crotalus* was rooted as a sister taxon to a clade consisting of *Trimeresurus* and the *Bothrops/Bothriopsis* clade in the unrooted KITSCH tree.

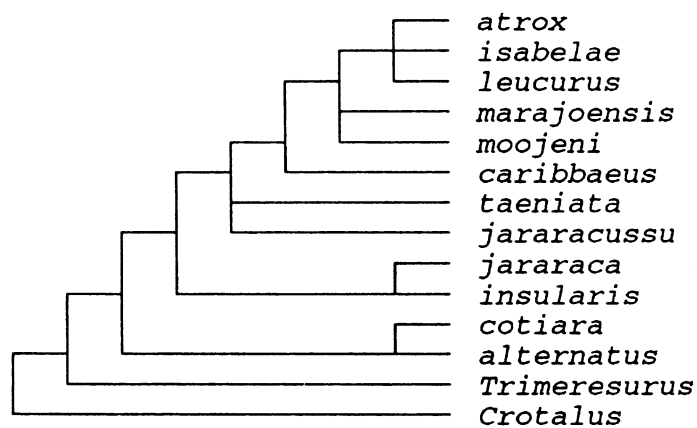


Fig. 2. Semistrict consensus tree of the trees obtained by the four algorithms used in this study. Note that no conclusions regarding the status of the taxa *atrox*, *isabelae*, *leucurus*, *marajoensis* and *moojeni* should be inferred from this analysis, as there is no evidence that these taxa do in fact represent real species. See Wüster *et al.* (this volume pp. 99–113) for a discussion of the systematics of the *Bothrops atrox* species group.

Discussion

Although the evolutionary relationships among pitvipers have formed the subject of intensive research for many decades, studies using advanced methods of phylogenetic inference and molecular systematics have been performed only recently.

The phylogenetic trees resulting from the analyses of our cytochrome *b* sequence data provide a new perspective on the systematics of this group. Comparison with the analyses of Pesantes (1989) and Werman (1992, this volume pp. 79–88) allows the identification of points of agreement and disagreement. The principal points to emerge from our analyses are discussed below with reference to these previous studies.

The status of the *Bothrops atrox* group and *B. jararacussu*

In all our analyses, the sequences representing the nominal species of the *B. atrox* group (*B. atrox*, *isabelae*, *leucurus*, *marajoensis* and *moojeni*) formed a mono-

phyletic group to the exclusion of all other taxa included in the study. This agrees with Pesantes (1989), but conflicts with the results of Werman (1992), in which a clade consisting of *B. jararacussu* and *B. brazili* was rooted within the *B. atrox* group. Our results consistently show *B. jararacussu* as an outgroup to the clade consisting of the *B. atrox* group and *B. caribbaeus*. The mtDNA results are unsurprising, as *B. jararacussu* is morphologically highly distinct from the relatively homogeneous *B. atrox* group. Unfortunately, we do not have sufficient sequence information for *B. brazili* to include this species in the present study. However, preliminary phylogenetic analysis of a 400 bp region of the cytochrome *b* gene suggests that this species is also rooted outside the *Bothrops atrox* complex (unpublished data). In Werman's (1992) study, which spanned all Neotropical crotalines as opposed to just *Bothrops sensu stricto*, the branch lengths were extremely short, indicating that the characters used perhaps lack the resolution required for a phylogenetic analysis of these relatively closely related species. Our results also differ from Pesantes' (1989) results of UPGMA analysis of hemipenis morphology and total plasma protein electrophoresis in that *B. jararacussu* does not form a sister taxon to *B. jararaca*.

The status of *Bothrops insularis*

In all analyses, *B. insularis* was identified as the sister taxon to our sample of *B. jararaca*, from the southern Brazilian state of Santa Catarina. The same sister-group relationship was obtained by Pesantes (1989) through the use of total plasma protein electrophoresis and hemipenial morphology. This indicates a relatively recent common ancestor for the two species, despite profound morphological (Amaral 1921), ecological (Duarte, Puerto & Franco 1995) and venom differences between these taxa. It should be noted that *B. jararaca* is a morphologically very heterogeneous species which displays considerable geographic variation; it is entirely conceivable that it may represent a complex of several species, and that *B. insularis* may be rooted within this complex, leaving *B. jararaca*, as currently understood, paraphyletic.

The status of the genus *Bothriopsis*

All our analyses show *B. taeniata*, the type species of the genus *Bothriopsis*, to be rooted within the genus *Bothrops*, although the precise rooting point differed between analyses. This is in agreement with the results of Werman (1992; this volume pp. 79–88), who found an arrangement which can be represented as (*alternatus* group (*jararaca* (*taeniata*(*atrox* group, *brazili*, *jararacussu*))). Recognition of *Bothriopsis* would therefore leave the genus *Bothrops* paraphyletic. Albumin immunological distance analysis (Cadle 1992) also showed *Bothriopsis taeniata* to be closer to *B. atrox* than *B. alternatus* was.

The avoidance of paraphyly of the genus *Bothrops* would require either the splitting of that genus, or synonymizing *Bothriopsis* with *Bothrops*. We argue that the latter solution would be preferable for a classification which is consistent

stability in the zoological nomenclature. We base this conclusion on the following considerations:

1. The precise rooting of *B. taeniata* within *Bothrops* is unclear. It would therefore be impossible to propose a genus-level classification for the species currently assigned to *Bothrops*.
2. No phylogenetic analysis has ever been carried out to test whether *Bothriopsis* is monophyletic. The various species of this genus form a heterogeneous group, and no phylogenetic analysis has ever included more than one species of the genus. Assigning all species of this genus to *Bothrops* avoids the creation of further instability which would result from the possible future discovery that the genus may be para- or polyphyletic.
3. A number of hitherto unstudied species of *Bothrops* (e.g., *B. lojanus*, *B. andianus*, *B. pictus*, *B. barnetti*) have uncertain affinities, and any further splitting of *Bothrops* would leave them *incertae sedis*.
4. The various species of *Bothriopsis* are relatively obscure and rarely discussed outside herpetological systematics, and were in any case generally classified as *Bothrops* until the 1980s. On the other hand, many of the species of *Bothrops* which would be involved in name changes in the event of a split of this genus to retain *Bothriopsis* (e.g., *B. alternatus*, *B. jararaca*, *B. neuwiedi*) are of considerable medical importance, and are frequently discussed in the biomedical literature. Changing the generic status of these species would therefore create considerable confusion. Researchers working with animals of medical importance have a particular responsibility to maintain the greatest possible stability compatible with systematic principles.

Finally, it is appropriate at this point to discuss the work of Schätti *et al.* (1990) and Schätti & Kramer (1993), who regarded *Bothriopsis* as a synonym of *Bothriechis*. No evidence of synapomorphies uniting the species of *Bothriopsis* and *Bothriechis* has ever been presented. A superposition of the phylogenetic trees for Central and South American pitvipers published in Werman (1992) clearly shows the branching order (*Bothriechis*(*Porthidium*(*Bothrops*, *Bothriopsis*))). This clearly contradicts the synonymization of *Bothriopsis* with *Bothriechis*. Unfortunately, we do not yet have any sequence information for any species of *Bothriechis*. However, in view of Werman's results, we do not question the status of the genus *Bothriechis* as a genus distinct from *Bothrops*, whereas we hereby synonymize *Bothriopsis* with *Bothrops*.

The *Bothrops alternatus* group

Werman (1992) found a basal dichotomy in the genus *Bothrops*, which separates the species with a lacunolabial scale (e.g., *B. atrox*, *B. jararaca*, *Bothriopsis taeniata*) from species without a lacunolabial (e.g., *B. alternatus*, *B. neuwiedi*). The same results, with different species, were obtained by Pesantes (1989). Our analysis only included two species without a lacunolabial, *B. alternatus* and *B. cotiara*, which consistently formed a monophyletic group, and were consistently rooted as the sister group of all species with an entire lacunolabial scale.

Conclusions: consensus and problems in Neotropical pitviper phylogeny

The present study is the third recent analysis of the systematics and phylogeny of Neotropical pitvipers of the genus *Bothrops* and related genera, after those of Pesantes (1989) and Werman (1992; this volume pp. 79–88). As these three studies have been carried out using different character systems (hemipenis morphology and plasma serum electrophoresis — Pesantes 1989; isozymes and morphology — Werman 1992 and this volume pp. 79–88), a comparison of the results provides an excellent opportunity to take stock of the situation and identify points of agreement as well as remaining problem areas within the systematics of the group.

Principal points of agreement:

- Within the genus *Bothrops*, *sensu* Burger (1971) and Campbell & Lamar (1989), a basal dichotomy between those species with a lacunolabial scale and those without has emerged in all three studies under consideration.
- The *Bothrops atrox* group, defined here as *B. atrox*, *B. isabellae*, *B. leucurus*, *B. moojeni* and *B. marajoensis*, is almost certainly monophyletic. The inclusion of *B. jararacussu* and *B. brazili* in this group in Werman's analysis appears unlikely in view of our results and those of Pesantes (1989), and also on the basis of the morphological distinctness of both these species from the *B. atrox* group.
- Bothrops insularis* and *B. jararaca* group as sister species in Pesantes' and our analyses.
- Werman's results (1992; this volume pp. 79–88) and ours both show *Bothriopsis taeniata* to be rooted within *Bothrops sensu stricto*, and there is therefore strong evidence in support of our decision to synonymize *Bothriopsis* with *Bothrops*.

Remaining problematic areas:

- Monophyly of the New World pitvipers. Our KITSCH tree suggests that *Trimeresurus* + *Bothrops* form a clade, with *Crotalus* as a sister taxon, suggesting multiple colonization of the New World by pitvipers.
- The phylogenetic affinities of the remaining species formerly classified as *Bothriopsis*.
- The phylogenetic position of *B. jararacussu*, on which there was disagreement between the three studies discussed here.
- The phylogenetic position of a number of other species of *Bothrops* not included here, including *B. lojanus*, *B. venezuelensis*, *B. pictus*, *B. barnetti* and *B. andianus*.
- The phylogenetic position of some species recently excluded from *Bothrops* by Schätti & Kramer (1993), namely *B. pulcher*/*B. campbelli*/*Porthidium almawebi* and *B./Porthidium microphthalmus/um*, as well as the superficially similar *Porthidium hyoprora*, which, in terms of pattern and scalation, appears more similar to *Bothrops* than to *Porthidium*.

The resolution of these problems will come from an integration of further multi-

... of different character systems and techniques

With the ever-increasing interest in pitviper systematics and phylogeny, there is now considerable hope that this will eventually be realized.

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