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MtDNA Phylogeny of Neotropical Pitvipers of the Genus *Bothrops* (Squamata: Serpentes: Viperidae)

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Zusammenfassung

Die Gattung *Bothrops* (sensu lato) umfaßt über 60 Arten von Grubenottern aus der gesamten neotropischen Region. Obwohl der Systematik dieser Gruppe in den letzten Jahren beträchtliche Aufmerksamkeit gewidmet wurde, wurde bis heute kein Konsens über die Untergliederung in Gattungen erreicht. In dieser Studie nutzen wir eine phylogenetische Analyse mitochondrialer DNA-Sequenzdaten (Cytochrom b), um die Phylogenie der Gruppe zu klären, mit Schwerpunkt auf den nominellen Gattungen *Bothrops* (sensu stricto), *Bothriechis* und „*Bothriopsis*“. *Bothriechis* zeigte sich als Schwestergruppe von *Crotalus*, wodurch *Bothrops* sensu lato nicht mehr monophyletisch ist. Die Arten *bilineata* und *taeniata*, die früher zu *Bothriopsis* gerechnet wurden, sind nicht näher mit *Bothriechis* verwandt, sondern scheinen eine monophyletische Gruppe zu bilden, die innerhalb von *Bothrops* wurzelt. Der *Bothrops atrox* Komplex (mit den nominellen Arten *atrox*, *moojeni*, *marajoensis*, *leucurus*, *pradoi* und *colombiensis*) ist monophyletisch, und seine Schwestergruppe besteht aus den beiden karibischen Inselarten *Bothrops caribbaeus* und *B. lanceolatus*. Unsere Ergebnisse zeigen auch die mehrfach unabhängige Entwicklung arborealer Lebensweise bei *Bothrops* (sensu lato).

Abstract

The genus *Bothrops* (sensu lato) comprises more than 60 species of pitvipers from throughout the Neotropical Region. Although the systematics of this group have received a considerable amount of attention in recent years, no consensus as to its generic classification has been reached so far. In this study, we use the phylogenetic analysis of mitochondrial DNA sequence information (cytochrome b) to investigate the phylogeny of the group, with particular emphasis on *Bothriechis*, *Bothrops* (sensu stricto) and „*Bothriopsis*“. *Bothriechis* was found to be the sister group of *Crotalus*, rendering *Bothrops* sensu lato non-monophyletic. The species *bilineata* and *taeniata*, previously assigned to *Bothriopsis*, appear to form a monophyletic group rooted within *Bothrops*, and are not associated with *Bothriechis*. The *B. atrox* complex (including the nominal species *atrox*, *moojeni*, *marajoensis*, *leucurus*, *pradoi* and *colombiensis*) is monophyletic, and its sister clade consists of the two Caribbean island species *Bothrops caribbaeus* and *Bothrops lanceolatus*. Our results also show the multiple origin of arboreality within *Bothrops* (sensu lato).

Introduction

Crotaline snakes are characterised by the synapomorphy of a loreal pit. Within this subfamily, there are about seventeen genera, distributed in both Old and New World. Their status and phylogenetic relationships remain controversial despite all the attention they have received in the last decades. This is largely due to the variety in morphology and the differences in habits and habitats present within this group (HOGE & ROMANO-HOGE 1978/79, FERRAREZZI 1994).

Traditionally, all rattle-less New World pitvipers with divided head shields, with the exception of *Lachesis muta*, were assigned to the genus *Bothrops* (e.g. PETERS & OREJAS-MIRANDA 1970). The *Bothrops* group includes about 60 species distributed primarily throughout the tropical regions of the New World, one third of them in Central America and the remaining two thirds in South America (HOGE

& ROMANO-HOGE 1978/79). The representatives of *Bothrops* occupy a vast variety of habitats, from deserts to lowland forests and up to mountains, including both arboreal and terrestrial forms (CAMPBELL & LAMAR 1989).

Morphology and habits have been extensively used as characters to subdivide this genus. BRATTSTROM (1964) divided this taxon into a group of large and terrestrial forms (*Bothrops*) and another group comprising smaller and arboreal forms (*Bothriechis*). BURGER (1971) investigated the morphology of the crotalines, with particular emphasis on the osteology of the *Bothrops* complex, and proposed that this genus should be split into five, namely *Bothriechis* (the Central American arboreal forms), *Bothriopsis* (the South American arboreal forms), *Bothrops* sensu stricto (the South American terrestrial forms), *Ophryacus* (a monotypic Central

American taxon) and *Porthidium* (Central American terrestrial representatives).

Since the late seventies, some or all of BURGER's genera have been recognised by various researchers (e.g. HOGE & ROMANO-HOGE 1978/79, SAVAGE 1980, PÉREZ-HIGAREDA et al. 1985, CAMPBELL & LAMAR 1989, SCHÄTTI et al. 1990), but none of them proposed a clear hypothesis of relationship to support such a division.

The first phylogenetic hypothesis for *Bothrops* (sensu lato), based on morphological characters and allozymes, was proposed by WERMAN (1992). His results showed *Porthidium* as a paraphyletic group, which led him to describe a new genus, *Atropoides*. Based on the same study, CAMPBELL & LAMAR (1992) described another new genus, *Cerrophidion*, to include three Mexican and Central American montane species.

WERMAN (1992) used a combination of morphological and allozyme data to investigate phylogenetic relationships within *Bothrops* sensu stricto, and found a basal dichotomy between species with an undivided lacunolabial scale (e.g. the *B. atrox* group) and those with a divided lacunolabial (e.g. *B. alternatus*). Furthermore, he found *Bothriopsis taeniata* (the type species of *Bothriopsis*) to be rooted within the clade of *Bothrops* sensu stricto with an undivided lacunolabial scale. This basal dichotomy between species with and without an undivided lacunolabial was also found by PESANTES (1989), based on phenetic analyses of hemipenial morphology and total plasma proteins.

SCHÄTTI et al. (1990) synonymised *Bothriopsis* with *Bothriechis*, arguing that the characters purported to distinguish them show gradual rather than categorical variation. However, no detailed analysis of character states and their variation within and between the two genera was presented, nor was any evidence given for the monophyly of these arboreal pitvipers, leaving the rationale for synonymising *Bothriopsis* with *Bothriechis* unclear.

Recently, the use of molecular data, particularly mitochondrial DNA sequence information, has helped to clarify some aspects of the phylogeny of many different groups of vertebrates, or to reveal cryptic species in complex groups, such as the Asiatic cobras (WÜSTER & THORPE 1994, WÜSTER et al. 1995).

Among Neotropical pitvipers, the use of mtDNA sequencing has provided evidence for the paraphyly of the *Bothrops* group (KRAUS et al. 1996, VIDAL et al. 1997) and the rooting of *Bothriopsis* within *Bothrops* sensu stricto, which resulted in the synonymisation of the former with the latter (SALOMÃO et al. 1997).

Due to the lack of agreement concerning the generic classification of *Bothrops* (sensu lato) and the high medical importance of these snakes

(BOLAÑOS 1984, CAMPOS et al. 1986, Ministério da Saúde 1990, CARDOSO 1992, OTERO et al. 1992), we carried out further investigations on the phylogenetic relationships of some taxa included within this group, using mitochondrial DNA sequencing in order to answer some questions related mainly to the monophyly of the entire group, the monophyly of the arboreal forms and finally the monophyly of the *atrox* group.

Material and methods

Total DNA was obtained from tissue and/or blood samples preserved in 70-100% ethanol of several taxa from different localities (Tab. 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) and 5'-GGC AAA TAG GAA GTA TCA TTC TG-3' (H-strand). PCR was carried out in 50 µl of a solution of 20 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.52 µM 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.

Chain-termination sequencing (SANGER et al. 1977) was carried out manually, using standard 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, and a homologous region of 580 base pairs available for all taxa of interest was analysed using maximum parsimony (PAUP 3.1.1 - SWOFFORD 1993) and neighbour-joining (MEGA 1.02 - KUMAR et al. 1993). A sequence of *Naja sputatrix* was used as an outgroup for tree rooting and sequences of *Trimeresurus albolabris*, *Tropidolaemus wagleri* (A. Malhotra, pers. comm.) and *Crotalus durissus* were included for comparative purposes.

Sequences for several specimens of many species were available; where these differed, the monophyly of the haplotypes of each species was checked. In all cases where the different haplotypes for each species formed monophyletic groups (everywhere except the *Bothrops atrox* group), only

Table 1: List of species included, with localities and sample sizes.

Taxon	Locality	Sample size
<i>B. schlegelii</i>	Costa Rica	1
<i>B. bilineatus</i>	Macuma, Morona Santiago, Ecuador	2
<i>B. taeniatus</i>	Macuma, Centro Pañyai, Morona Santiago, Ecuador	1
<i>B. alternatus</i>	Santa Rita do Passaquatro, São Paulo, Brazil and Pinhão, Paraná, Brazil	2
<i>B. atrox</i> 3	Itacoatiara, Amazonas, Brazil	1
<i>B. atrox</i> 1	Rio Branco, Acre, Brazil	2
<i>B. atrox</i> 2	Tucuruí, Pará, Brazil	3
<i>B. brazili</i>	Macuma, Morona Santiago, Ecuador	1
<i>B. caribbaeus</i>	Saint Lucia	2
<i>B. colombiensis</i>	Altagracia de Orituco, Guárico, Venezuela	1
<i>B. cotiara</i>	Herval D'Oeste, Santa Catarina, Brazil	1
<i>B. fonsecai</i>	Campos do Jordão, São Paulo, Brazil	1
<i>B. insularis</i>	Ilha da Queimada Grande, São Paulo, Brazil	2
<i>B. isabelae</i>	Guanare, Portuguesa, Venezuela	1
<i>B. jararaca</i>	São Bento do Sul, Santa Catarina, Brazil	2
<i>B. jararacussu</i>	Cananeia, Jujuitiba and Miracatu, São Paulo, Brazil	4
<i>B. lanceolatus</i>	Martinique, French Antilles	1
<i>B. leucurus</i>	Porto Seguro, Bahia, Brazil	1
<i>B. marajoensis</i>	Marajó Island, Pará, Brazil	2
<i>B. moojeni</i>	Lençóis Paulista, São Paulo, Brazil	2
<i>B. pradoi</i>	Domingos Martins, Espírito Santo, Brazil	1
<i>C. durissus</i>	Pindamonhangaba, São Paulo, Brazil	2
<i>N. sputatrix</i>	West Java	1
<i>T. albolabris</i>	Pantar Island, Indonesia	1
<i>T. wagleri</i>	Malayan Peninsula	1

one haplotype per species was selected for the final analysis.

The parsimony analysis used the heuristic search option of PAUP, using tree bisection-reconnection branch swapping. Ten replicates with randomised taxon input order were carried out. Bootstrap analysis involved the same search algorithm, with 100 bootstrap replicates with randomised taxon input order each. Neighbour-joining analysis was based on a Jukes-Cantor distance matrix, which takes into account the probability of multiple hits. Bootstrap analysis involved 100 replicates.

Results

The analysis of the region of 580 base pairs showed that 241 (41.6%) of the sites were variable and 154 (26.6%) were informative under the parsimony criterion. Uncorrected pairwise divergences among the ingroup taxa (Neotropical crotalines) ranged from 0.9 to 17.7%. Among sequences of *Bothrops* and "*Bothriopsis*", there was no indication of a negative correlation between the total base-pair divergence and the transition/transversion ratio, suggesting no saturation of transition sites in the data set. However, comparisons of ingroup and outgroup sequences showed a reduced transition/transversion ratio, suggesting partial saturation of transition sites (SALOMÃO et al. 1997). In order

to determine the effect of this, the parsimony analysis was carried out twice, once on the unweighted data and once with transversions weighted twice as heavily as transitions.

The parsimony analysis of unweighted data revealed a single most parsimonious tree of 623 steps (Fig. 1), whereas analysis of the weighted data revealed four equally most parsimonious trees of 799 steps (Fig. 2), differing in the branching positions of the *caribbaeus/lanceolatus*, *jararacussu/brazili* and *taeniatus/bilineatus* groups. The parsimony bootstrap trees are shown in Fig. 3. The neighbour-joining tree with bootstrap values is shown in Fig. 4.

All trees show *Bothriechis* as sister group of *Crotalus durissus*, albeit with weak bootstrap support. The monophyly of *Bothrops* + *Bothriopsis* has strong bootstrap support in all trees. Within *Bothrops* sensu stricto, the most parsimonious trees obtained under both weighting regimes support a basal dichotomy between the forms with a divided lacunolabial scale (represented by *B. alternatus*, *B. cotiara* and *B. fonsecai*) and the remaining species with an undivided lacunolabial, but bootstrap support for this is low. The neighbour-joining tree groups the *jararaca/insularis* clade as the sister group of the forms with a divided lacunolabial, but there is no bootstrap support for this grouping.

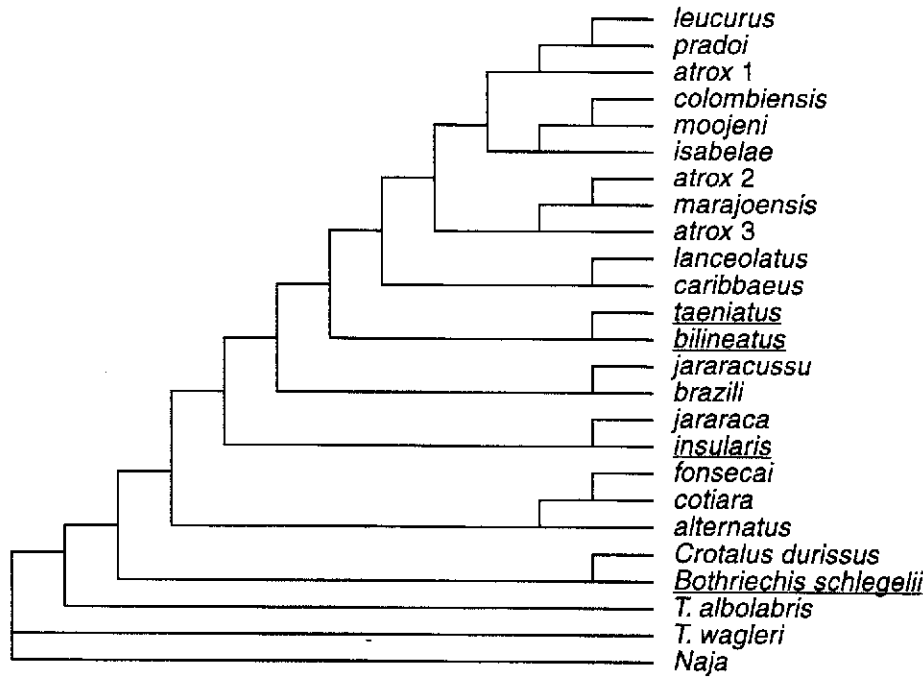


Fig. 1: Phylogenetic tree for the species of *Bothrops* (sensu lato) using maximum parsimony algorithm on the unweighted data. Underlining indicates arboreal taxa. Consistency index = 0.531; Homoplasy index = 0.469 and Retention index = 0.566.

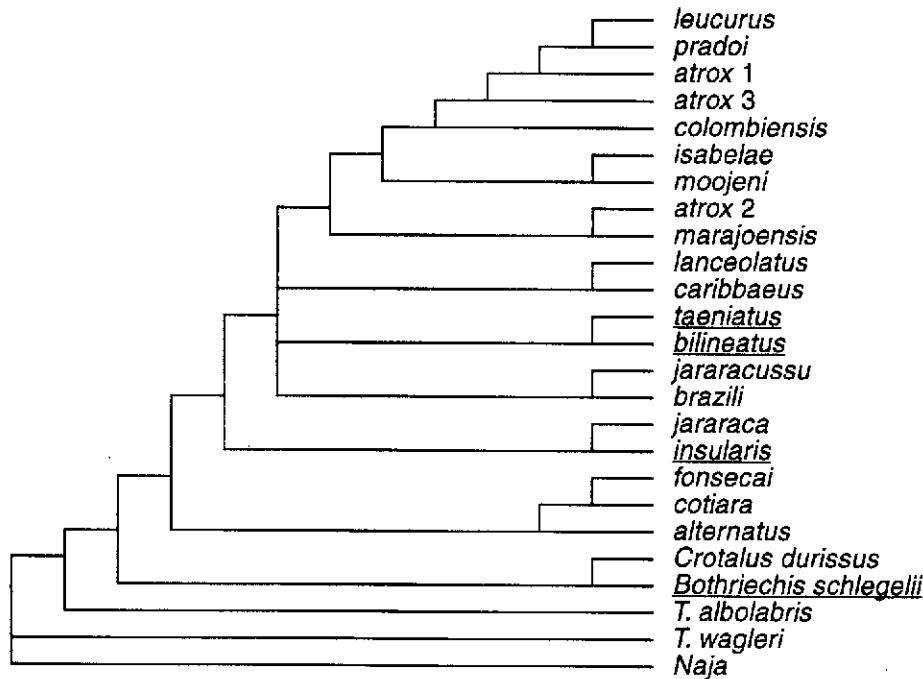
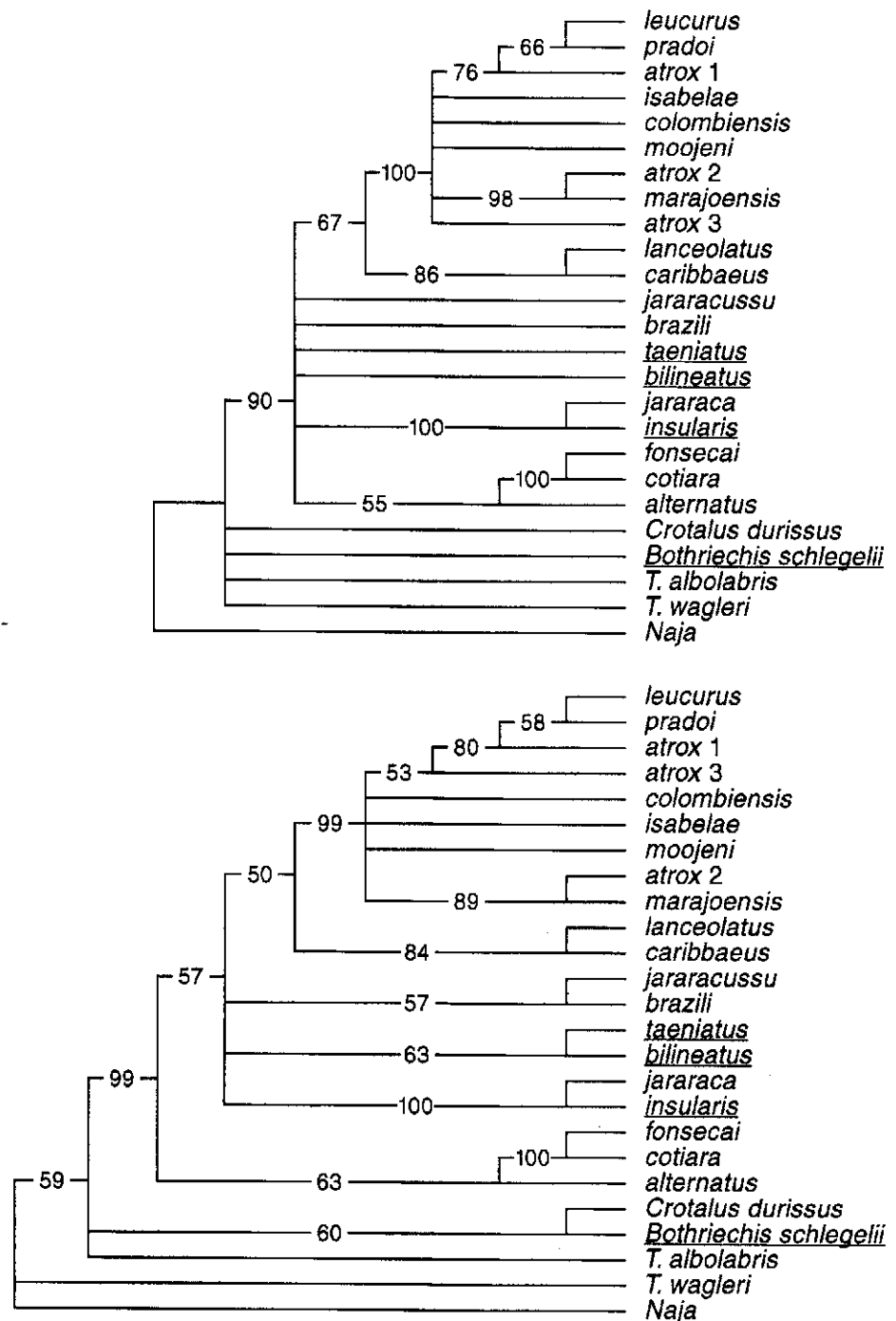


Fig. 2: Strict consensus of four equally most parsimonious trees for the species of *Bothrops* (sensu lato), resulting from analysis of the data with transversions double-weighted.

The sister group relationship between *B. jararaca* and *B. insularis* is strongly supported by all analyses and receives strong bootstrap support. The two species of "*Bothriopsis*" (*B. taeniata* and *B. bilineata*) are sister species forming a monophyletic group rooted within *Bothrops* sensu stricto, although bootstrap support for their monophyly is unconvincing in the parsimony analyses, and their exact rooting position within the phylogeny is highly unstable between analyses.

All analyses supported a sister group relationship between *B. brazili* and *B. jararacussu*, although with low levels of bootstrap support. The two Antillean species, *B. caribbaeus* and *B. lanceolatus*, are sister species and this is strongly supported by all bootstrap analyses. Most analyses grouped them as the sister taxon of the *Bothrops atrox* complex (which is strongly supported as monophyletic in all analyses), and this is especially supported by the bootstrapped neighbour-joining tree.

Fig. 3: Bootstrap 50% majority-rule consensus trees for unweighted parsimony analysis (top) and parsimony analysis with transversions double-weighted (bottom).



Discussion

The results presented here show that the molecular phylogeny is consistent with the morphological phenotypes observed in *Bothrops* and congruent with some past results based on anatomical characters combined with biochemical data (WERMANN 1992, 1997).

Our analyses showed *Bothriechis* to be the sister group of *Crotalus durissus*, leaving *Bothrops* sensu lato paraphyletic. This agrees with the results of VIDAL et al. (1997) who also found *Bothriechis* as sister group of *Crotalus*, but contradicts the findings of WERMANN (1992, 1997).

The remaining taxa of the *Bothrops* complex included here form a natural group. Our results support the basal dichotomy between the species with a divided lacunolabial and those with an undivided lacunolabial, which agrees with the findings of PESANTES (1989), WERMANN (1992, 1997) and SALOMÃO et al. (1997). However, bootstrap support for this grouping is minimal, and sequences from a number of other species without lacunolabial (e.g. *B. newwiedi*, *B. erythromelas*, *B. itapetini-gae*) were not yet available at the time of writing.

Within the *B. alternatus* group, *B. fonscecai* and *B. cotiara* are sister taxa, having *B. alternatus* as their

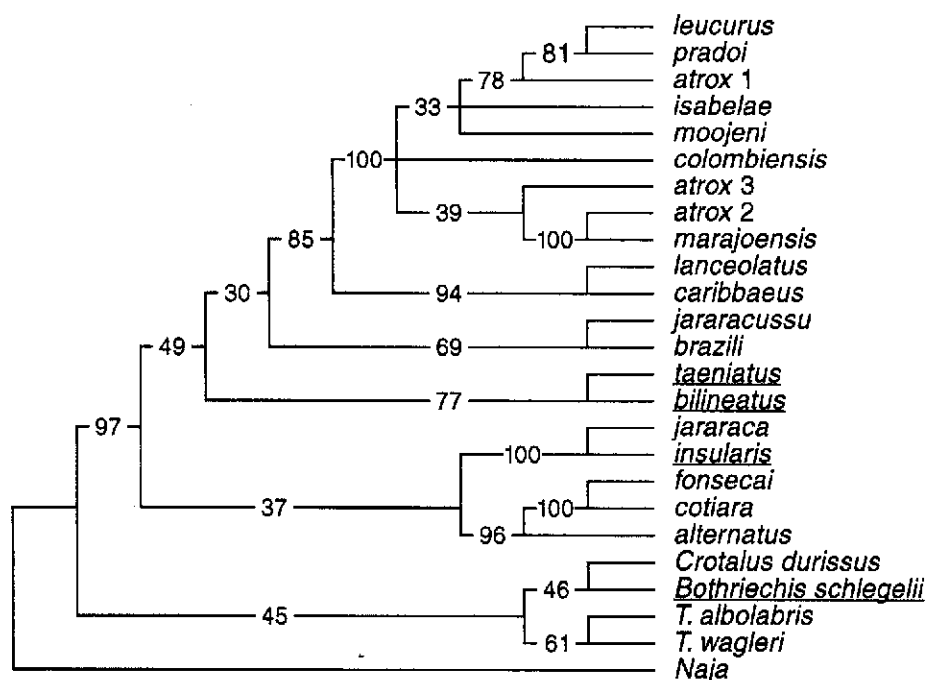


Fig. 4: Bootstrapped neighbour-joining tree. The bootstrapped tree is topologically identical to the original tree, hence all nodes, including those with less than 50% bootstrap support, are shown.

sister group. These results disagree with those of PESANTES (1989) who found *cotiara* and *alternatus* more closely related, with *fonsecai* as sister group. *Bothrops fonsecai* and *B. cotiara* are superficially very similar species and occupy remnant *Araucaria* forest habitats in southern and south-eastern Brazil. The sister group relationship between these two species suggests that they are vicariant remnants of a more widespread ancestral taxon occupying this specialised habitat.

Among the forms with an undivided lacunolabial scale, *B. jararaca* and *B. insularis* are sister taxa, despite all the differences in morphology, habits, habitats and venom composition (AMARAL 1921, COGO 1991, DUARTE et al. 1995). The clade consisting of these two species forms the sister clade to all other species with an undivided lacunolabial in the parsimony analyses. The same relationship was found by WERMAN (1992). Its position as a sister group to the *B. alternatus* group, as supported by the neighbour-joining tree, has negligible bootstrap support. Further research on the population systematics of *B. jararaca*, a morphologically highly variable species, is needed. In particular, it appears that *B. jararaca* may be paraphyletic with respect to *B. insularis* (WÜSTER et al., unpublished data).

The two arboreal species formerly assigned to *Bothriopsis*, *Bothrops bilineatus* and *B. taeniatus*, form a monophyletic group, which is rooted consistently within *Bothrops* sensu stricto, as already shown by CADLE (1992), WERMAN (1992, 1997) and SALOMÃO et al. (1997). However, the exact rooting point of the group remains uncertain, particularly with respect to the *B. jararacussu/brazili* clade.

These findings contradict the unsupported synonymisation of *Bothriopsis* with *Bothriechis* proposed by SCHÄTTI et al. (1990). Despite superficial similarities in habits, these arboreal forms do not form a monophyletic group.

Bothrops jararacussu and *B. brazili* appear to form a monophyletic group. Their exact rooting point remains uncertain, but there is no support for PESANTES' (1989) hypothesis, in which *B. jararacussu* formed the sister species of *B. jararaca* and *B. insularis*. Moreover, WERMAN'S (1992) results, in which *B. jararacussu* and *B. brazili* were rooted within the *B. atrox* group, are strongly contradicted by the very high support for the monophyly of the *Bothrops atrox* group obtained in this study. *Bothrops jararacussu* and *B. brazili* are morphologically similar, moderately heavy-bodied terrestrial snakes, with large heads and eight supralabials, and appear to be specialised forest dwellers. As a group, they are highly distinct from the more slender, smaller-headed members of the *B. atrox* group, which have only seven supralabials, a likely synapomorphy.

The Antillean species, *Bothrops caribbaeus* and *B. lanceolatus*, form a well-supported clade. Most analyses support the position of this complex as the sister group of the *Bothrops atrox* group, although bootstrap support is variable.

Finally, the *B. atrox* group, which includes the nominal species *atrox*, *colombiensis*, *isabelae*, *leucurus*, *marajoensis*, *moojeni* and *pradoi*, forms a strongly supported monophyletic group. *Bothrops atrox* is shown to be a heterogeneous taxon: different populations are represented in different parts of the cladogram, grouping with populations representing other nominal species of the group. The

problems of the systematics of this group have been discussed elsewhere (WÜSTER et al. 1996, 1997, this volume).

Overall conclusions

This study, based on mtDNA sequencing, has provided some additional information about the phylogeny and systematics of *Bothrops* (sensu lato). In particular, our results show that:

- *Bothrops* (sensu lato) is a paraphyletic group, as already pointed out by KRAUS et al. (1996) and VIDAL et al. (1997); in particular, *Bothriechis* appears to share a more recent common ancestor with *Crotalus* than with *Bothrops* sensu stricto, as also pointed out by VIDAL et al. (1997)
- *B. cotiara* and *B. fonsecai* are sister species, contrary to the results of PESANTES (1989), who found *B. cotiara* and *B. alternatus* to be sister species
- *B. bilineatus* and *B. taeniatus*, formerly assigned to *Bothriopsis*, form a monophyletic group rooted within *Bothrops* and are not associated with *Bothriechis*, contrary to SCHÄTTI et al. (1990)
- Arboreality evolved multiple times within *Bothrops* (sensu lato); as a consequence, characters associated with this facet of natural history should be interpreted cautiously and not be assumed to provide evidence of shared common ancestry
- The Caribbean island taxa, *Bothrops caribbaeus* and *B. lanceolatus*, form a monophyletic group which constitutes the sister clade of the South American *B. atrox* group
- The *B. atrox* complex forms a monophyletic group, but does not include *B. jararacussu* and *B. brazili*, contrary to the results of WERMAN (1992)

Other aspects of *Bothrops* phylogeny, however, still require attention, such as the affinities among other rare species assigned to *Bothrops* (*andianus*, *barnetti*, *lojanus*, *pictus* and *venezuelensis*), the other species formerly assigned to *Bothriopsis*, some other forms of uncertain affinities (e.g. *B. microphthalmus*, *B. colombianus*, *B. campbelli*, *Porthidium hyoprora*), and other genera (e.g. *Atropoides*, *Cerrophidion* and *Porthidium*) which have not been found to be monophyletic in some recent studies (KRAUS et al. 1996). Furthermore, although the monophyly of many species groups within *Bothrops* sensu stricto is strongly supported in this study, there is relatively little bootstrap support for the interrelationships among these species groups within *Bothrops*. Additional data are clearly needed to further test the phylogenetic hypotheses formulated here.

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