

Combining mitochondrial DNA sequences and morphological data to infer species boundaries: phylogeography of lanceheaded pitvipers in the Brazilian Atlantic forest, and the status of *Bothrops pradoi* (Squamata: Serpentes: Viperidae)

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Abstract

Phylogeographic studies using mitochondrial DNA sequence information are frequently used as the principal source of evidence to infer species boundaries. However, a critical analysis of further evidence is essential to test whether different haplotype clades identify different species. We demonstrate a hypothesis-testing approach, using a combination of phylogeographic methods, multivariate morphometrics and matrix association tests, to investigate species boundaries in eastern Brazilian pitvipers conventionally assigned to the species *Bothrops leucurus* and *B. pradoi*. Two basal haplotype clades with partly overlapping geographical distributions are identified, which could either represent two partly sympatric species, or multiple haplotypes within one organismal lineage. We use partial Mantel matrix association tests to verify whether generalized morphology, or any of four supposedly diagnostic characters for the two species, show any association with mtDNA variation. Negative results lead to the conclusion that the haplotype clades do not denote independently evolving organismal lineages, and do not constitute separate species under any criterion.

Introduction

Species are the basic units of biodiversity. Since much of evolutionary biology focuses on the process of speciation, understanding species limits is clearly a fundamental requirement. In addition, an accurate estimate of species limits is a key factor in improving the accuracy and validity of biodiversity assessment. This is of particular importance in areas of high conservation interest, such as zones of endemism. The Atlantic forests of north-eastern

Brazil constitute a particularly clear example of such a region, with high levels of endemism, and the presence of many clades of organisms highly differentiated from their Amazonian relatives (e.g. da Silva & Patton, 1998). At the same time, these forests are of great conservation concern, as only 2–8% of the original forest cover now remains (Tabarelli *et al.*, 1999).

In recent years, mitochondrial DNA has become the most widely used molecular marker in animal systematics, particularly at low taxonomic levels, due to its ease of isolation and interpretation (Avise *et al.*, 1987). Furthermore, due to its smaller effective population size, mtDNA will show lineage coalescence more rapidly than a nuclear marker; thus, a resolved mtDNA gene phylogeny is more likely to represent organismal phylogeny than a tree based on any specific nuclear sequence (Moore, 1995).

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Mitochondrial DNA sequences have been used particularly intensively for phylogeographic studies, in which the distribution of mtDNA haplotype clades across the range of a species or species complex is used to infer the history of that distribution. Phylogeographic studies across several unrelated taxa can reveal concordant phylogeographic patterns, and thus identify areas that were important in the genetic differentiation of sets of populations, and which may thus merit special conservation attention (e.g. da Silva & Patton, 1998).

Additionally, phylogeographic studies have often been used to infer species boundaries in species complexes (e.g. Zamudio & Greene, 1997; da Silva & Patton, 1998; Rodríguez-Robles & De Jesús-Escobar, 2000; Slowinski & Wüster, 2000). In general, the conclusion of more than one species being present was drawn from the presence of allopatric, or in some cases sympatric mtDNA haplotype clades, combined with the presence of morphological differences. However, the relationship between mtDNA and morphological variation was not always fully explored. Often, morphological data for the various populations were taken from the literature as a *post hoc* justification for the definition of species, but without using them critically to test species boundaries established on the basis of mtDNA sequence variation. Moreover, the morphological data were often pooled observations for the entire range of the putative species, thus eliminating any possibility of assessing intraspecific variation. Furthermore, the specimens used for the morphological study were not usually those providing the mtDNA samples, so that a direct link between mtDNA and morphological variation cannot be established.

Sites & Crandall (1997) emphasized the importance of viewing the diagnosis of species boundaries as a hypothesis-testing procedure, with clear statements of the criteria used. This is complicated by the fact that different species concepts use different criteria for the recognition of sets of populations as distinct species. de Queiroz (1998) emphasized the distinction between concepts of the nature of species, and the criteria used to diagnose them. He noted furthermore that all species concepts are variations on the single theme of species as evolutionary lineages, that different criteria provide information on different phenomena associated with the separation of lineages, and that different researchers emphasize different criteria, based on their research priorities. Consequently, instead of relying on one criterion as the single pivotal factor, it would be more useful to test the observed data against the predictions of all the various criteria used, to determine if they fulfil any or all of them.

The various criteria for the diagnosis of species were summarized by de Queiroz (1998). Each makes requirements and predictions about patterns of variation to be observed in morphology, mtDNA or other genetic markers, or both (terminology according to de Queiroz, 1998): (i) the isolation and recognition criteria (Mayr, 1963; Paterson, 1985) require a reproductive discontinuity

between species, i.e. a lack of intergradation; in terms of phenotype, this leads to the prediction that species should be separated by a clear disjunction, at least in the characters used to diagnose them; (ii) the niche criterion (Van Valen, 1976) relies on the occupancy of a different adaptive zone as the basis for recognizing sets of populations as different species; (iii) the phenetic cluster criterion (Sokal & Sneath, 1963), in which species are minimally distinguishable as separate clusters in multivariate analyses; (iv) the diagnosability criterion, according to which species are diagnosed on the basis of 'unique combinations of primitive and derived characters' (phylogenetic species concept *sensu* Cracraft, 1989); to qualify as separate species, sets of populations must show fixed differences in some character; (v) the apomorphy criterion (e.g. Baum, 1992), which, operationally, represents a more stringent version of the diagnosability criterion, in which populations constituting a species must share a unique derived character state not possessed by other such groups; (vi) the concordant coalescence criterion (Baum & Shaw, 1995), in which species are characterized by concordant coalescence of gene genealogies, i.e. all gene trees show the alleles or haplotypes present within species to form clades; Moritz's (1994) 'Evolutionarily Significant Units' (ESUs) are conceptually related to this notion, and are based principally on the presence of monophyletic mtDNA haplotype clades within units; although Moritz did not regard his ESUs as synonymous with species, these criteria were used for diagnosing species by da Silva & Patton (1998).

Using mtDNA sequence variation alone to infer species boundaries carries several potential pitfalls. First, due to the matrilineal, nonrecombining mode of inheritance of mtDNA, this molecule always displays a phylogenetic pattern of transmission, even within populations, where patterns of organismal ancestor–descendant relationships are tokogenetic rather than phylogenetic (Davis, 1996). Consequently, the existence of multiple haplotype clades does not necessarily imply the existence of multiple organismal lineages (e.g. pattern V of Avise *et al.*, 1987). A similar pattern can also arise due to introgressive hybridization (e.g. Echelle & Echelle, 1994). Furthermore, in some cases, a strong geographical pattern in mtDNA haplotype distribution may mask the fact that the populations involved are in fact part of one and the same lineage (e.g. Palumbi & Baker, 1994; Thorpe *et al.*, 1996), with extensive male-mediated gene flow between them.

The consequence of this is that the existence of multiple mtDNA haplotype clades alone is neither sufficient nor necessary for the recognition of species under any criterion. Multiple haplotype clades within a locality may either represent the existence of sympatric species, or may co-occur within one lineage. While one might expect the existence of sympatric species to be immediately obvious, this is not necessarily the case, due to the potential existence of morphologically identical, 'cryptic'

species. However, although genetic studies often discover such 'cryptic' species, the basis for the assumption of morphological identity is rarely stated, and often simply refers to the absence of diagnostic, discrete character states. However, studies employing more appropriate methods, in particular multivariate morphometrics, have generally found such 'cryptic' species to be identifiable by morphology alone (e.g. Masters & Bragg, 2000), at least in vertebrates. Consequently, a critical comparison of morphological and mtDNA variation is required to determine whether distinct haplotype clades denote separately evolving lineages, or represent gene lineages evolving within a single organismal lineage.

For studies of geographical variation in morphology, multivariate methods are particularly useful, as they can summarize information on variation in a number of characters simultaneously, and can thus reveal subtle patterns of variation in the general phenotype. Furthermore, ordination techniques that do not require specimens to be grouped prior to analysis, such as principal components analysis, allow patterns of variation to be elucidated without the bias of *a priori* allocation of specimens to specific taxonomic units. These methods have been used extensively in snake systematics (e.g. Lenk & Wüster, 1999).

In order to relate morphological variation to mtDNA variation, it may be necessary to exclude other intercorrelated associations, such as sex or geographical variation unrelated to the mtDNA affinities of the animals. In recent years, partial matrix correspondence (Mantel) tests have become the method of choice for this (Thorpe *et al.*, 1994; Daltry *et al.*, 1996), as they allow observed patterns of variation (e.g. in morphological characters) to be tested simultaneously for significant association with hypothesized causes, such as mtDNA affinities and environmental variables, without the confounding effects of intercorrelation between alternative hypotheses.

In this paper, we address the question of species limits in the pitvipers of the *Bothrops atrox* Linnaeus, 1758 complex in the Atlantic forests of Brazil. The *B. atrox* complex consists of a series of populations of pitvipers widespread throughout the tropical parts of South America east of the Andes. The complex has been divided into between five and seven species, depending on author, but these are poorly defined, and the status of many is questionable (Wüster *et al.*, 1999). The populations from the Atlantic coastal forests of Brazil have a particularly confused history (partially summarized by Hoogmoed & Gruber, 1983). Until 1947, these populations were considered to be conspecific with *Bothrops atrox*, the common lancehead of Amazonia and the Guyanas (e.g. Boulenger, 1896). Hoge (1947) described a population from Pau Gigante (now Ibiracú), in southern Espírito Santo State, as *Trimeresurus pradoi*, but without discussing the status of populations from further north along the coast. These were assigned to *Bothrops megaera* Wagler, 1824 by Hoge (1965), and then to *B. leucurus*

Wagler, 1824 by Hoge & Romano (1972). In both cases, no justification for separating these populations from either *B. atrox* or *B. pradoi* was given. Doubts about the status of *B. leucurus* and *B. pradoi* have surfaced repeatedly. Campbell & Lamar (1989) suggested that they might be conspecific. Ripa (1997) stated that *B. leucurus* and *B. pradoi* were found sympatrically around 'São Pauline' (correct spelling: São Paulinho), in southern Bahia State, but also noted interbreeding between them in captivity, as well as the role of ontogeny and sexual dimorphism in determining the presence or absence of supposedly diagnostic characters.

The aim of this paper is to use a novel approach, consisting of a combination of mtDNA phylogeography, multivariate morphometric methods and numerical hypothesis-testing techniques, to provide a more detailed study of the population systematics of the *Bothrops atrox* species complex along the Atlantic seaboard of Brazil. The observed pattern of variation will be tested against different species criteria to determine whether these populations constitute one or more species.

Materials and methods

Multivariate morphometrics

We recorded 68 morphological characters from 104 preserved specimens, spanning the range of the complex from southern Espírito Santo State north to Alagoas State. All specimens were examined by the same observer (G.P.) to avoid observer bias (Lee, 1990).

In order to describe the position or size of characters along the body, the ventral scales were numbered according to Dowling (1951). The position of a character along the body was scored as the number of the ventral scale opposite which it was situated. In order to compensate for different ventral scale counts, this was converted to percentage ventral scale count (%VS). Similarly, the position of scale reductions along the tail was recorded as percentage subcaudal scale count (%CS), and the height or position of characters across the longitudinal axis of the body as percentage dorsal scale row count (%DS).

We used two-way analysis of variance (BMDP 2V; Dixon, 1991) to test for the presence of significant among-locality variation in each character. For this purpose, the specimens were grouped by locality and sex. Characters found to display significant among-locality variation are listed in Table 1. Characters that do not show significant geographical variation were not considered further. The remaining characters were a mixture of discrete, meristic and continuous variables. However, none of the continuous variables was a linear measurement. As a result, size did not affect the analysis.

Patterns of geographical variation were investigated by means of principal components analysis (PCA), run on a between-character covariance matrix, using a program

Table 1 Characters used and their eigenvector coefficients in relation to the first and second principal components of the male and female PCAs.

	PC1 (males)	PC2 (males)	PC1 (females)	PC2 (females)
1. %CS position of reduction from 13 to 12 rows	0.315	-0.133	0.223	0.184
2. %CS pos. of red. from 12 to 11 rows	0.298	-0.125	0.316	0.176
3. %CS pos. of red. from 11 to 10 rows	0.302	-0.262	0.349	0.135
4. %CS pos. of red. from 10 to 9 rows	0.347	-0.132	0.337	0.206
5. %CS pos. of red. from 9 to 8 rows	0.36	-0.103	0.343	0.138
6. Number of infralabials	0.186	0.235	0.11	0.411
7. Number of scales contacting 3rd to last supralabial	0.145	-0.22	0.12	0.027
8. Black dot surrounding nostril (0 = absent, 1 = faint, 2 = conspicuous)	0.052	0.407	0.077	-0.099
9. %VS width of 3 half bands at 50% VS length at 2nd paravertebral scale row	0.211	-0.036	0.181	-0.449
10. Maximum percentage VS width of 3 half bands at 50% VS length	0.29	-0.061	0.188	-0.459
11. %DS lowest scale row encroached on by 3 half bands at 50% VS length	-0.189	-0.442	-0.269	0.085
12. %DS separation between lower and upper parts of half band	-0.179	-0.285	-0.221	-0.171
13. %VS width of lower parts of half band	0.276	0.254	0.311	-0.038
14. %VS width of inner part of half bands	0.29	-0.138	0.208	-0.433
15. %VS position of posterior end of first half-band	0.031	-0.254	0	-0.075
16. %DS height of highest scale row involved in secondary markings	0.003	0.248	-0.059	-0.11
17. %VS position of anterior thyroid edge	-0.186	-0.224	-0.274	0.11
18. %VS position of anterior liver tip	-0.143	-0.244	-0.238	0.097

written by R. G. Davies and extensively modified by R. S. Thorpe. Since the species of the *Bothrops atrox* group show significant sexual dimorphism in many characters, the data from male and female specimens were analysed separately in parallel analyses. All characters were standardized to zero mean and unit standard deviation. Two of the specimens from which DNA sequence information was available had truncated tail tips, as a result of which characters relating to the scale row reductions along the tail could not be standardized. In order to relate mtDNA variation to morphological variations, the PCAs were re-run, excluding characters 1–5 (Table 1).

Molecular methods

Tissue (liver) or blood samples were collected from 18 specimens of *Bothrops leucurus* and *B. pradoi*, and additionally from three specimens of *Bothrops atrox* originating from Suriname and Acre State, Brazil. Tissue samples were stored refrigerated in absolute alcohol. Blood samples (0.1–0.3 mL) were taken from the caudal vein with a hypodermic syringe, collected initially into a solution of 0.5 M EDTA, and then diluted to a final concentration of 0.1 M EDTA, 0.1 M Tris, and 2% SDS, which was then stored refrigerated. Sequences were submitted to GenBank, and are available under the accession numbers AF246267–246286. Detailed information on specimens examined for both the morphological and the molecular parts of this study can be found at: <http://www.blackwell-science.com/products/journals/suppmat/JEB/JEB313/JEB313sm.htm>

Total DNA was extracted from blood and tissues by standard methods (Hillis *et al.*, 1996). Two regions of the

mitochondrial DNA molecule were amplified using the polymerase chain reaction (PCR): a 767-base-pair (bp) section of the gene for cytochrome *b* (*cytb*), and a 900-bp region of the gene for NADH dehydrogenase subunit 4 (ND4) and adjoining tRNAs. Details of primers, PCR and sequencing protocols are given in Pook *et al.* (2000).

Sequences were aligned by eye. Unadjusted pairwise sequence divergences were calculated using the Program MEGA (Kumar *et al.*, 1993).

We used both maximum parsimony (MP) and maximum likelihood (ML) methods to infer the haplotype phylogeny in these populations. Parsimony analysis (exhaustive search) was implemented using the program PAUP*4b6 (Swofford, 2001). No weighting was used, since the low levels of observed sequence divergence suggest that saturation of transitions or third codon positions is exceedingly unlikely to have occurred. In the absence of evidence of saturation, transition positions are likely to be no less informative than transversions. The sequence data were assayed for the presence of a significant phylogenetic signal by means of the *g*1 tree skewness statistic (Hillis & Huelsenbeck, 1992). The robustness of the results was assessed by means of bootstrap analysis (Felsenstein, 1985), using 1000 pseudo-replicates and branch-and-bound searching. Bremer branch support values (Bremer, 1994) were calculated by repeating the exhaustive analysis while retaining successively longer trees, until all nodes were collapsed. To test whether breaking up any particular node in the tree would result in a significant increase in tree length, for each node we constrained the analysis to retain only trees incompatible with that node, using the converse constraint option of PAUP*. The most parsimonious trees compatible with the constraint were tested for significant

differences in tree length against the unconstrained MP tree by means of Wilcoxon signed-ranks tests (Templeton, 1983).

In order to determine the best model of sequence evolution for maximum likelihood (ML) analysis, we used the program Modeltest 3.0 (Posada & Crandall, 1998). We used the parameters estimated by Modeltest in a branch-and-bound search in PAUP*. We then re-estimated the parameters from the resulting tree, and ran a further branch-and-bound search using these settings. Bootstrap analysis was run, using 500 pseudoreplicates, heuristic search, tree bisection-reconnection (TBR) branch swapping, and a NJ starting tree. We tested whether breaking up any particular node would lead to a significant decrease in the likelihood score of the constrained tree by comparing constrained ML trees to the unconstrained tree by means of the Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999).

Since this work compared recently diverged haplotypes, and some of these may thus be the direct ancestors of others, we also constructed a minimum spanning network for the Brazilian east coast haplotypes, using the program Arlequin (Schneider *et al.*, 2000).

Numerical hypothesis-testing

In order to test whether morphological and mtDNA variation were associated among specimens, we used both pairwise and simultaneous partial matrix correspondence (Mantel) tests (Thorpe *et al.*, 1994, 1996; Daltry *et al.*, 1996). Pairwise tests can be used to reveal the significance of the correlation between matrices representing observed patterns and individual matrices representing hypothesized causes or correlates of the observed pattern. With simultaneous tests, one can test for the association between observed data and the pattern predicted for multiple causal hypotheses, free of the effect of intercorrelation between the predicted patterns. In this case, the aim was to test for the significance of association between morphological variation and mtDNA sequence variation among individual specimens, while eliminating the confounding effects of geographical distance and sexual dimorphism. We used the morphological data collected from 15 specimens with known mtDNA sequences, and constructed between-specimen Euclidean distance matrices to summarize patterns of generalized morphological differentiation. For this purpose, all characters were transformed to zero mean and unit standard deviation. Characters 1–5 (tail scale row reductions – Table 1) were not included, as several of the specimens with DNA data had incomplete tails. The resulting matrix of observed distances was then regressed against distance matrices constructed to represent the following potential causal hypotheses for the observed pattern: (i) patristic distance along each of the equally most parsimonious trees constructed from the analysis of the 15 DNA sequences; (ii) sex of each

specimen; (iii) geographical distance between localities of origin. This is based on the prediction that if two species are present, these should be characterized by possession of different mtDNA clades, and specimens belonging to either species should show patterns of morphological variation congruent with species identity as revealed by mtDNA sequence variation.

In addition, we also constructed matrices of observed between-specimen differences for individual characters stated to be diagnostic for differentiating between *B. pradoi* and *B. leucurus*, namely the presence/absence of a black blotch surrounding the nostril, supralabial pigmentation, infralabial pigmentation, and number of intersupraocular scales. The extent of labial scale pigmentation was scored on a scale from 1 to 5, to represent the percentage of the scales covered by dark pigment, where 1 denotes 0–20% coverage, 2 denotes 21–40%, etc. Matrices representing individual characters were regressed against the matrices representing potential causal hypotheses, as stated in the previous paragraph. All Mantel tests were run using 10 000 randomizations of the matrix of observed distances.

Results

Multivariate morphometrics

The principal components analyses with and without the tail scale row reduction characters gave similar results. The ordination of the individual specimens along the first two principal components, resulting from the two analyses using all characters, is shown in Fig. 1, and the eigenvector coefficients for the individual characters are shown in Table 1. The ordination plots show an indication of geographical variation, with populations from southern Espírito Santo having, on average, higher first principal component scores than more northerly specimens. However, the plots do not show the existence of discrete clusters of individuals.

In order to visualize the pattern of variation in morphology, and relate it to mtDNA variation, we plotted the first principal component scores of individual specimens from the analyses including all specimens with DNA information against their position along a latitudinal transect along the Brazilian coast. The resulting pattern is suggestive of clinal variation in the southern part of the region (northern Espírito Santo and southern Bahia), whereas there appears to be less geographical pattern to variation in the central and northern parts of Bahia and further north (Fig. 2).

mtDNA sequence analysis

A total of 1401 base pairs of mtDNA (693 bp of ND4, 708 bp of *cytb*) were aligned. The 18 specimens of *B. leucurus* and *B. pradoi* revealed eight unique haplotypes. Fifty-six base pair positions were variable, and 20

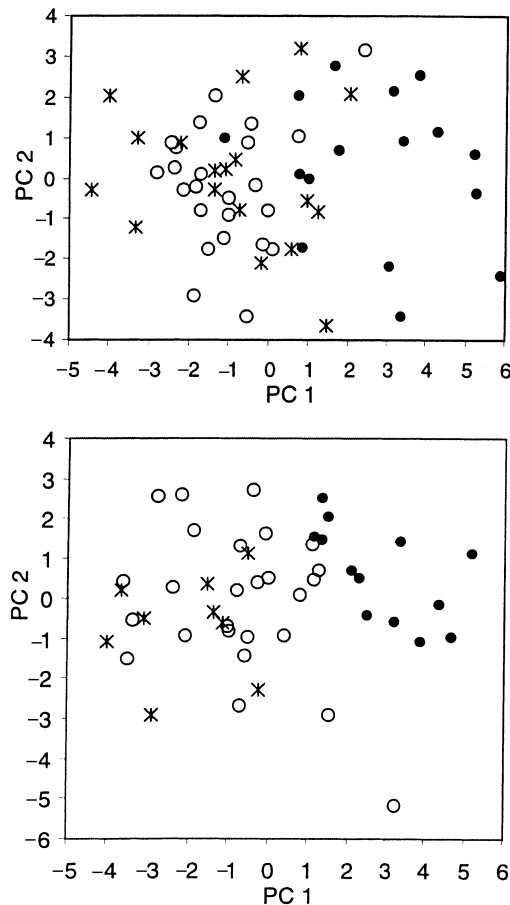


Fig. 1 Ordination of male (top) and female (bottom) specimens along the first two principal components of their respective principal components analyses. In males, the first and second principal components account for 31.3% and 14.4% of the total variance, respectively, and in females 30.6% and 15.4%. For the purposes of illustrating geographical variation, the specimens were grouped into three regions: southern Espírito Santo (south of the Rio Doce) (●); northern Espírito Santo and southern coastal Bahia (○); and central and northern coastal Bahia (from Ilhéus northward), Sergipe and Alagoas (crosses).

informative under the parsimony criterion. Unadjusted pairwise *p*-distances between the ingroup haplotypes were very small (0.0007–0.0087) (Table 2). Distances between the ingroup sequences and the *B. atrox* outgroup sequences ranged from 0.0207 to 0.0245.

Parsimony analysis using the exhaustive search algorithm resulted in two equally most parsimonious trees (Length = 59 steps, CI = 0.9661, HI = 0.0339, RI = 0.9429) (Fig. 3A). The *g*₁ statistic of tree length distribution skewness was -1.374 , which suggests that the database contains significant phylogenetic information ($P < 0.01$) (Hillis & Huelsenbeck, 1992).

In both trees, the Atlantic coast haplotypes are divided into two basal clades: one contains haplotype sequences

from the predominantly southern localities of Domingos Martins, Nova Venécia, São Paulinho, Teixeira de Freitas and Mucuri; the other contains haplotypes from the generally more northern localities of Teixeira de Freitas, Porto Seguro, and Salvador. The only difference between the two trees is that in one, the São Paulinho haplotype represented the sister group to the other northern haplotypes, whereas relationships within the northern clade were entirely unresolved in the other. Bootstrap support for the two main clades is high (83 and 91%, respectively), but Bremer support less so (two for both).

The final ML tree, calculated under the TrN + I model identified as optimal by Modeltest, had a log-likelihood score of $-\ln L = 2228.75612$, and was topologically identical to the strict consensus MP tree (not shown). Bootstrap support values for individual nodes were similar to those from the MP analysis (Fig. 3A).

In the MP analyses, the only node which would lead to a significant increase in tree length when broken up was the monophyly of the *leucurus/pradoi* haplotypes. In the case of the ML trees, a significant decrease in log-likelihood score in Shimodaira Hasegawa tests resulted from breaking up the same clade, and also the clade consisting of the haplotypes TdFreitas/PSeguro1 & 2 and PSeguro. The other nodes, when broken up, did not lead to a statistically significant increase in tree length or decrease in tree likelihood score (Table 3).

Figure 3(B) shows the relationship between geographical position and haplotype relationships as portrayed in a minimum spanning network. The relationship between morphological variation, geography and mtDNA sequence variation is shown in Fig. 2. The two mtDNA clades overlap in geographical distribution around Teixeira de Freitas and São Paulinho, and the zone where they overlap corresponds to specimens of intermediate phenotype. Moreover, specimens with mtDNA haplotypes of the northern and southern clades do not show consistent differences in PC 1 scores.

Numerical hypothesis testing

The pairwise correlation between distance matrices derived from individual characters and potential causal hypotheses was universally low (Table 4A), and only the correlation between supralabial pigmentation and sex was statistically significant. In partial Mantel tests, neither generalized morphology nor any of the supposedly diagnostic characters were associated with any of the two equally most parsimonious trees resulting from our analysis of the mtDNA sequences (Table 4B). Similarly, none showed significant association with geographical distance, and only supralabial pigmentation was significantly associated with sex (before but not after row-wise Bonferroni correction for the number of hypotheses tested) across our limited mixed mtDNA/morphology sample.

Fig. 2 Plot of first principal component scores of individual specimens against latitude, and mtDNA haplotype. Triangles and diamonds indicate the morphology of the actual specimens used for mtDNA analysis against latitude. Note the lack of consistent differentiation of specimens carrying the northern and southern mtDNA haplotype lineages. Two-letter codes indicate localities from which sequence information was available: DM = Domingos Martins; NV = Nova Venécia; MU = Mucuri; TF = Teixeira de Freitas; SP = São Paulinho; PS = Porto Seguro; SAL = Salvador.

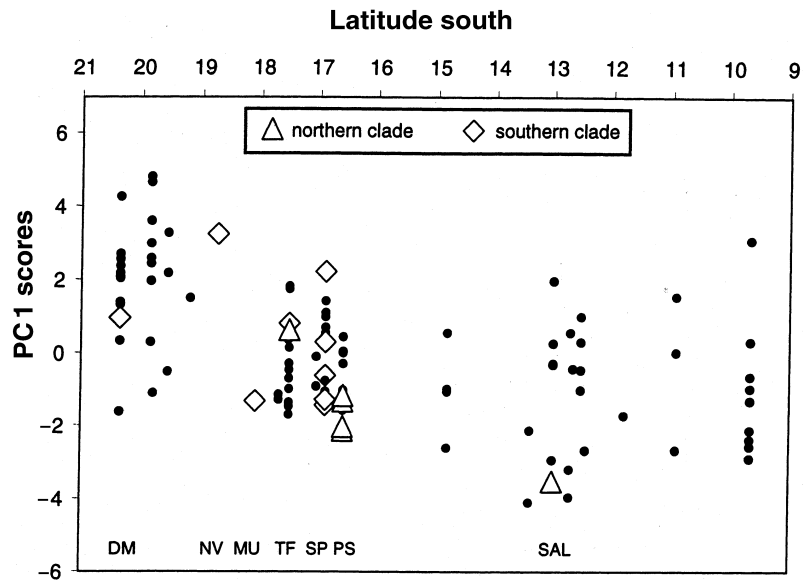


Table 2 Sequence divergence among the haplotypes found in this study. Upper-right matrix: absolute distances (number of substitutions). Lower-left matrix: uncorrected p-distances, expressed as percentage (e.g. 2.21 = p-distance of 0.021).

	Sur	Acre	DMart	NVen	MucTF	TFPS1	TFPS2	SP	PS	Sal
Suriname	–	31	31	32	30	32	34	29	33	29
Acre	2.21	–	33	34	32	32	34	31	33	31
DMartins	2.22	2.36	–	3	1	9	11	2	10	6
NVenecia	2.31	2.45	0.22	–	2	10	12	3	11	7
MucuriTFreitas	2.14	2.28	0.07	0.14	–	8	10	1	9	5
TFreitasPSeguro1	2.29	2.28	0.64	0.72	0.57	–	2	9	1	3
TFreitasPSeguro1	2.43	2.43	0.79	0.86	0.71	0.14	–	11	3	5
SPaulinho	2.07	2.21	0.14	0.22	0.07	0.64	0.78	–	10	6
PSeguro	2.36	2.37	0.72	0.79	0.64	0.07	0.21	0.71	–	4
Salvador	2.07	2.21	0.43	0.50	0.36	0.21	0.36	0.43	0.29	–

Discussion

This study demonstrates the importance of taking evidence beyond mtDNA phylogeography into account when attempting to diagnose species limits. In the eastern Brazilian *Bothrops atrox* complex, the discovery of two geographically overlapping haplotype clades is consistent both with the hypothesis of sympatry of separate organismal lineages and with the hypothesis of the coexistence of haplotype clades within a single organismal lineage. The use of morphological analysis is instrumental in resolving this issue, in particular when related to mtDNA variation by means of the Mantel matrix association tests. Of critical importance in cases involving sympatry between mtDNA haplotype lineages is the fact that mtDNA and morphological evidence is available from the same specimens: only this allows the association of mtDNA and morphological variation to be tested rigorously.

Our results show a lack of strong morphological or phylogeographic structure within the *Bothrops atrox*

complex along the eastern coast of Brazil. Morphological data do not reveal strong discontinuities. Instead, the pattern of variation is more suggestive of clinal variation. No single character will diagnose different populations or groups of populations in this complex. Although the mtDNA haplotypes of these populations group into two separate clades, the support for most of the internal phylogenetic structure among the Atlantic coast haplotypes is not statistically significant. This is in itself remarkable, in view of the considerable amount of sequence of relatively fast-evolving mitochondrial genes used in these analyses.

The geographical separation between the basal haplotype clades is incomplete. In the absence of further data, this could be taken as evidence of either conspecificity, in which case the two mtDNA lineages would coexist within a single evolutionary lineage, or of sympatry between two distinct species. However, these two scenarios lead to different predictions for observed patterns of morphological variation. In the former case, one

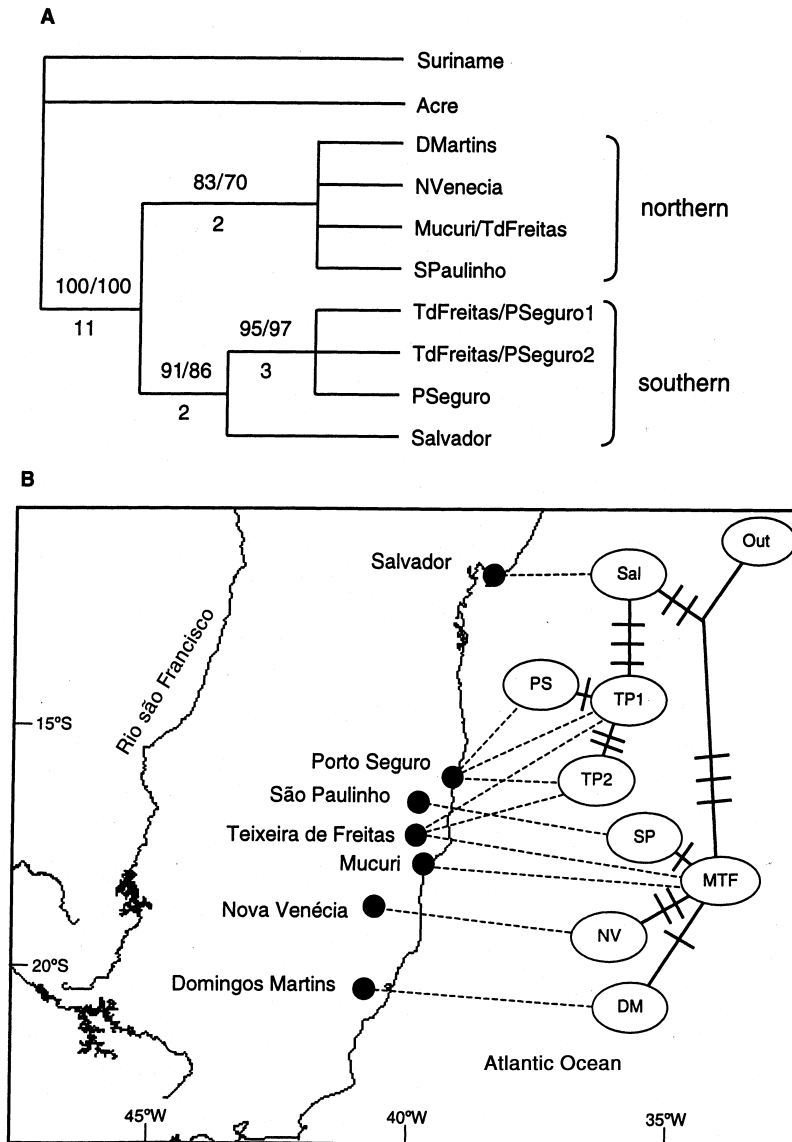


Fig. 3 (A) Bootstrap 50% majority rule consensus of two equally parsimonious trees based on exhaustive parsimony analysis of 1401 base pairs of cytochrome *b* and ND4 sequence information. Numbers above branches indicate bootstrap support: numbers before the slash indicate MP bootstrap support, numbers after the slash, ML bootstrap support. Numbers below branches indicate Bremer branch support. (B) Minimum spanning tree of Brazilian Atlantic coast haplotypes in relation to geography. Each crossbar indicates one base pair substitution. Haplotype codes as related to Fig. 3(A): Out = branch to outgroup taxa (base pair substitutions not shown); Sal = Salvador; TP1 = TdFreitas/PSeguro1; TP2 = TdFreitas/PSeguro2; PS = PSeguro; SP = São Paulinho; MTF = Mucuri/TdFreitas; NV = NVenecia; DM = DMartins.

would predict an absence of association between mtDNA and morphological variation within the zone of sympatry, whereas in the latter case, one would expect morphological variation to be strongly associated with the mtDNA affinities of the specimens.

Figure 2 shows that the morphology of specimens from the zone where the two mtDNA lineages intermingle is entirely unrelated to the haplotype lineage carried by the specimens. Our matrix correspondence tests further demonstrate the lack of significant association between morphological variation and the mtDNA affinities of the specimens. Although our sample size for combined mtDNA and morphological data was small, it was large enough to result in a significant association between supralabial pigmentation and sex; moreover, the pairwise correlations between any of the morphological characters

and patristic distance were uniformly low (<0.1), and all partial Mantel tests were very clearly insignificant. We are therefore confident that the lack of significant association between morphology and mtDNA is not due to insufficient statistical power by the Mantel tests. Consequently, we reject the hypothesis that the two mtDNA haplotype clades characterize two partly sympatric species. Instead, they appear to coexist within a single organismal lineage in southern Bahia. In this case, the use of morphological and DNA data from the same specimens has been of critical importance in discriminating between the alternative hypotheses of conspecificity and sympatry.

The characters conventionally used to distinguish between *B. leucurus* and *B. pradoi* (Hoge & Romano, 1972; Campbell & Lamar, 1989; Grantsau, 1991) are

Table 3 Differences in tree length or likelihood, statistics, and their significance, between the most parsimonious or the most likely trees, and trees constrained to be compatible with alternative phylogenetic or biogeographical hypotheses, as calculated through Wilcoxon signed-ranks tests (for parsimony) and Shimodaira–Hasegawa tests for maximum likelihood.

Hypothesis	Wilcoxon signed-ranks			Shimodaira–Hasegawa	
	d(Steps)	–z	P	d(–lnL)	P
Monophyly of northern clade	2	1.4142	0.1572	2.4332	0.181
Monophyly of southern clade	2	1.0000	0.3173	1.5477	0.304
Monophyly of haplotype clade TdFreitas/ PSeguro1 & 2 and Pseguro	3	1.7321	0.0833	7.9262	0.046*
Monophyly of all <i>leucurus/pradoi</i> haplotypes	11	3.3166	0.0009*	12.4749	0.035*

*Indicates significance.

Table 4 A. Correlation coefficients between distance matrices of observed data and individual causal hypotheses. B. Significance of association between observed patterns and hypothesized causes. Numbers represent P-values for the null hypothesis of no significant association between observed variation and the potential cause. We have only included the results of one tree, as the results for the two trees are essentially identical.

	Geographic distance	Patristic distance	Sex
A. Pairwise correlation coefficients			
General phenotype	0.269	0.032	0.117
Intersupraoculars	–0.124	–0.077	0.071
Dot around nostril	–0.044	0.098	0.018
Infralabial pigmentation	–0.165	0.038	–0.005
Supralabial pigmentation	0.035	–0.084	0.227*
B. Simultaneous Mantel test P-values			
General phenotype	0.141	0.882	0.225
Intersupraoculars	0.550	0.602	0.410
Dot around nostril	0.828	0.219	0.617
Infralabial pigmentation	0.592	0.661	0.995
Supralabial pigmentation	0.900	0.425	0.037*

*Indicates significance prior to Bonferroni correction for the number of hypotheses tested for each variable.

inadequate for this purpose. None was significantly associated with mtDNA haplotype lineages in our Mantel tests, none is fixed within any of our samples, and only the presence or absence of a dot around the nostril showed significant among-sample variation in our ANOVA. Supralabial, infralabial and chin coloration show extensive ontogenetic and sexual variation, as well as some geographical variation, as previously noted by Ripa (1997). Juveniles from the entire Atlantic coast of Brazil generally have extensive dark pigmentation on the sides of the head. This is lost early during growth in females, whereas males retain this pigmentation for longer. The quoted differences in pattern between these forms are thus clearly a matter of ontogeny and sexual dimorphism rather than qualitative genetic differences.

The results of this study suggest that *Bothrops leucurus* and *B. pradoi* cannot be regarded as separate species under any of the diagnostic criteria outlined in the introduction. The existence of extensive clinal variation, and the absence of stepped variation, suggest lack of reproductive isolation among all populations, which is

incompatible with the isolation/recognition criteria. There is no indication that the different populations along the Atlantic coast occupy different adaptive zones; if anything, the constituent populations of the *B. atrox* complex can be characterized as generalists. There are no clearly distinct phenetic clusters among our samples, which is incompatible with the phenetic criterion. The absence of diagnostic characters, coupled with the very broad clinal variation displayed, is also incompatible with the diagnosability criterion, and thus also with the apomorphy criterion. Finally, the absence of consistent differentiation between specimens carrying different mtDNA clades is inconsistent with the genealogical concordance criterion: from a phylogeographic perspective, there are no allopatric or parapatric sets of populations with reciprocally monophyletic mtDNA haplotype clades. Consequently, *B. pradoi* and *B. leucurus* would not even qualify as Evolutionarily Significant Units according to the criteria of Moritz (1994).

In view of these results, we regard the Atlantic coast populations of the *Bothrops atrox* complex as part of one

single species. The oldest available name for these populations is *Bothrops leucurus* Wagler, 1824. *Bothrops pradoi* (Hoge, 1947) thus becomes a subjective junior synonym of *B. leucurus*.

Biogeography and conservation

The monophyly of the Atlantic coast haplotypes of the *Bothrops atrox* complex, the minimal levels of sequence divergence within this clade, and its rooting among clades of Amazonian populations of the *Bothrops atrox* complex (Wüster *et al.*, 1999), are all consistent with the hypothesis that these populations represent a recent (Pleistocene) colonization of the Atlantic forests of Brazil from an Amazonian origin. Recent estimates of rates of mtDNA sequence evolution in squamates of $\sim 1.4\%$ Myr⁻¹ for cytochrome *b* and ND4 (Wüster *et al.*, in press) suggest a divergence among Atlantic coast haplotypes within the last 1 Myr. The same phylogeographic pattern is also found in *Lachesis* (Zamudio & Greene, 1997). This is likely due to a formerly continuous zone of mesic vegetation between the present-day rainforests of the Amazon Basin and the Atlantic coast (Lynch, 1988).

The existence of northern and southern haplotype clades is consistent with a hypothesis of Pleistocene fragmentation of the forests of the Atlantic coast of north-eastern Brazil, as suggested by some authors (Brown, 1987; Lynch, 1988). However, this must be considered cautiously in view of the low levels of support for these clades and the limited sampling in this study.

Contrary to Campbell & Lamar (1989), *Bothrops leucurus* (including *B. pradoi*) is amply distributed along the eastern coast of Brazil, from southern Espírito Santo north to at least Alagoas. Greene & Campbell (1992) listed both *B. leucurus* and *B. pradoi* as potentially vulnerable due to their supposedly small, fragmented range. In reality, *B. leucurus* is very common in many parts of eastern Brazil, and adapts well to agricultural areas (Ripa, 1997) as well as urban environments, and remains a frequent cause of snakebites even within metropolitan areas such as Salvador (Lira-da Silva & Brazil Nunes, 1992). Conservation concern for this species thus appears unwarranted.

Conclusions

In conclusion, this study has demonstrated the importance of considering sources of evidence beyond mtDNA phylogeography in the diagnosis of species boundaries, particularly where there is overlap between the ranges of mtDNA clades. Additional data, either from independent genetic markers, or from morphology, are required. Evidence in favour of mtDNA haplotypes denoting separate species includes genealogical concordance with other genetic markers (Baum & Shaw, 1995), or a clear association between morphology and haplotype lineage. Using the same specimens for all sources of evidence may

be crucial. The use of partial matrix association tests to relate patterns of morphological variation to that of mtDNA sequence variation was shown to be useful to test some of the commonly advocated criteria for species status, and to discriminate among alternative interpretations drawn from the mtDNA evidence alone. The practice of using pooled literature data on morphology to back up species inferred solely on the basis of mtDNA phylogeography is potentially highly misleading, as it may mask patterns of variation of morphology that may be present within the putative species. We caution against the uncritical use of phylogeographic studies unsupported by the critical analysis of patterns of variation in other characters in the inference of species boundaries.

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