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Morphology, phylogeny and taxonomy of South American bothropoid pitvipers (Serpentes, Viperidae)

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South American bothropoids comprise a monophyletic and greatly diverse group of pitvipers that were initially included in the genus *Bothrops* and later assigned to five genera. Until recently, most phylogenetic analyses of bothropoids used exclusively mitochondrial DNA sequences, whereas few of them have included morphological traits. Moreover, the systematic affinities of some species remain unclear. In this study, we performed a parsimony analysis of morphological data obtained from the examination of 111 characters related to lepidosis, colour pattern, osteology, and hemipenial morphology of 35 of the 48 species that compose the bothropoid group. The morphological data analysed contain novel information about several species, including the *incertae sedis*. Morphology was analysed separately and combined with 2393 molecular characters obtained from published sequences of four mitochondrial genes. Five characters of the ecology were also included. A sensitivity analysis was performed using different weighting criteria for the characters. The congruence among different sources of evidence was evaluated through partitioned and total evidence analyses, the analyses of reduced datasets and the use of incongruence length difference test. With few exceptions, results showed groups of species similar to those obtained in previous studies; however, incongruences between morphological and molecular characters, and within the molecular partition, were revealed. This conflict affects the relationship between particular groups of species, leading to alternative phylogenetic hypotheses for bothropoids: hierarchical radiation or two major lineages within the group. The results also showed that *Bothrops sensu stricto* is paraphyletic. We discuss previous taxonomic approaches and, considering both phylogenetic hypotheses, we propose an arrangement that rectifies the paraphyly of *Bothrops*: maintaining *Bothrocophias*, assigning *Bothrops andianus* to this genus; and recognising the sister clade as *Bothrops*, synonymising *Bothriopsis*, *Bothropoides* and *Rhinocerophis*.

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Introduction

Viperidae is a group of snakes characterised by a buccal apparatus highly specialised in venom injection and, given that accidental envenomation by snakebite requires antivenom therapy, it is considered of medical importance. Phylogenetic analyses of these snakes, like studies on their venom toxins, may contribute to the understanding of the variability in viperid venoms (Wüster *et al.* 2008 and

references therein), which may be the result of selective pressures, e.g., for different diets (Barlow *et al.* 2009).

The major radiation of Viperidae occurs in Crotalinae ('pitvipers'), a widely distributed subfamily whose most notable synapomorphy is the presence of a loreal pit. An important reference for the biodiversity of crotalines in the Americas is the work of Campbell & Lamar (2004), which provides descriptions and images of pitvipers and other venomous reptiles present in the continent. In their study, these authors included North American pitvipers in the genera *Agkistrodon* Palisot de Beauvois, 1799, *Crotalus* Linnaeus, 1758 and *Sistrurus* Garman, 1884; and Neotropical pitvipers in the genera proposed by Burger (1971) (*Bothrops* Wagler, 1824; *Bothriechis* Peters, 1859; *Bothriopsis* Peters, 1861; *Porthidium* Cope, 1871; *Ophryacus* Cope, 1887) with the additional genera *Atropoides* Werman, 1992; *Cerrophidion* Campbell & Lamar, 1992, and *Bothrocophias* Gutberlet & Campbell, 2001. In a revision of the systematic findings of pitvipers in the Americas, Gutberlet & Harvey (2004) recognised that *Bothrops*, which includes most South American species, was a conflictive genus inasmuch as all the available evidence indicated its paraphyly.

Bothrops is greatly diverse in its morphology and ecological traits, and its species inhabit a wide spectrum of habitats across South America: from tropical and subtropical forests to arid and semiarid regions, and from altitudes of more than 3000 m to sea level, including islands. Some species of *Bothrops* with relatively wide distributions are the main cause of ophidic accidents in the continent (Salomão *et al.* 1997; Warrel 2004). Other species are present in relatively restricted areas, and their general biology is poorly known. Thus, the enormous diversity within *Bothrops* and the relative rareness of some of its species have led to a complex systematics.

The first cladistic analysis of *Bothrops* was conducted by Werman (1992), based on morphology (lepidosis and cranial osteology), isozymes and allozymes, and highlighted the paraphyly of *Bothrops* with respect to the arboreal genus *Bothriopsis*. This finding was further supported by several studies based on mitochondrial DNA sequences (Cadle 1992; Kraus *et al.* 1996; Salomão *et al.* 1997, 1999; Vidal *et al.* 1997; Parkinson 1999; Parkinson *et al.* 2002; Wüster *et al.* 2002; Castoe & Parkinson 2006); however, proposals differed in how to rectify the paraphyly. Salomão *et al.* (1997) recommended synonymising *Bothriopsis* with *Bothrops*. Wüster *et al.* (2002) agreed with them, and based on the results of other studies (e.g. Gutberlet 1998) also decided to treat *Bothrocophias* as a synonym of *Bothrops*. The results of Wüster *et al.* (2002) showed the monophyly of *Bothrocophias* unsupported, and the species of *Bothriopsis* rooted within *Bothrops*. The authors suggested maintaining *Bothrops* as a single, diverse genus, arguing that its morphological and ecological diversity might be the result of a single radiation event that occurred in South America, and that splitting the genus would obscure this biogeographic pattern. Other authors (Parkinson 1999; Gutberlet & Campbell 2001; Parkinson *et al.* 2002; Harvey *et al.* 2005; Castoe & Parkinson 2006; Fenwick *et al.* 2009) proposed maintaining *Bothriopsis* and *Bothrocophias* and dividing *Bothrops* into monophyletic genera. Despite these different perspectives, most authors concur on the monophyly of the *Bothrops–Bothrocophias–Bothriopsis* group (hereafter 'bothropoids') (Parkinson *et al.* 2002; Wüster *et al.* 2002; Castoe & Parkinson 2006; Fenwick *et al.* 2009). Fenwick *et al.* (2009) analysed the relationships among bothropoids based on morphological and molecular evidence, and their study was the first to include an almost complete taxon sampling of *Bothrops*. The results of Fenwick *et al.* (2009) showed mostly the same groups of species within the genus as those obtained in previous molecular analyses (e.g. Salomão *et al.* 1997; Parkinson *et al.* 2002; Wüster *et al.* 2002), and the authors proposed maintaining *Bothrocophias* and *Bothriopsis*, and splitting *Bothrops* into three genera: *Rhinocerophis* Garman, 1881, for the *Bothrops alternatus* group; *Bothropoides gen. n.*, for the *Bothrops jararaca* and *Bothrops neuwiedi* groups; and *Bothrops sensu stricto*, for the *Bothrops jararacussu* and *Bothrops atrox* groups; whereas four species remained *incertae sedis* (*Bothrops pictus*, *B. roedingeri*, *B. barnetti* and *B. lojanus*). Carrasco *et al.* (2009) found variation in the character that Fenwick *et al.* (2009) reported as a morphological synapomorphy of *Rhinocerophis*, and Carrasco *et al.* (2010) made a morphological revision and a redescription of the type species of that genus, maintaining the traditional taxonomy (*Bothrops ammodytoides*). Carrasco *et al.* (2010) also remarked that the classification proposed by Fenwick *et al.* (2009) did not resolve the paraphyly of *Bothrops*, because the generic assignment of some species (e.g. *Bothrops andianus*) was not consistent with phylogenetic results.

Most of the prior phylogenetic analyses included few species of the group, and not until recently have more comprehensive analyses been performed (Wüster *et al.* 2002; Fenwick *et al.* 2009). Additionally, phylogenetic relationships within bothropoids are known mainly from molecular evidence, because very few analyses combined molecular with morphological characters (Werman 1992; Fenwick *et al.* 2009). Fenwick *et al.* (2009) combined both types of data into analyses that included several species for the first time, and the genera they proposed were diagnosed with characters from mitochondrial genes, and (except *Bothropoides*) osteological characters within unique phenotypic synapomorphies. However, the morphological data analysed by these authors lacked osteological information for most of the ingroup taxa (Fenwick *et al.* 2009:

Appendix 2). Some cranial elements are directly involved in venom injection and ingestion of prey, and this relevant information has yet to be analysed for most bothropoids. Hence, more comprehensive morphological analyses may be necessary for a robust phylogenetic classification of these snakes. Furthermore, the uncertain or unclear position of some relatively rare taxa (e.g. some species distributed in the Andean region) and the paraphyly of *Bothrops* remain to be resolved.

Here, we present a parsimony analysis of morphological data of 35 of the 48 species that compose the South American bothropoid group. We assembled a morphological matrix based on the examination of 111 characters (some of which are used for the first time) of lepidosis, colour pattern, cranial osteology and hemipenial morphology. These morphological data contain new information (particularly regarding cranium and hemipenis) of several species, including the *incertae sedis*. We added ecological characters to the morphological matrix and analysed this dataset separately as well as in combination with molecular characters obtained from published mitochondrial DNA sequences. A sensitivity analysis (Wheeler 1995; Giribet 2003) was performed using different weighting schemes for the characters, and the congruence between different types of data was evaluated through partitioned and total evidence analyses, analyses of reduced datasets, and an incongruence length difference (ILD) test. The goal of this study was to explore the following issues: phylogenetic information of different types of morphological characters; the level of congruence among different sources of evidence (morphological, molecular and ecological); and the monophyly of and relationships within the bothropoid group. Finally, after evaluating previous taxonomic proposals, we suggest a rearrangement that rectifies the paraphyly of *Bothrops* while attending taxonomic stability.

Material and methods

Ingroup and outgroup taxa

The most recent taxonomic proposal for South American bothropoids is that of Fenwick *et al.* (2009); however, as mentioned above, this classification does not resolve the paraphyly of *Bothrops*. Therefore, in the present study, we followed Wüster *et al.* (2002) and considered bothropoids as a single monophyletic genus *Bothrops*. Nevertheless, to illustrate discussions on monophyly, we adopted the taxonomy of Fenwick *et al.* (2009) in the cladograms, using the specific epithet between quotation marks for the species *incertae sedis*. In the text, we employed this designation for informal names previously used for groups of species (e.g. '*neuwiedi*').

The ingroup is comprised of 35 taxa (Table 1). All species groups previously recognised within bothropoids were widely represented. The ingroup included the species that Fenwick *et al.* (2009) considered *incertae sedis* (*Bothrops pictus*, *B. roedingeri*, *B. barnetti*, *B. lojanus*). For the present study, we also considered two additional species (*B. andianus* and *B. venezuelensis*) to be *incertae sedis*. The ingroup was also comprised of some species that were rarely included in phylogenetic analyses (e.g. *B. jonathani*, *B. sanctaecrucis*, *B. mattogrossensis*, *B. oligolepis*). Some species were not available for this study (*B. alcatraz*, *B. caribbaeus*,

Table 1 Ingroup and outgroup taxa analyzed in this study

	Wüster *et al.* (2002)	Fenwick *et al.* (2009)
Ingroup taxa		
'*microphthalmus*' group	*Bothrops microphthalmus*	*Bothrocophias microphthalmus*
	Bothrops hyoprora	*Bothrocophias hyoprora*
'*taeniata*' group	*Bothrops taeniata*	*Bothriopsis taeniata*
	Bothrops chloromelas	*Bothriopsis chloromelas*
	Bothrops pulchra	*Bothriopsis pulchra*
	Bothrops bilineata	*Bothriopsis bilineata*
	Bothrops oligolepis	*Bothriopsis oligolepis*
'*alternatus*' group	*Bothrops alternatus*	*Rhinocerophis alternatus*
	Bothrops jonathani	*Rhinocerophis jonathani*
	Bothrops cotiara	*Rhinocerophis cotiara*
	Bothrops fonsecai	*Rhinocerophis fonsecai*
	Bothrops itapetiningae	*Rhinocerophis itapetiningae*
	Bothrops ammodytoides	*Rhinocerophis ammodytoides*
'*neuwiedi*' group	*Bothrops neuwiedi*	*Bothropoides neuwiedi*
	Bothrops mattogrossensis	*Bothropoides mattogrossensis*
	Bothrops diporus	*Bothropoides diporus*
	Bothrops pauloensis	*Bothropoides pauloensis*
	Bothrops lutzi	*Bothropoides lutzi*
	Bothrops erythromelas	*Bothropoides erythromelas*
'*jararaca*' group	*Bothrops jararaca*	*Bothropoides jararaca*
	Bothrops insularis	*Bothropoides insularis*
'*jararacussu*' group	*Bothrops jararacussu*	*Bothrops jararacussu*
	Bothrops brazili	*Bothrops brazili*
'*atrox*' group	*Bothrops atrox*	*Bothrops atrox*
	Bothrops moojeni	*Bothrops moojeni*
	Bothrops asper	*Bothrops asper*
	Bothrops leucurus	*Bothrops leucurus*
	Bothrops lanceolatus	*Bothrops lanceolatus*
	Bothrops sanctaecrucis	*Bothrops sanctaecrucis*
	Bothrops andianus	*Bothrops andianus*
	Bothrops venezuelensis	*Bothrops venezuelensis*
	Bothrops pictus	*Incertae sedis*
	Bothrops roedingeri	*Incertae sedis*
	Bothrops barnetti	*Incertae sedis*
	Bothrops lojanus	*Incertae sedis*
Outgroup taxa		
	Bothriechis schlegelii	
	Atropoides nummifer	
	Cerrophidion godmani	
	Porthidium lansbergii	
	Porthidium nasutus	
	Lachesis muta	
	Crotalus durissus	

B. campbelli, *B. colombianus*, *B. marajoensis*, *B. marmoratus*, *B. medusa*, *B. muriciensis*, *B. myersi*, *B. osbornei*, *B. pirajai*, *B. pubescens* and *B. punctatus*), but there is general consensus regarding the systematic affinities of most of these species (e.g. Salomão *et al.* 1997, 1999; Wüster *et al.* 2002; Fenwick *et al.* 2009). The outgroup comprised selected species of other crotaline genera (Table 1). Werman (1992) and Fenwick *et al.* (2009) used the genus *Agkistrodon* as an outgroup in their analyses. Werman (1999) suggested that another potential outgroup for bothropoid taxa would be the genus *Crotalus*. In the present study, *Crotalus durissus terrificus* was used for rooting the trees.

Morphological study and morphological characters

The specimens examined and their origins are detailed in Appendix S1. Morphological techniques and terminology used followed Carrasco *et al.* (2009, 2010). With few exceptions (see Appendix S1), we examined the external morphology, cranial osteology and hemipenial morphology of most taxa. For *Bothrops lojanus* and *B. lanceolatus*, we examined skulls only; information regarding external morphology for these species was taken from Campbell & Lamar (2004). A total of 111 morphological characters were analysed (Appendix S2), 32 of which are proposed for the first time. Several characters were taken from Werman (1992), Wüster *et al.* (1996), Gutberlet & Harvey (2002) and Fenwick *et al.* (2009). Some of these characters were reinterpreted and redefined to provide new morphological information. Additional characters were adapted from Campbell & Lamar (2004) and Harvey *et al.* (2005). Most hemipenial characters were adapted from Pesantes (1989). Characters 0–34 are continuous, and characters 35–110 are discrete. Morphometric data were treated as continuous characters without discretisation. Methods to discretise morphometric data have been questioned (Farris 1990) as the same state may be assigned to significantly different taxa, or vice versa (i.e. different states assigned to taxa that do not differ significantly). We followed Goloboff *et al.* (2006), who demonstrated that when continuous characters are treated as simply additive characters, they can be optimised with algorithms such as Farris (1970), implemented in TNT program (Goloboff *et al.* 2008a), and analysed without discretisation. Continuous characters were represented as ranges of two standard deviations around the mean; thus, given a normal distribution, two terminals overlap when their means are not significantly different (and vice versa) (Goloboff *et al.* 2006). Each continuous character was standardised to the same range (between 0 and 2) to avoid scaling problems associated with the direct use of measures of different scales. Characters were standardised with TNT 1.1 (Goloboff *et al.* 2008a) using a macro script provided by Pablo Goloboff (INSUE, San Miguel de Tucumán, Argentina). Polymorphic discrete characters were coded under the majority criterion (see Wiens 1999). All discrete characters were considered unordered.

Ecological characters

Among previous studies on Neotropical pitvipers, Gutberlet & Harvey (2002) and Martins *et al.* (2002) were the first to include ecological characters in a phylogenetic analysis of these snakes. Gutberlet & Harvey (2002) included two characters related to arboreality, which provided evidence that arboreality evolved independently in *Bothriechis* and *Bothriopsis*. Martins *et al.* (2002) provided a description of the diversity of feeding habits in *Bothrops* and used a phylogenetic hypothesis to explore evolutionary aspects of these traits and their relation with morphology, micro- and macrohabitat. Here, we included five ecological characters (Appendix S2): one from Gutberlet & Harvey (2002) and four adapted from the ecological and phylogenetic correlation of Martins *et al.* (2002).

Molecular characters

Molecular characters were obtained from published sequences of four mitochondrial genes (12S and 16S rRNA, NADH4 and cytochrome *b*) retrieved from GenBank (Appendix S3). In this study, we primarily used the same sequences analysed by Fenwick *et al.* (2009). However, unlike those authors, we chose to use the sequence obtained from a single specimen (choosing the one with the highest number of the genes sequenced) for each species, given that fusing all the available data into a majority-rule consensus sequence for one species may lead to chimerical constructions (e.g. *Bothrocophias microphthalmus* cyt-*b* sequence AY223594 at GenBank, used in combination with other sequences by Fenwick *et al.* 2009, appears to come from a misidentified *Bothriopsis bilineata smaragdina* or a contaminated sample. Blast searches we performed showed 100% identity with sequence AY223591 belonging to *B. b. smaragdina*). Ribosomal sequences (12S and 16S) were aligned using MAFFT 5.3 (Katho *et al.* 2002), available online at http://align.bmr.kyushu-u.ac.jp/mafft/software/. Algorithm G-ins-i, a scoring matrix 20 PAM/k = 2, gap opening cost of 1.53 and offset value 0.1 were used for both alignments. Protein-coding sequences (NDAH4 and cyt-*b*), of trivial alignment, were aligned and edited in BIOEDIT 7.05.3 (Hall 1999).

The molecular dataset had 2393 aligned sites (12S: 414 bp, 16S: 503 bp, cyt- *b*: 782 bp and NADH4: 694 bp), with 666 parsimony-informative characters. Sequences were not available from GenBank (accessed in May 2011) for *Bothrops jonathani*, *B. andianus*, *B. lojanus*, *B. barnetti*, *B. roedingeri*, *B. mattogrossensis*, *B. lutzi*, *B. sanctaecrucis*,

B. venezuelensis and *B. oligolepis*. Ribosomal 12S and 16S sequences were also absent for *B. pictus*, *B. fonsecai*, *B. neuwiedi*, *B. lanceolatus*, *B. pulchra* and *Crotalus durissus terrificus*. Although we preferred to avoid creating chimerical (composite) terminals, this approach was applied to *C. d. terrificus*, given that the species was selected to root the trees and that the absence of 12S and 16S sequences for this taxon could strongly affect the results. Thus, we performed an additional round of analyses using 12S and 16S sequences of *C. d. vegrandis* (closely related to *C. d. terrificus*, see Wüster *et al.* 2002: fig. 2), together with morphology, cyt-*b* and NADH sequences of *C. d. terrificus*. Nevertheless, when using the sequences of *C. d. vegrandis*, we recovered the same topologies for the bothropoid group (with one exception in the outgroups, detailed below) as those obtained when using the sequences of *C. d. terrificus* only (the latter results are shown in the present study). Gaps were treated as a fifth state, instead of missing data, as they can carry useful phylogenetic information (Simmons & Ochoterena 2000; Ogden & Rosenberg 2007; Simmons *et al.* 2008).

Assembly of matrices

The total evidence matrix was composed of six blocks, 2504 characters and 42 taxa. We assembled the morphological matrix in two blocks: one for continuous characters (Goloboff *et al.* 2006) and one for discrete characters. The ecological characters were added to the block of discrete characters of the morphological matrix. The molecular matrix was composed of four blocks, one for each mitochondrial gene. The total evidence matrix was constructed adding the four blocks of molecular characters to the morphological–ecological matrix.

Cladistic analysis

Parsimony analyses were conducted using the program TNT 1.1 (Goloboff *et al.* 2008a; available at http://www.zmuc.dk/public/phylogeny/TNT/). Optimal trees were searched using random addition sequences of Wagner trees, followed by the TBR algorithm, making 500 replications and saving up to 10 trees per replicate (command sequence: 'hold 5000; mult = tbr replic 500 hold 10;'). The resulting trees were used as starting points for a round of TBR branch swapping (command: 'bbreak = TBR'). Support values were estimated using group frequencies under jackknifing (see Goloboff *et al.* 2003). We used a probability of elimination of $P = 0.36$ for jackknife calculations performing 500 pseudoreplicates of 10 random addition sequences each, followed by TBR swapping, saving up to 10 trees (string of commands 'mult: noratchet repl 10 tbr hold 10; resample jak repl 1000').

The morphological–ecological and molecular matrices were analysed separately and combined. For each matrix (morphological–ecological, molecular and total evidence matrices), characters were analysed using two weighting criteria: equal weights (EW) and implied weights (IW). The implied weighting method (Goloboff 1993), implemented in TNT, uses a concave function (k) that gives low weights to those characters with high levels of homoplasy (see Goloboff *et al.* 2008b for discussion on this weighting method). In the present study, we used eight values for the concavity constant k: 3–10 for the morphological–ecological matrix and 8–15 for molecular and total evidence matrices (given that substitution rates and homoplasy are much higher in mitochondrial protein-coding genes than in morphology of closely related species, the use of mild to strong weighting functions is not recommended, Goloboff *et al.* 2008b).

The combination of alternative weighting criteria, concavity constant (k) values and different datasets resulted in the alternative analyses that are detailed in Table 2. Additionally, we explored the inclusion vs. exclusion of the ten species lacking published DNA sequences in the combined evidence analyses (following Fenwick *et al.* 2009).

Congruence of the dataset

Considering the diversity of the data analysed, we explored the degree of congruence among different sources of evidence primarily in two ways: by comparing the results of the separate analysis of the morphological–ecological and molecular matrices, and through the ILD test (Farris *et al.* 1995). While some issues have been raised regarding the ILD test (reviewed in Ramírez 2006), it remains a useful tool in assessing the contrasting phylogenetic signals of different partitions in an analysis. For the ILD test, we used a script provided in the TNT program, modified by

Table 2 Analyses resulting from the combination of alternative weighting criteria, k values and different datasets

Analysis of morphological and ecological evidence (AMEE)
AMEE, EW
AMEE, IW (k = 3–10)
Analysis of molecular evidence (AMLE)
AMLE, EW
AMLE, IW (k = 8–15)
Analysis of total evidence (ATE)
ATE, EW
ATE, IW (k = 8–15)
Analysis of reduced datasets
ATE, excluding taxa without molecular data, EW
ATE, excluding taxa without molecular data, IW (k = 8–15)
ATE, excluding characters from cyt-*b* and NADH4 genes (see results of ILD test), EW
ATE, excluding characters from cyt-*b* and NADH4 genes, IW (k = 8–15)

Table 3 Partitions of the different matrices analysed through the ILD test

Matrix	Partitions
Morphology–ecology	Continuous vs. discrete characters
Molecules	Ribosomal genes (12S-16S) vs. protein-coding genes (cyt-*b*-NADH4)
Total evidence	Morphology–ecology vs. molecules
	Morphology–ecology vs. 12S-16S
	Morphology–ecology vs. cyt-*b*-NADH4

Ramírez (2006), and evaluated the congruence between the partitions detailed in Table 3.

Results

Sensitivity analysis

In the analysis of morphology (AMEE), we obtained a single most parsimonious tree using both EW and IW (for each *k* value) (Fig. 1). The results of the alternative weighting criteria differed in the relationships within some terminal groups. In the analysis of molecules (AMLE), we obtained seven equally parsimonious trees under EW (Fig. 2) and a single most parsimonious tree under IW (for each *k* value). We obtained similar results at the terminal group level, except for the polytomy obtained under EW, under both weighting criteria. The analysis of all taxa and all characters [analysis of total evidence (ATE)] showed the same most parsimonious tree under both EW and IW (for each *k* value) (Fig. 3), except for the position of *Bothrops lojanus*, which showed a slight variation. In the analysis excluding taxa without molecular data, we obtained a single most parsimonious tree under both EW and IW (for each *k* value) (Fig. 4); results obtained under different weights varied in the position of '*taeniata*'.

Phylogenetic results

Bothropoids formed a monophyletic group in all analyses. Relationships between bothropoids and the outgroup

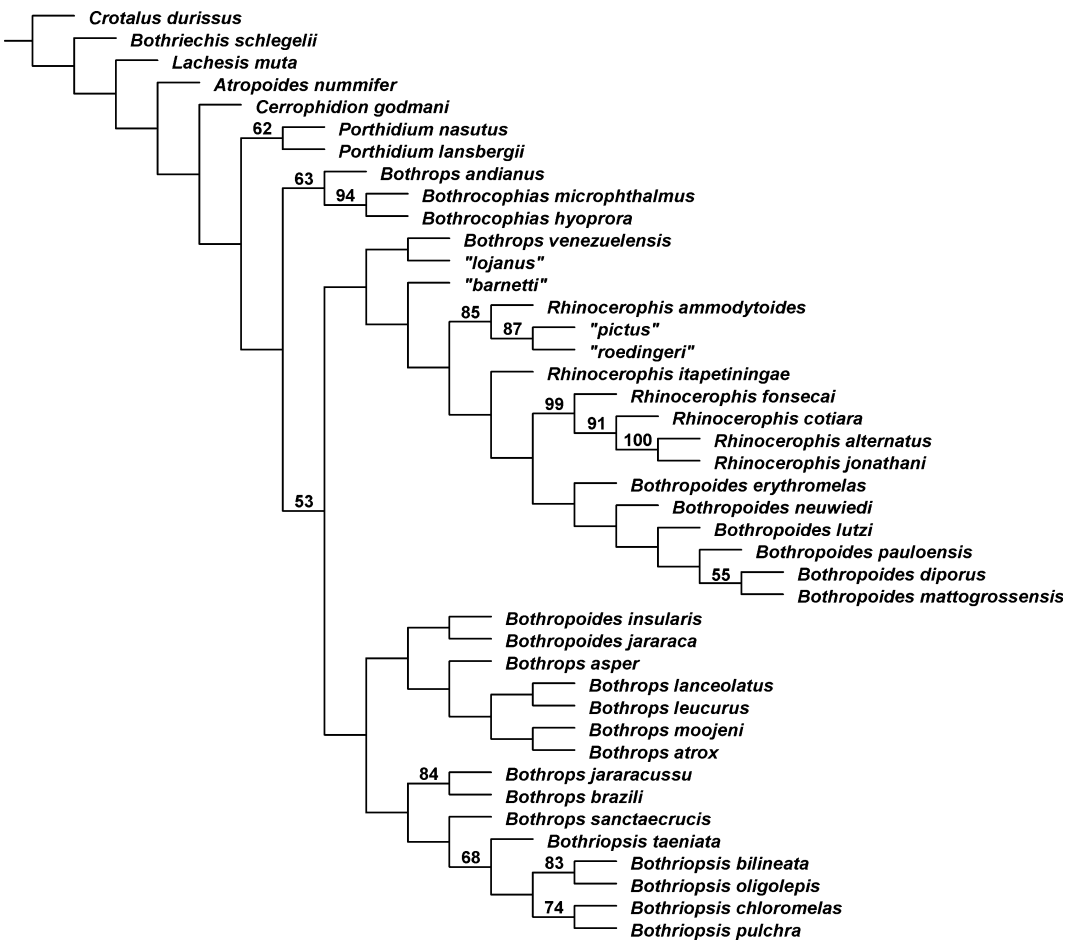

Fig. 1 Single cladogram obtained in the analysis of morphology under implied weights (*k* = 7) (Length = 550 069; Fit = 32,25). Above nodes, Jackknife proportions.

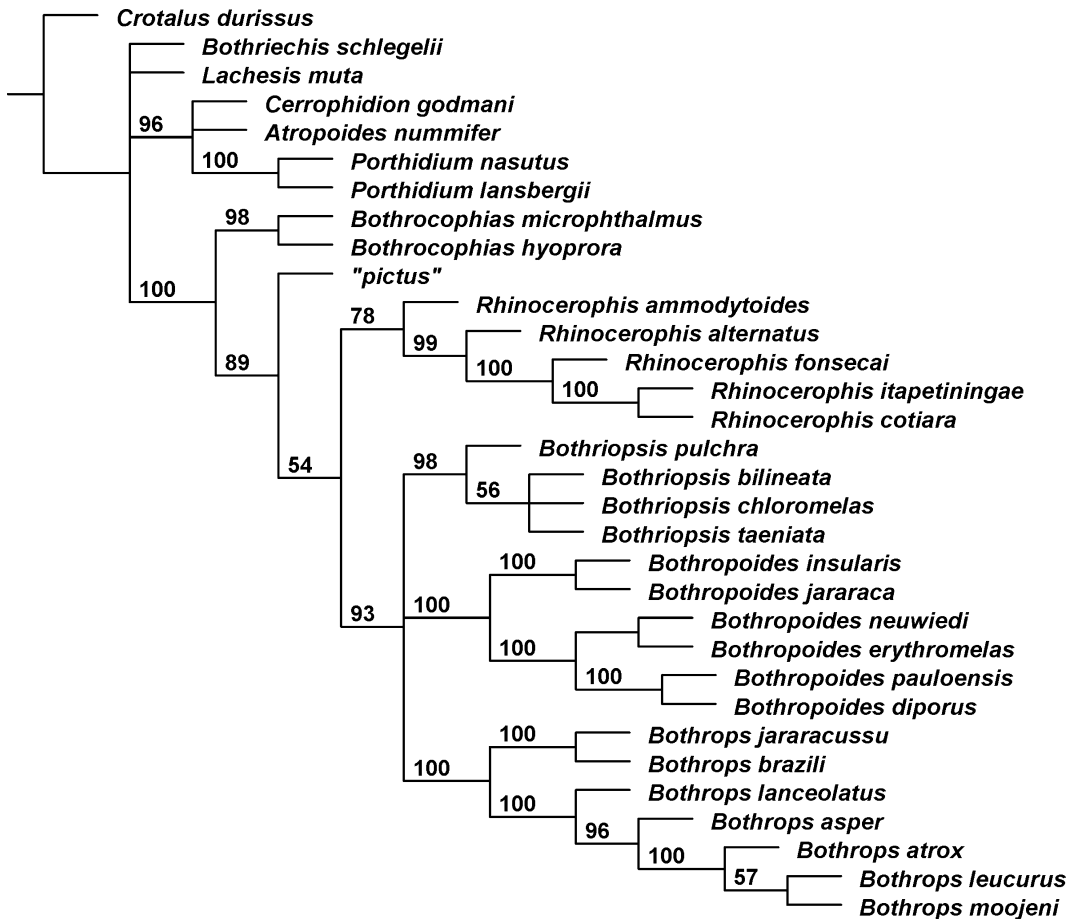

Fig. 2 Strict consensus of seven cladograms obtained in the analysis of molecules under equal weights (Length = 2691). Above nodes, Jackknife proportions.

resulted unstable, as the taxa that were closest to the group varied. Alternative sister taxa for bothropoids were *Porthidium*, *Bothriechis schlegelii* or the *Porthidium* complex (*sensu* Parkinson *et al.* 2002: *Atropoides*, *Cerrophidion* and *Porthidium*).

All groups of bothropoid species were monophyletic, except for the non-monophyly of '*alternatus*' and '*neuwiedi*' in the AMEE. The '*jararacussu*' group was related to '*taeniata*' in the AMEE, whereas it was the sister group of '*atrox*' in the remaining analyses. The AMEE and the AMLE showed different relationships within '*atrox*' and '*taeniata*'. *Bothrops sanctaecrucis* was rooted with '*taeniata*', except in the AMEE under strongest *k* values, where the species was related to '*jararacussu*'. The position of most species *incertae sedis* was stable throughout the analyses: *Bothrops andianus* was rooted with '*microphthalmus*', with whom it conformed the sister group of the rest of bothropoids; *B. barnetti* was related to '*alternatus*'; and *B. pictus* and *B. roedingeri* were sister taxa (which was expected as both species are phenotypically quite similar), and, except in the AMLE, both species were related to '*alternatus*'. The remaining species *incertae sedis* (*B. lojanus* and *B. venezuelensis*) were recovered in alternative positions.

Congruence among data

The separate analyses of the morphological–ecological and molecular matrices yielded similar results regarding groups of species, but showed alternative placements for two groups: '*neuwiedi*' and '*jararaca*'. In the AMEE, '*neuwiedi*' formed a clade with '*alternatus*' and some species *incertae sedis*, whereas the sister group of that clade included '*jararaca*'. In the AMLE, '*neuwiedi*' and '*jararaca*' formed a distinct clade.

The results of the ILD test showed congruence between partitions of the morphological–ecological matrix (continuous vs. discrete characters, non-significantly incongruent, 51.4%). Ribosomal vs. protein-coding genes partitions presented significant incongruence (99.7% confidence

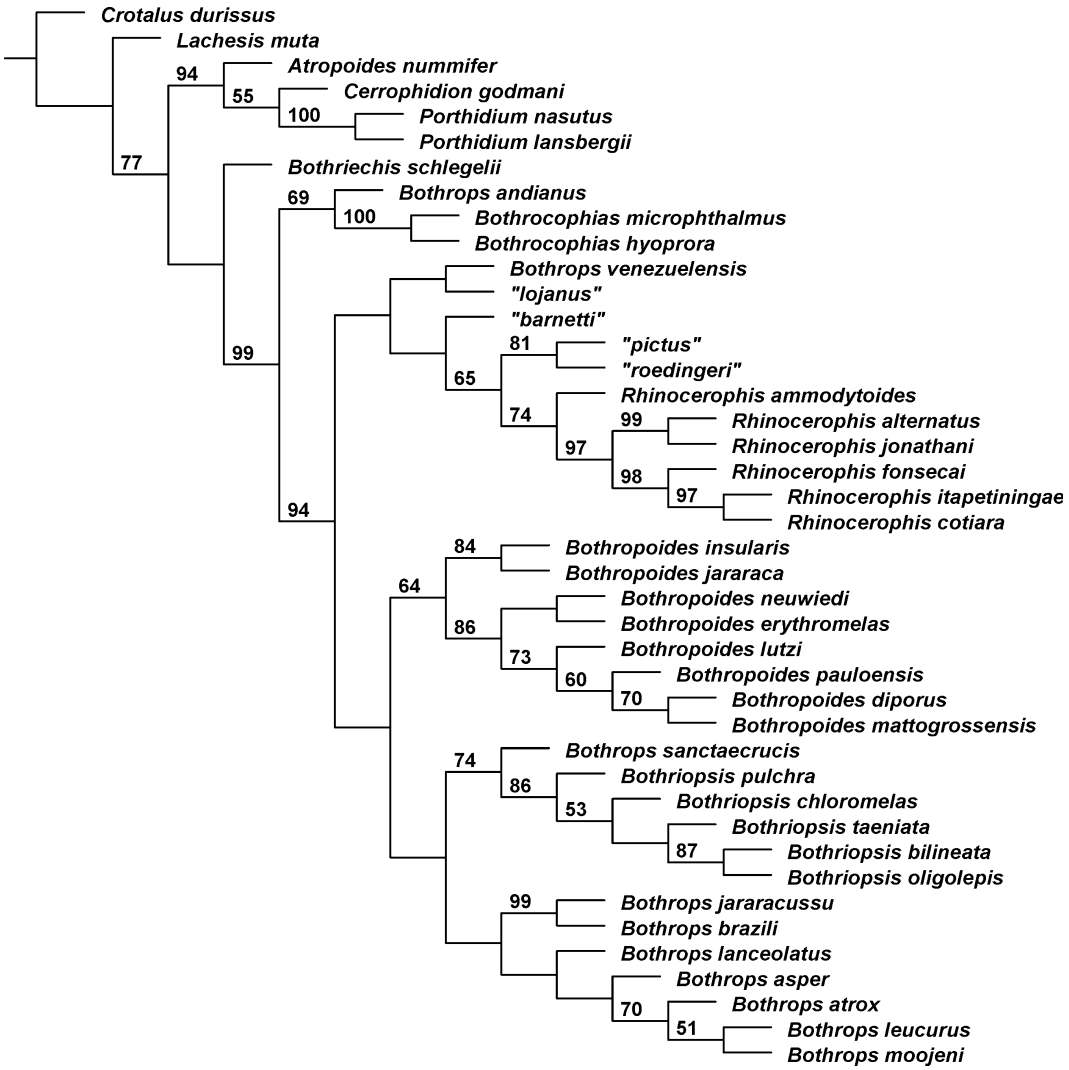

Fig. 3 Single cladogram obtained in the analysis of total evidence under equal weights (Length = 3 270 996). Above nodes, Jackknife proportions.

level) within the molecular matrix. The test found significant incongruence between morphological–ecological vs. molecular matrices (99.9% confidence level). The morphological–ecological matrix was congruent with two of the four genes of the molecular matrix (morphology–ecology vs. 12S-16S, incongruence not significant at 42% confidence level). Finally, we found that the morphological–ecological-12S-16S genes vs. cyt-*b*-NADH4 genes partitions presented significant incongruence (99.8% confidence level). Considering the latter results, we performed an analysis that included all taxa and data of morphology, ecology and the ribosomal genes, excluding the protein-coding genes. The resulting cladogram is shown in Fig. 5 (the same topology was obtained under EW and IW, for all *k* values). This analysis was the only one affected by the inclusion of sequences of *C. d. vegrandis*, *Bothriechis schlegelii* was recovered as the sister taxon of the bothropoid clade, instead of the *Porthidium* complex.

Discussion

Phylogenetic information of different types of morphological characters

The results of this study provide a framework for the interpretation of evolutionary patterns in morphological characters of bothropoids (see Sereno 2009 and Wirkner & Richter 2010), and discussions will be provided elsewhere. Here, we briefly address the information contained in different sources of morphological data.

Characters from external morphology (lepidosis and colour pattern) were found to be informative at all levels

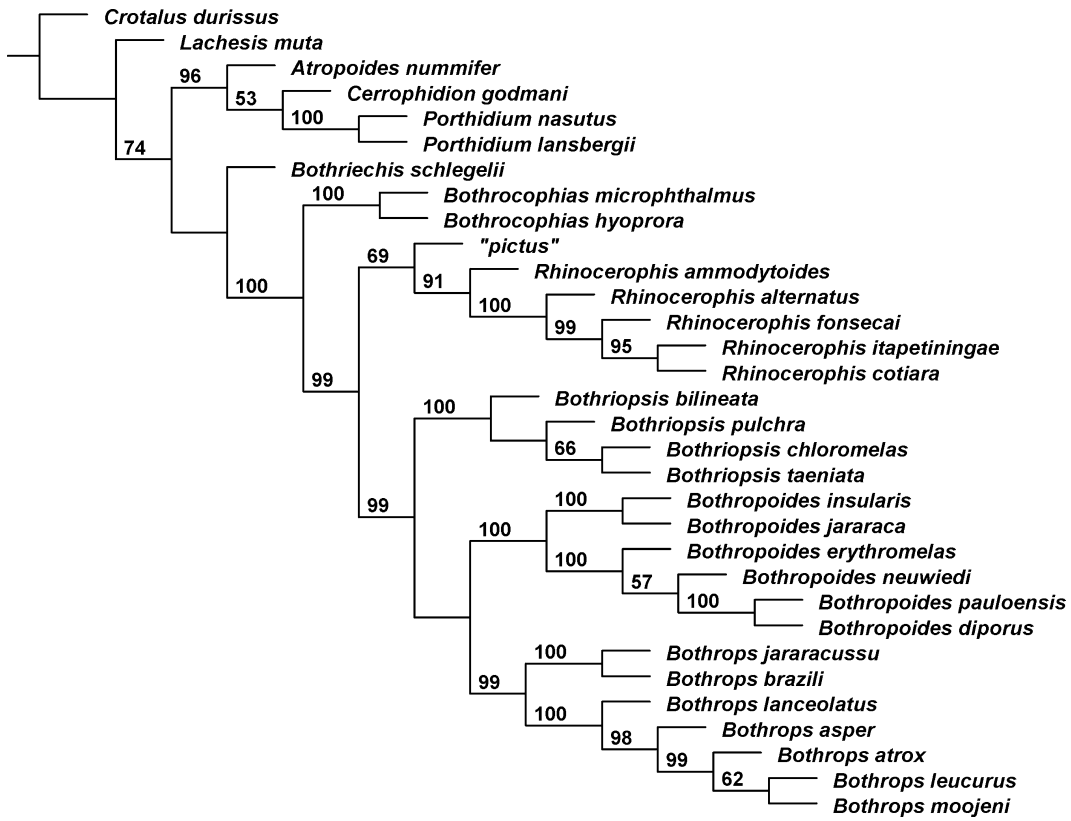

Fig. 4 Single cladogram obtained in the analysis of total evidence, excluding taxa without molecular data, under equal weights (Length = 3 177 578). Above nodes, Jackknife proportions.

of the cladograms, as almost every node was supported by at least one synapomorphy from this type of data. Some character states of the hemipenial morphology were synapomorphic in groups of species that share the same general structure of the hemipenis, e.g. '*neuwiedi*' (see Da Silva & Rodrigues 2008). It has been hypothesised that male genitalia are under sexual selection and evolve at a species-specific level (Songa & Bucheli 2010 and references therein). This hypothesis and the results of the present study suggest that hemipenial characters of bothropoids become informative at terminal groups. Characters of cranial osteology were informative at different levels of the cladograms, particularly characters related to the palatomaxillary arch (pterygoid, ectopterygoid, palatine, maxilla and fangs), a group of osteological elements involved in fang erection and ingestion of prey (Werman 1999). Fenwick *et al.* (2009) reported features of the palatine as synapomorphies of some terminal groups (e.g. '*alternatus*', '*taeniata*'). In the present work, we found that palatine characters were more informative at deeper nodes in the cladograms (i.e. as non-homoplastic synapomorphies of some internal nodes). Finally, we observed that continuous characters (which included information of all morphological sources) supported most, if not all, nodes and that most of them showed hierarchical patterns in the resulting trees. In their phylogenetic analysis of several New World pitvipers, Gutberlet & Harvey (2002) noted that inclusion or exclusion of overlapping meristic characters did not influence the robustness of terminal clades, but rather affected resolution at deeper nodes. Our results agree with their conclusion in that, e.g., three continuous characters were determined to be synapomorphies of the bothropoid clade.

Phylogeny

The monophyly of the bothropoid group is well supported in this study and consistent with previous analyses. Parkinson (1999), Parkinson *et al.* (2002), Wüster *et al.* (2002) and Castoe & Parkinson (2006) found molecular characters supporting the group and Estol (1981) determined a character of dorsal scale microstructure to be a morphological synapomorphy. In the present study, we found additional morphological synapomorphies for the bothropoid clade. The relationship between bothropoids and other crotalines showed to be unstable in our analyses, although the alternative sister taxa we obtained for bothropoids were

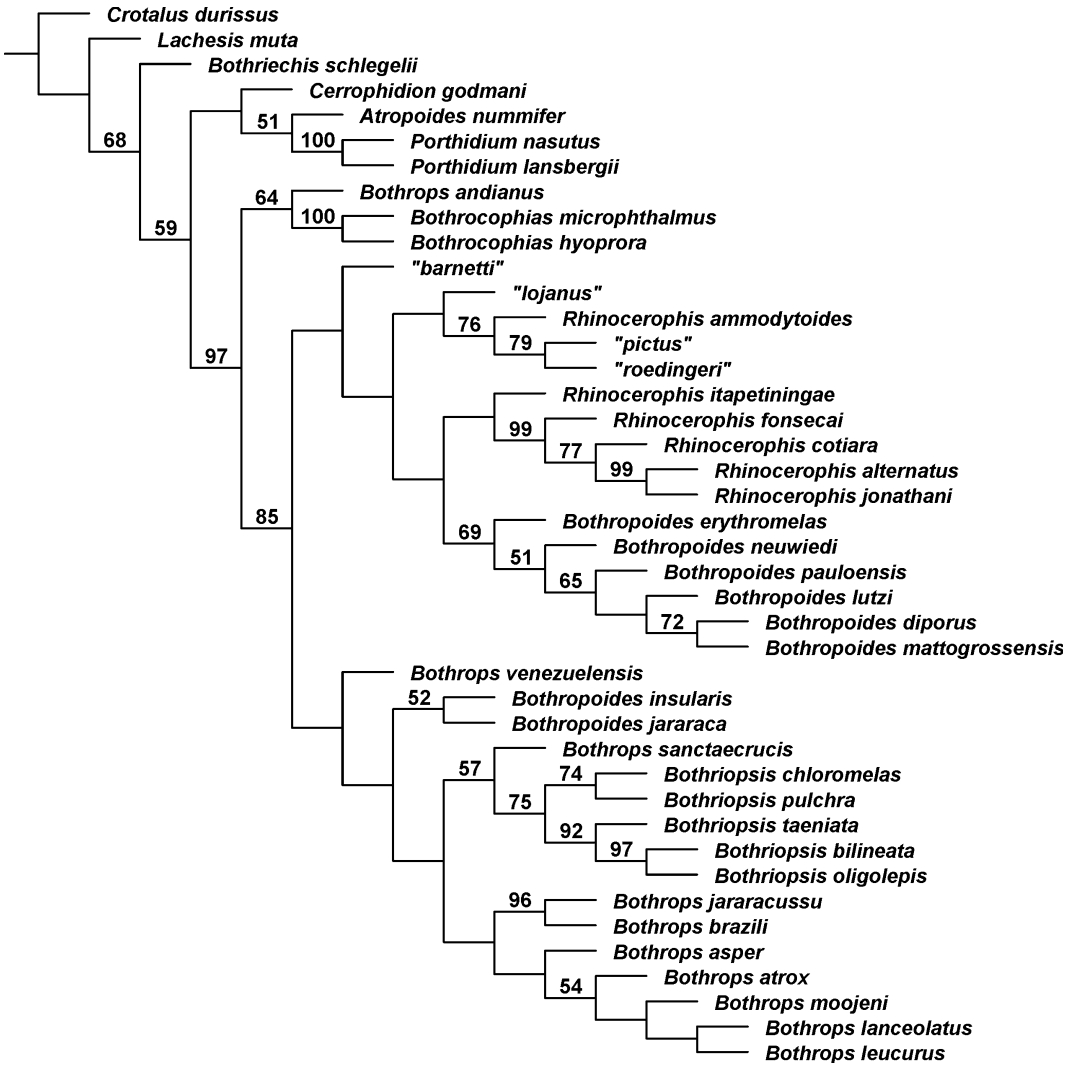

Fig. 5 Single cladogram obtained in the analysis of total evidence, excluding characters of NADH4 and cyt-*b* genes (see results of ILD test), under equal weights (Length = 986 880). Above nodes, Jackknife proportions.

also obtained by other authors (*Porthidium*: Werman 1992; *Bothriechis schlegelii*: Parkinson 1999; *Porthidium* complex: Parkinson *et al.* 2002, Castoe *et al.* 2005, Castoe & Parkinson 2006). It has also been suggested that the taxa closest to bothropoids may be *Lachesis* (see Wüster *et al.* 2002 and Gutberlet & Harvey 2004); however, in the present study, *Lachesis muta* was found to be distantly related to bothropoids. Determining which taxon is most closely related to bothropoids requires further research.

Within the bothropoid clade, the results showed the same groups of related species recognised in previous studies, and these groups were recovered as monophyletic in most cases. Results obtained for '*alternatus*' were different, apparently due to instability in the position of '*neuwiedi*', which may be related to some species of '*alternatus*' or to '*jararaca*'. Instability at those nodes leads to different hypotheses for the relationship among the groups of species (see further discussion on congruence). Although the *incertae sedis Bothrops lojanus* appeared to be related to '*alternatus*', too few characters for this taxon were included in the analyses to consider its position sufficiently resolved. *Bothrops venezuelensis* was the sister taxon of *B. lojanus* in some instances, although the species may actually be more closely related to taxa topologically distant from *B. lojanus* and '*alternatus*' (e.g. '*atrox*'). Thus, we conclude that *B. lojanus* and *B. venezuelensis* remain *incertae sedis*. *Bothrops sanctaecrucis* was related to '*taeniata*' in most of the results, and this relation was supported by moderate to high jackknife values and synapomorphies of both external and internal morphologies. However, these

synapomorphies were homoplastic (i.e. the features were independently present in other bothropoid taxa). Specimens of *B. sanctaecrucis* show phenotypic similarities with '*jararacussu*' (W. Wüster personal communication; P.A. Carrasco personal observation), which was supported by some results obtained in the AMEE. Therefore, we consider that determining which taxa are most closely related to *B. sanctaecrucis* will need to be re-evaluated through the analysis of additional data for this species, particularly molecular data.

Regarding group support, we observed that the highest jackknife values were obtained in the AMLE and, when taxa without molecular data were excluded, in the ATE. In the AMEE, jackknife values decreased (except for some terminal clades that were supported by jackknifes' values >70 in the totality of the analyses, e.g., the clade conformed by *Bothrops alternatus*, *B. jonathani*, *B. fonsecai* and *B. cotiara*). In the present study, we considered jackknife values as an additional measure to use in data exploration, e.g., the distribution of homoplasy (Chen *et al.* 2003; Jenner 2004). We observed, through mapping morphological characters in the topologies obtained in the AMEE, that several reversions occurred and that some clades were mostly supported by homoplastic synapomorphies, which may have had a 'negative' impact on jackknife values in the AMEE. However, we do not consider that those low values make the morphology of bothropoids less reliable for phylogenetic reconstruction (for discussions on measures of support, see Grant & Kluge 2003; Giribet 2003; Ramírez 2005; Egan 2006; Grant & Kluge 2008; Freudenstein & Davis 2010). We also consider that homoplasy may be useful for evolutionary interpretations and diagnoses (de Carvalho 1996; Assis 2009). On the other hand, some apparently well-supported clades obtained in the AMLE and the ATE may reflect strong convergent base-compositional similarities among some taxa, rather than strong historical signal (Naylor & Brown 1998). It has been reported in previous studies that non-randomly distributed homoplasy occurs in DNA sequences, and that taxa that share a strong compositional bias in a gene may be erroneously grouped in a tree, independently of the method employed for phylogenetic inference (e.g. maximum parsimony or model-based methods) (Chen *et al.* 2003 and references therein). In this study, we do not discard the possibility of that artifact, particularly because results showed discrepancies among different sources of evidence.

Congruence among different sources of evidence and alternative phylogenetic hypothesis for South American bothropoids

The comparison of topologies revealed not only that morphological and molecular data are mostly congruent regarding terminal groups (although some within-group relationships varied among the analyses), but also that conflict exists at most internal nodes of the bothropoid clade. The results of the AMEE showed most bothropoids included in two major sister groups: one group including '*alternatus*', '*neuwiedi*' and the species *incertae sedis*, and the other group including '*jararaca*', '*jararacussu*', '*atrox*' and '*taeniata*'. The AMLE showed a hierarchical radiation, apparently due to the recovery of a clade composed of '*neuwiedi*' and '*jararaca*'. This is a resolution drastically different from that obtained in the AMEE where '*neuwiedi*' and '*jararaca*' were not related but rather separated by eight nodes. Another evidence of this conflict is the result of the ILD test, which showed significant incongruence between the morphological–ecological and molecular matrices. Conflicting hypotheses from morphological and molecular data are common in systematic studies (see Pisani *et al.* 2007 and references therein), and several authors have demonstrated a synergistic effect in some cases where both types of data are analysed in combination (Assis 2009 and references therein). However, in the present study, the results of the ATE showed identical resolution between groups of species as the AMLE, which coincides with the results of Fenwick *et al.* (2009). Chen *et al.* (2003: 263) referred to the possibility that the contribution of different datasets be disproportionate in simultaneous analyses and illustrated this with a case for teleostean phylogeny. The simultaneous analysis of data from two nuclear genes, two mitochondrial ribosomal genes and a protein-coding gene (rhodopsin) showed a topology similar to the one obtained by rhodopsin alone. The authors attributed the high GC content in rhodopsin to a phylogenetic bias, which may have been eclipsing the simultaneous analysis. Such results beg the question: Is molecular data obscuring or 'swamping' (*sensu* Kitching *et al.* 1998: 160) some phylogenetic signal from morphology in analyses of total evidence of bothropoids? Even if this were the case, morphological data are only partially incongruent with molecular data. Fenwick *et al.* (2009: 620) stated that they found no supported incongruence among different gene trees. However, in the present study, the ILD test showed significant incongruence between ribosomal and protein-coding genes partitions; yet, it revealed congruence between the morphological–ecological and ribosomal genes partitions. This was confirmed with a posteriori analysis that excluded the cyt-*b* and NADH4 genes (Fig. 5). The topology obtained was similar to that obtained in the AMEE in that it retrieved the same major sister groups; nonetheless, some synergistic effect was observed (e.g. the position of '*jararacussu*' and '*atrox*' in this topology in contrast with the results of the AMEE and AMLE), and some novel relationships were

recovered (e.g. for *B. itapetiningae* and *B. venezuelensis*). These results agree with findings reported by Werman (1992), who obtained similar sister groups based on characters from external morphology, cranial osteology, isozymes and allozymes of 11 bothropoid species. Interestingly, each of these sister groups includes species that are cohesive, both morphologically and ecologically. While the species of one group (e.g. '*alternatus*' and '*neuwiedi*') are terrestrial and most of them inhabit open and xerophilous areas, the species of the other group (e.g. '*jararaca*', '*taeniata*' and '*atrox*') inhabit forests, and several of these species are semiarboreal or arboreal (Martins *et al.* 2002; Campbell & Lamar 2004). Many would argue that this resolution may be the result of convergence (homoplasy) owing to similar selective pressures; however, as Szucsich & Wirkner (2007: 283) mentioned, shared extrinsic causes (selective pressures) are not 'sufficient to refute homology hypotheses, because a common origin logically implies selective pressure and shared developmental constraints during origination', and '[t]he necessary condition for a homoplasy hypothesis is the multiple origination of a pattern'. As demonstrated in the present study, the main incongruence between the information from morphology–ecology–ribosomal genes and cyt-*b*-NADH4 genes is that, when the latter genes are included in the analyses, '*neuwiedi*' and '*jararaca*' form a distinct clade (Wüster *et al.* 2002; Fenwick *et al.* 2009; the present study). Thus, a hierarchical radiation is obtained for bothropoids, where '*alternatus*' becomes a basal group. In this topology, we found that all synapomorphies of ('*jararaca*' + '*neuwiedi*') were homoplastic (in agreement with Fenwick *et al.* 2009, who found no unique phenotypic synapomorphies for the clade). Furthermore, we found that most synapomorphies of '*alternatus*' (e.g. number of palatine teeth) also resulted homoplastic as the features were also present in '*neuwiedi*'.

We consider the robustness of the alternative hypothesis (two major lineages within bothropoids) based on its stability to variation in phylogenetic inference procedures (Giribet 2003), and a critical analysis of the overall congruence of the evidence (Assis 2009). Unlike the remaining analyses, the ATE that excluded the cyt-*b*-NADH4 partition recovered the same optimal topology (Fig. 5) under the different parameters employed (EW and the complete range of *k* values used in IW). In addition, this hypothesis is supported by most of the available evidence (including the most character-comprehensive morphological dataset of bothropoids use to date) and the congruence among different type of data analysed (morphology, ecology and DNA sequences). The main sister groups within bothropoids were weakly supported by jackknife values, but we do not consider group support values to be indicators of the accuracy of a phylogenetic hypothesis (Chen *et al.* 2003; Egan 2006 and references therein). Giribet (2003) provided examples where low nodal support values were related to highly stable clades, the scenario shown in the present study. Furthermore, this hypothesis is consistent with that proposed by Werman (1992), which provides an additional source of supporting evidence: isozymes and allozymes.

We acknowledge that excluding data (cyt-*b* and NADH4 sequences) from the analysis is contradictory to the fundamentals of total evidence (Eernisse & Kluge 1993; Kluge 1998), and we are not encouraging this kind of approach nor disregarding the phylogenetic information contained in those genes. However, the exclusion of this data, based upon the previously discussed criteria, resulted useful in revealing which nodes were in conflict. Consequently, this conflict affects taxonomic decisions and hypothesis of diversification processes within bothropoids.

We recommend testing these alternative phylogenetic hypotheses through the evaluation of the molecular data supporting ('*jararaca*' + '*neuwiedi*') and/or inclusion of additional data in combined analyses (e.g. new morphological and molecular data, information of venom traits).

Taxonomy of South American bothropoids

The proposal of Wüster *et al.* (2002) to recognise South American bothropoids as a single genus *Bothrops* is consistent with the monophyly of the group, and is supported by more than one source of evidence and by several studies, including the present work (Table 4). Fenwick *et al.* (2009) proposed an alternative taxonomic rearrangement, splitting *Bothrops* in five genera; yet, we found this classification to be unstable (Table 4) and some aspects of this rearrangement, questionable. The taxonomic decisions made by Fenwick *et al.* (2009) were based on the result of a combined evidence analysis that excluded taxa without molecular data (Fig. 4; Fenwick *et al.* 2009: fig. 1). However, their analyses, as well as ours, demonstrate that including these taxa affects the resulting topologies at certain nodes and may lead to paraphyly of genera; e.g., including *B. andianus* may result in *Bothrops* being paraphyletic with respect to *Bothrocophias* (Fig 1, 3, 5; Fenwick *et al.*: fig. S7), including *Bothrops mattogrossensis* may result in *Bothropoides* being paraphyletic with respect to *Rhinocerophis* (Fenwick *et al.*: figs S4 and S9), including *Bothrops sanctaecrucis* may result in *Bothrops* being paraphyletic with respect to *Bothriopsis* (Figs 1, 3 and 5) or *Bothropoides* (Fenwick *et al.*: fig. 2), and including *Bothrops venezuelensis* may result in *Bothrops* being paraphyletic with respect to *Bothrocophias* (Fenwick *et al.*: fig. 2) or *Rhinocerophis* (Fig. 3). These results show that some phylogenetic information is missing when taxa without molecular data are excluded from the analysis, and that the generic assign-

Table 4 South American bothropoids and genera proposed by Fenwick *et al*. (2009) recovered as monophyletic (×) or non-monophyletic (–) in the different analyses of the present study

Analysis	*South American bothropoids*	*Bothrocophias*	*Rhinocerophis*	*Bothropoides*	*Bothrops (s.s.)*	*Bothriopsis*
AMEE, EW	X	X	–	–	–	X
AMEE, IW (*k* = 3–10)	X	X	–	–	–	X
AMLE, EW	X	X	X	X	X	X
AMLE, IW (*k* = 8–15)	X	X	X	X	X	X
ATE, EW	X	X	X	X	–	X
ATE, IW (*k* = 8–15)	X	X	X	X	–	X
Analysis excluding taxa without molecular data, EW	X	X	X	X	X	X
Analysis excluding taxa without molecular data, IW (*k* = 8–15)	X	X	X	X	X	X
Analysis excluding characters from cyt-*b* and NADH4 genes, EW	X	X	–	–	–	X
Analysis excluding characters from cyt-*b* and NADH4 genes, IW (*k* = 8–15)	X	X	–	–	–	X

ment of some of these species made by Fenwick *et al.* (2009) is somewhat arbitrary. These authors argued that their generic rearrangement 'recognises evolutionarily, ecologically and morphologically distinct lineages' (619), but this is not entirely correct. In their rearrangment, ('*jararaca*' + '*neuwiedi*') was assigned to the new genus *Bothropoides*, based on 38 molecular characters. The '*neuwiedi*' and '*jararaca*' groups are greatly different phenotypically, a fact already remarked by Martins *et al.* (2002: 308). Furthermore, both groups also differ in ecological features: the species of '*jararaca*' are mostly semiarboreal and inhabit Atlantic forests, whereas the species of '*neuwiedi*' are terrestrial, most of them inhabit open areas, and one of them (*B. neuwiedi*) presents the same apomorphic state for diet (mammal specialist) as some species of '*alternatus*' (*Bothrops alternatus*, *B. cotiara*, *B. fonsecai*; Martins *et al.* 2002). Hence, *Bothropoides* appears only supported by molecular evidence, whereas morphology and ecology do not warrant the recognition of the genus.

Considering the medical importance of bothropoids (see Salomão *et al.* 1997), we believe that splitting *Bothrops* into genera actually may be premature, based on the above-described unresolved issues: the relationship of '*neuwiedi*' and '*jararaca*' with other groups, and the systematic affinities of some species (e.g. *Bothrops lojanus*, *B. venezuelensis*, *B. sanctaecrucis*, *Bothrops muriciensis*, *B. punctatus*, *B. medusa* and *B. osbornei*). Therefore, based on the results of the present study, we propose resolving the paraphyly of *Bothrops* with the following taxonomic rearrangement.

Maintaining Bothrocophias Gutberlet & Campbell, 2001, *assigning Bothrops andianus to this genus.* We found sufficient evidence (Fenwick *et al.* 2009; the present study) to conclude that *B. andianus* is related to the *Bothrocophias* group ('*microphthalmus*'), and thereby propose maintaining *Bothrocophias*, including *andianus* in this genus. While *Bothrocophias andianus* does not exhibit the white spots in infralabial and gular scales diagnosed by Gutberlet & Campbell (2001) as a synapomorphy of the genus, they do share a similar pattern of dorsal blotches (compare Plate 496 with Plates 475–76 in Campbell & Lamar 2004). Another feature recognised by Gutberlet & Campbell (2001) as a synapomorphy of *Bothrocophias* is the presence of smooth intrasupraocular scales. Most specimens of *B. andianus* examined had slightly keeled intrasupraoculars, but smooth intrasupraoculars were also observed. During examinations at museum collections, we found some specimens of *B. andianus* mistakenly catalogued as *B. microphthalmus* (which reflects their phenotypic similarity) or as *Bothriopsis oligolepis*. In the latter case, the specimens of *Bothrocophias andianus* presented greenish coloration, a feature already reported by Harvey *et al.* (2005) for some specimens of *B. andianus* from Bolivia. Lucindo Gonzales (personal communication) noticed that populations of *B. andianus* from Peru and Bolivia may show other phenotypic differences. The presence of greenish coloration in *B. andianus* also reveals that this feature is not exclusive of '*taeniata*' (=*Bothriopsis*), as was proposed by Fenwick *et al.* (2009). Specimens of three species of *Bothrocophias* (*B. myersi*, *B. campbelli*, and *B. colombianus*) were not available for the present study, and our results would justify the recognition of the bothropoid clade as a single monophyletic genus (*Bothrops*), as recommended by Wüster *et al.* (2002). We prefer to maintain *Bothrocophias*, given that the clade [*B. andianus* (*B. microphthalmus* + *B. hyoprora*)]

was the sister group of the rest of bothropoids in the totality of the results and recommend further evaluation of assigning *B. andianus* to the genus through the analysis of additional data for this taxon (particularly DNA sequences), and/or through the analysis of the relationship between *B. andianus* and the three species of the genus that were not included in the present study. The clade [*B. andianus* (*B. microphthalmus* + *B. hyoprora*)] is supported by seven synapomorphies, two of them non-homoplastic: quadrangular rostral scale (ch. 53), and tuberculate keels in mid-posterior dorsal scales (ch. 60) (the latter character is polymorphic in *B. andianus*). Content of *Bothrocophias*: *B. hyoprora*, *B. microphthalmus*, *B. myersi*, *B. campbelli*, *B. colombianus*, *B. andianus*.

Recognising the remaining bothropoid taxa as Bothrops Wagler, 1824, synonymising Bothriopsis Peters, 1861, Rhinocerophis Garman, 1881, and Bothropoides Fenwick , Gutberlet, Evans & Parkinson, 2009. Following the recommendation of Salomão *et al.* (1997) and Wüster *et al.* (2002) of synonymising *Bothriopsis* with *Bothrops*, and synonymising the additional genera proposed by Fenwick *et al.* (2009), *Bothrops* shows to be a monophyletic genus supported by five synapomorphies, four of them non-homoplastic: interorbital space/maximum head width (ch. 18), minimum width between frontals/length of frontal (ch. 23), internasals in contact (ch. 51) and quadrangular shaped parietal (ch. 86). Content of *Bothrops*: *B. alcatraz*, *B. alternatus*, *B. ammodytoides*, *B. asper*, *B. atrox*, *B. barnetti*, *B. bilineata*, *B. brazili*, *B. caribbaeus*, *B. chloromelas*, *B. cotiara*, *B. diporus*, *B. erythromelas*, *B. fonsecai*, *B. insularis*, *B. itapetiningae*, *B. jararaca*, *B. jararacussu*, *B. jonathani*, *B. lanceolatus*, *B. leucurus*, *B. lojanus*, *B. lutzi*, *B. marajoensis*, *B. marmoratus*, *B. mattogrossensis*, *B. medusa*, *B. moojeni*, *B. muriciensis*, *B. neuwiedi*, *B. oligolepis*, *B. osbornei*, *B. pauloensis*, *B. pictus*, *B. pirajai*, *B. pubescens*, *B. pulchra*, *B. punctatus*, *B. roedingeri*, *B. sanctaecrucis*, *B. taeniata*, *B. venezuelensis*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Material examined.

Appendix S2. Morphological and ecological characters.

Appendix S3. GenBank accession numbers for each taxon used in the analysis.

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