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Dissertação de Mestrado

ESTUDO FILOGEOGRÁFICO DE *Bothrops jararaca* (WIED, 1824) BASEADO NO DNA MITOCONDRIAL (SQUAMATA: SERPENTES: VIPERIDAE).

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Resumo

Bothrops jararaca distribui-se na região da Mata Atlântica, concentrando-se nos estados do sul e sudeste do Brasil. Possui grande variação nos padrões de desenho e cor, entretanto sua história evolutiva é desconhecida. Visando avaliar a variabilidade do gene mitocondrial cyt-*b* sequenciamos aproximadamente 700pb de 154 exemplares de 96 localidades (abrangendo sete estados brasileiros) mais cinco espécimes insulares (duas *B. insularis* e três *B. alcatraz*). As relações filogenéticas foram estimadas utilizando três métodos: Máxima-Parcimônia; Neighbor-Joining e Maximum-likelihood com seqüências de *B. atrox* e *B. erythromelas* como grupos externos. Estas análises demonstram, com alto suporte, a formação de dois clados: um majoritariamente composto de indivíduos do sudeste (SEC) e outro com indivíduos do sul (SC). Comparativamente a diversidade média dentro de *B. jararaca* (2,9%) é maior que as espécies de *Bothrops* até o momento avaliadas. SEC possui 30 haplótipos e diversidade nucleotídica de 0,01, SC possui 14 haplótipos e diversidade nucleotídica de 0,007. Nas análises baseadas em simulaçôes de anelamento (SAMOVA) não foi possível encontrar o agrupamento com o maior F_{CT}, pois este aumentou em função do número de grupos selecionados. Contudo foram definidos dois grupos populacionais: sudeste e sul, baseado nas árvores filogenéticas e nos networks inferidos utilizando Median-joining e Parcimônia Estatística. Em todas as análises os grupos mantiveram-se com apenas dois indivíduos migrantes, sendo que sua estruturação apresentou F_{ST} de 0,70 e Nm de 0,21. O limite aproximado da divisão entre os grupos relaciona-se com o leito do Rio Paranapanema, entretanto não existe fundamentação para afirmar que este rio tenha sido a barreira para o fluxo gênico. O tempo de divergência entre os dois grupos foi estimado entre 3,6 e 1,0 milhões de anos o que situaria o evento cladogênico no final do Plioceno e inicio do Peistoceno, período de grandes mudanças climáticas que poderiam ter fragmentado a população ancestral.

Os resultados do NCA (Nested Clade Analisys) confirmam uma fragmentação passada como explicação para a estruturação entre os dois grandes clados, além de fluxo gênico restrito para a formação da maioria dos subclados. Análises de Aucorrelação espacial contrariamente indicam com alta significância uma variação clinal, contudo este padrão poderia ser gerado pela grande diferença entre os clados. Testes de neutralidade foram significantes apenas para os subclados do SC, assim como, o padrão unimodal nas análises de mismatch distrution, sugerindo um bottleneck moderado seguido de uma expansão populacional para SC.

As espécies insulares: *B. alcatraz* e *B. insularis* agrupam-se no SEC e possuem haplótipos continentais. Contudo provem de subclados distintos dentro de SEC. Estes dados sugerem que a colonização das ilhas ocorreu a poucos milhares de anos, e podem estar associados a flutuação do mar durante o Pleistoceno.

PHYLOGEOGRAPHIC STUDY OF *Bothrops jararaca* (WIED, 1824) BASED ON MITOCHONDRIAL DNA (SQUAMATA: SERPENTES: VIPERIDAE).

Abstract

Bothrops jararaca is a pitviper distributed across the Brazilian Atlantic Forest, manly in the south and southeastern regions of Brazil, being one of the main causes of the more than 10,000 registered snakebites in Brazil. Large variation in color and drawn patterns has been described, however noting is known about the population genetic and evolutionary history. We analyzed the sequence variation at the mitochondrial gene cytocrome *b* in 154 individuals from 96 localities and five specimens from two closely related insular species (*B. insularis* and *B. alcatraz*). Phylogenetic and network analyses reveled the existence of a southern and a southeastern clade with high support for all methods. The boundary between the two clades is near to where is today the Paranapanema River, between the São Paulo and the Paraná State, although its seems that this river is not a dispersion barrier to this species. The divergence time for these two clades was estimated at 3.5 to 1.0 millions years BP at the late Pliocene or early Pleistocene, when the tropical rain forest was fragmented by large climatic changes. Our data suggest that the SOC subclade underwent a moderate bottleneck followed by a large size expansion while SEC showed signals of range and size expansion, and absence of a strong bottleneck. The origin of the insular *B. alcatraz* and *B. insularis* occurred in the Late Pleistocene by individuals from the actual *B. jararaca* species, and its origins may be associated with the fluctuation of the sea level at that period.

APRESENTAÇÃO

1. HISTÓRICO DO PROBLEMA

1.1 Introdução

Bothrops jararaca (Wied, 1824) é uma serpente amplamente distribuída nas áreas mais povoadas do Brasil, não demonstrando problemas em habitar áreas degradadas. Sua distribuição estende-se pelo que representaria a área original da Mata Atlântica (Campbell & Lamar 1989), concentrando-se nos estados do sul e sudeste do Brasil. É juntamente com *B. atrox* a maior causadora de acidentes ofídicos (Sazima 1992), bem como, é frequentemente a *Bothrops* mais abundante nos institutos de pesquisa dentro de sua área de dispersão.

Sua variação morfológica é muito grande, principalmente na coloração de fundo e no padrão dos desenhos (Lema 1994). Entretanto, nada se sabe sobre a variabilidade genética das populações de *B. jararaca*, como também é desconhecida a história evolutiva destas populações.

Os estudos filogeográficos descortinam a história evolutiva de uma linhagem, relacionando-a com sua distribuição geográfica, através, principalmente, das diferenças entre seqüências de DNA mitocondrial (Avise, 2000).

Este projeto tem por objetivo avaliar a variabilidade do gene mitocondrial citocromo b na espécie *B. jararaca*, em uma grande representatividade de sua distribuição, para compreender os processos filogeográficos e inferir sobre a história evolutiva de suas populações.

1.2. O gênero *Bothrops*

As serpentes do gênero *Bothrops* apresentam um alto interesse científico nos estudos da herpetofauna Sul-americana, pois são as maiores causadoras de acidentes ofídicos na América Latina (Campbell & Lamar 1989).

Este gênero contém por volta de 31 espécies, comumente chamadas de "jararacas" no Brasil e "lanceheads" nos EUA. A forma em lança da cabeça das serpentes deste gênero é sua característica mais distintiva. Ocorrem principalmente na América do Sul, com uma espécie apenas alcançando a América Central. Existem cinco espécies insulares (*B. caribbaeus, B. insularis*, *B. alcatraz* e *B. lanceolatus*) e as continentais exibem uma grande diversidade na

sua distribuição, com espécies estritamente ligadas à mata atlântica (*B. jararaca, B. jararacuçu, B. cotiara, B. fonsecai*) outras ligadas à floresta amazônica (*B. atrox, B. brazili*), ao cerrado (*B. moojeni, B. itapetiningae*), aos campos (*B. alternatus*) e estepes (*B. ammodytoides*) entre outros ecossistemas onde habitam serpentes deste gênero (Campbell & Lamar 1989).

Atualmente as espécies do gênero *Bothrops* encontram-se com suas relações filogenéticas em discussão devido aos diferentes resultados obtidos pelas várias metodologias utilizadas (Hoge & Romano-Roge 1981; Pesantes 1989; Cadle 1992; Wermam 1992; Wüster *et al.*. 1996; Salomão *et al.* 1997; Wüster *et al.*. 2002).

1.3. *Bothrops jararaca*

1.3.1. Descrição

Bothrops jararaca é uma serpente terrestre de tamanho médio. Possui em média 120 cm de comprimento, podendo chegar a 160 cm, sendo relativamente delgada.

O padrão de coloração desta espécie é extremamente variável. A coloração dorsal de fundo pode ser bronze, pardo, cinza, amarelo, oliva ou próximo ao marrom (Figura 1); é usualmente escura sobre a cabeça e as porções anterior e posterior do corpo. Uma proeminente faixa escura pós-orbital estende-se de trás dos olhos até o ângulo das mandíbulas. A íris é dourada para esverdeada com finas reticulações escuras (Campbell & Lamar 1989).

O padrão dorsal consiste em uma série de desenhos trapezoidais ou subtriangulares, onde os ápices estão sobre a linha vertebral (Campbell & Lamar 1989). Estes desenhos podem ser pardo-escuros marginados por uma linha negra ou totalmente negros (Lema 1994). Eles podem se apresentar opostos, um de cada lado da linha dorsal, ou justapostos. As variações destes desenhos são muitas, desde elongações, fusões, fragmentações e até em alguns indivíduos ausentes na região média do corpo. O ventre pode ser de um esverdeado pálido para um amarelo esbranquiçado, com manchas escuras ou não; e de um cinza alvacento ao preto irregularmente manchado (Campbell & Lamar 1989).

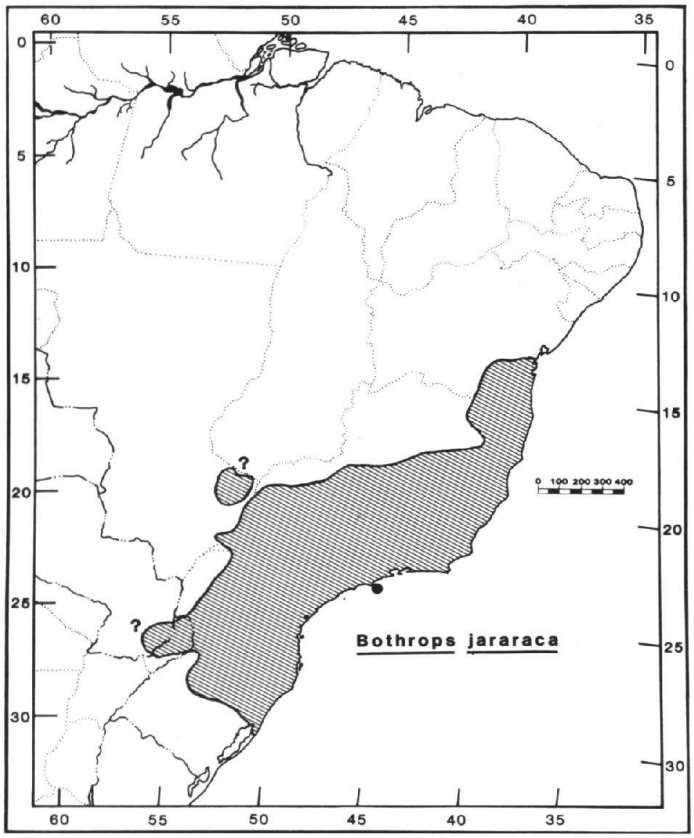
B. jararaca é caracterizada por folidose por possuir 5 – 12 intersupraoculares fracamente quilhadas; 7 – 9 supralabiais; 9 – 13 infralabiais; 20 – 27 fileiras de escamas dorsais no meio do corpo; 170 – 216 ventrais e 51 – 71 subcaudais (a maioria dividida) (Campbell & Lamar 1989).

Figura 1: Variação do padrão de coloração de *B. jararaca* (Retirado de Campbell & Lamar 1989).

1.3.2. Distribuição

Bothrops jararaca ocorre no Brasil, Paraguai e Argentina. No Brasil ela é encontrada no sudeste da Bahia, Espírito Santo, Rio de Janeiro, Minas Gerais, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul. Estende-se a oeste para o extremo leste de Mato Grosso. No Paraguai ocorre no nordeste e ao norte da Argentina (Misiones) (Hoge & Romano-Hoge 1981; Campbell & Lamar 1989). A distribuição segundo Campbell & Lamar (1989) pode ser observada na Figura 2.

Figura 2: Distribuição de *Bothrops jararaca* segundo Campbell & Lamar (1989). Ponto de interrogação representa possíveis áreas de ocorrência.



1.3.3. Hábitat

Uma diversidade de hábitats é ocupada por *B. jararaca*: florestas tropicais decíduas e savanas, bem como florestas semitropicais de altitude, campos abertos, regiões cultivadas e áreas impactadas. A distribuição da espécie coincide com o Domínio Morfoclimático Tropical Atlântico (Sazima 1992).

Bothrops jararaca pode ser encontrada nas grandes cidades brasileiras. Em dois anos de estudo na cidade de São Paulo, esta espécie representou 12% das serpentes amostradas (Sazima 1992).

1.3.4. Abundância

Dentro do gênero *Bothrops* a espécie *Bothrops jararaca* é freqüentemente a mais abundante em sua área de distribuição. De um total aproximadamente 490 mil serpentes recebidas pelo Instituto Butantan em 40 anos (1906-1945), por volta de 39% foram *B.*

jararaca, enquanto 24% foram *Crotalus durissus* correspondendo a uma taxa de 1,6 *B. jararaca* para cada *C. durissus* (Fonseca, 1949).

Entretanto durante o triênio de 1988, 1989 e 1990, esta taxa reduziu para 0,84:1. Estes números podem indicar uma tendência devido ao contínuo desmatamento do sudeste do Brasil, favorecendo a expansão de *C. durissus* (Sazima 1992).

Estes dados, contudo, não informam sobre a diminuição das populações de *B. jararaca.* A entrada de *B. jararaca* vem constantemente aumentando no Instituto Vital Brazil (A. Melgarejo, com pess.) e na Universidade de Caxias do Sul (P. J. Demeda, com. pess.) e mantendo-se inalterada no Instituto Butantan (F. L. Franco, com. pess).

1.3.5. História Natural

B. jararaca é mais ativa à noite, contudo alguma atividade diurna ocorre. É ativa durante a maior parte do ano, com pouca ou nenhuma atividade durante os meses frios (junho a agosto). As fêmeas grávidas tendem a manter áreas onde termorregulam e se protegem. Raramente estas serpentes se aquecem com luz do sol direta, e a noite utilizam o calor da superfície do solo (Sazima 1992).

As fêmeas tendem a ser maiores e mais volumosas que os machos. Ambos atingem o tamanho adulto por volta do terceiro ou quarto ano e provavelmente reproduzem na estação seguinte. Os nascimentos ocorrem durante a estação chuvosa e o tamanho da ninhada é de aproximadamente 8 –14 filhotes (Sazima 1992).

B. jararaca preda principalmente pequenos vertebrados, entretanto ocorre modificação ontogenética na dieta, quando jovem preda principalmente anuros e na fase adulta reoedores. Adultos de *B. jararaca* possuem poucos predadores, enquanto os juvenis são vulneráveis a diversos predadores como gaviões, gambás e serpentes ofiófagas (Sazima 1992).

1.4. Filogeografia

Filogeografia pode ser definido como o estudo dos princípios e processos que governam a distribuição geográfica de linhagens genealógicas (Avise 2000).

As bases históricas desta ciência estão intimamente ligadas aos estudos empíricos com o DNA mitocondrial de animais, iniciados nos anos setenta e que têm sido usadas intensivamente para estudos filogenéticos, nos quais a distribuição dos grupos de haplotipos de mtDNA através da área de uma espécie ou complexos de espécies é utilizado para inferir sobre a história das populações (Puorto *et al.*. 2001)

A filogeografia supre uma ponte empírica e conceitual entre a genética de populações e a biologia filogenética. E mesmo relações explicadas de forma estritamente ecológica, podem através de uma interpretação filogeográfica explicitar as bases filogenéticas para certas características da história natural de um grupo (ver Martins *et al.*. 2001).

A utilização da variação entre seqüências do DNA mitocondrial tem sido a metodologia mais amplamente utilizada para inferir sobre os padrões filgeográficos das espécies (Avise 2000). A mitocôndria, como organela aparentemente provinda de uma simbiose, possui um genoma próprio que se encontra fracamente envolvido com o genoma nuclear (Attardi 1985). A evolução de algumas regiões mitocondriais é extremamente rápida comparada ao DNA nuclear (Nedbal and Flynn 1998) o que habilita o mtDNA a ser utilizado como um marcador molecular para microevolução (Avise 2000).

Análises de padrões filogeográficos intraespecíficos conduzem a observação de barreiras e estruturas geográficas (Eizirik 2001) e levam a um maior avanço em nosso conhecimento dos processos históricos biogeográficos.

2. OBJETIVOS

O objetivo geral deste trabalho foi avaliar os processos filogeográficos de *Bothrops jararaca* através da variabilidade mitocondrial.

Para isso buscamos estimar a diversidade genética dentro da espécie utilizando a variabilidade de regiões do gene que codifica para o citocromo *b*. Através desta variabilidade avaliamos sua estruturação geográfica estimando grupos populacionais, bem como, o fluxo gênico entre estas populações, para desta forma inferir sobre os processos demográficos e históricos que moldaram a diversidade de *Bothrops jararaca*.

Este trabalho será submetido à revista **Molecular Ecology**.

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Phylogeography of the *Bothrops jararaca* complex (Serpentes: Viperidae): past fragmentation and island colonization in the Brazilian Atlantic Forest

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Running title: Phylogeography of *Bothrops jararaca* complex

Abstract

The Brazilian Atlantic Forest is one of the world major biodiversity hotspots defined by a remarkable richness of endemic species and threatened by a severe habitat loss but the evolution of patterns of endemism within that ecoregion are poorly understood. The jararaca (*Bothrops jararaca*) is a pitviper endemic of this region, distributed mainly in the southern and southeastern states of Brazil, responsible for the majority of about 10000 annual snakebites in Brazil. However, its evolutionary history and patterns of genetic diversity are largely unknown. Here we analyze sequence variation at the mitochondrial cytochrome *b* gene in 159 individuals from 96 localities and twelve individuals from two closely related insular species (*B. insularis* and *B. alcatraz*). Phylogenetic and network analyses of the forty-five haplotypes found herein revealed the existence of two well supported clades, exhibiting a southern and a northern range. The divergence time of these two clades was estimated at 1.0 to 3.5 million years ago, a period of intense climatic changes and frequent fragmentation of the tropical rain forest. Both clades show high haplotype and nucleotide diversity and our data suggest that the southern clade underwent a moderate bottleneck followed by a large size expansion while the northern clade shows signals of range expansion in the absence of a strong bottleneck. The insular species *B. alcatraz* and *B. insularis* share different haplotypes with mainland individuals of *B. jararaca*, their differentiation occurred very recently and most likely independently from costal *B. jararaca* populations, possibly associated with past sea level fluctuations.

Introduction

The *Bothrops jararaca* species complex comprises three closely related Atlantic Forest species of lanceheaded pitvipers (Marques *et al.* 2002): the mainland *B. jararaca* and the two insular species *B. insularis* and *B. alcatraz*. The distribution of this complex coincides with the southern and central areas of the Brazilian Atlantic Forest domain (Sazima 1992) and it occurs in all primary subdivisions of this domain (tropical evergreen mesophytic broadleaf forest, tropical semi-deciduous mesophytic broadleaf forest and a mixed forest or Araucaria forest (see Valladares-Padua *et al*. 2002 for Atlantic forest subdivision).

The jararaca (*B. jararaca*) is a semi-arboreal pitviper, with an average length of 120 cm distributed primarily in southern and southeastern Brazil (Fig. 1). It also occurs, but with small frequency in Misiones (northeastern Argentina) and northeastern Paraguay (Hoge & Romano-Hoge 1981; Campbell & Lamar 1989, Giraudo 2001). Most of the distribution of *B. jararaca* comprises the most populated areas of Brazil in which less than 7.5% of the original vegetation remains (Myers *et al.* 2000). Despite this fact *B. jararaca* is abundant in this area (Fonseca, 1949), being responsible for the major part of snake bite envenomation in Brazil (Ribeiro & Jorge 1990; Clissa 2002). *Bothrops jararaca* is a forest dweller and may be found in evergreen or semideciduous broadleaf forest, closed scrub and even highly degraded formations and cultivated fields. Jararacas are usually observed in proximity to vegetational cover, however may occasionally use open areas (Sazima 1992). The ventral and dorsal colors, patterns and number of scales are highly variable within and among populations, and also among some geographical regions (Hoge *et al.* 1977; Campbell & Lamar 1989; Lema 1994). The existence of some geographical structure in the morphology of *B. jararaca* prompted Salomão *et al.* (1997) to conclude that this taxon may represent a complex of several species.

Bothrops insularis, the golden lancehead, is restricted to Queimada Grande Island, about 30 km off the coast of São Paulo State (SP), southeastern Brazil. This species is more arboreal than mainland jararaca; its diet is predominately based on migrant passerine birds (Martins *et al.* 2001) and its venom has become more effective in killing bird prey (Cogo *et al.* 1993). This species was considered by Salomão *et al.* (1997) the sister taxon to *B. jararaca* but they noted that *B. insularis* may be placed within the *B. jararaca* species complex, rendering jararaca a paraphyletic taxon. The second insular species is the recently described *Bothrops alcatraz* (Marques *et al.* 2002), endemic to the Alcatrazes Island, also about 30 km off the coast of SP and more than 100 km north of Queimada Grande Island. *Bothrops alcatraz* is paedomorphic, has a smaller adult size and larger eyes than the continental *B. jararaca* and feeds primarily on ectothermic prey (centipedes and lizards) a characteristic of juvenile mainland vipers, and its venom is similar to that of juveniles of jararaca (Marques *et al.* 2002).

In spite of these intriguing features, the knowledge about the genetic diversity and the evolutionary history of *B. jararaca* and associated species is scarce. Additionally, although the Brazilian Atlantic Forest is considered one of the eight major biodiversity hotspots for conservation (Myers *et al.* 2000), the processes through which its striking diversity was formed are controversial, and the knowledge about patterns of endemism and endemic species phylogeography is poor in contrast to other areas (see Moritz *et al.* 2000). The aim of the present study was to evaluate the genetic variability of the mitochondrial cytochrome *b* gene in a large sample throughout most of the range of *B. jararaca* and the two insular species, to infer their genetic structure and evolutionary history and to contrast these findings to paleoclimatic processes reported for the Brazilian Atlantic Forest.

Materials and methods

Population sampling and molecular methods

A total of 159 specimens from 94 localities was sampled in the states of Rio Grande do Sul (RS), Santa Catarina (SC), and Paraná (PR) and the states of São Paulo (SP), Rio de Janeiro (RJ), Minas Gerais (MG), and Espírito Santo (ES), covering most of the species´ range (Fig. 1, Appendix 1), as well as 12 individuals of two insular endemic species (seven *B. insularis* and five *B. alcatraz*) belonging to the *jararaca* complex (Salomão *et al.* 1997, Martins *et al.* 2001, Marques *et al.* 2002). DNA was extracted from scales, blood, liver or shed skin following published protocols specific for each tissue (Hillis *et al.* 1996; Hillel *et al.* 1989; Bricker *et al.* 1996). Additionally DNA from formalin-fixed museum specimens was extracted using a modified protocol from Chatigny (2000).

A 726 bp region of the cytochrome *b* gene was amplified via PCR using the primers 703Bot (Kocher *et al.* 1989) and MVZ (Moritz *et al.* 1992), both modified by Pook *et al.* (2000) for increased efficiency in snakes. Amplicons were purified with shrimp alkaline phosphatase and exonuclease I (Amersham Biosciences) and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences), in a MegaBACE 1000 automated sequencer (Amersham Biosciences) following the manufacturer's protocols. Chromatograms were checked with the Chromas software (Technelysium) and sequences were manually edited using BioEdit 6.0.7 (Hall, 1999).

Sequence alignment and evolutionary models

Sequences were aligned using the ClustalX 1.83 program (Thompson *et al.* 1997) and corrected by eye. The level of saturation was assessed for all codon positions considering the degree of deviation from isometric lines in scatterplots of transition and transversion substitutions against the total genetic distance (TrN) using the program DAMBE (Xia & Xie

2001). Possible saturation effects causing bias in the phylogenetic analyses were assessed by comparing the trees found considering the whole data, without the third codon position, and using transversions only. These trees were reconstructed using a maximum-likelihood approach (ML) with a heuristic TBR search option and a neighbor-joining (NJ) starting tree using the Jukes-Cantor model (JC) as implemented in PAUP* 4.0b10 (Swofford 2001).

The model of evolution used for the phylogenetic reconstructions was estimated with the MODELTEST 3.06 program (Posada & Crandall 1998). This program uses a hierarchical likelihood-ratio test (hLRT) and minimum theoretical information criterion (AIC) to select the most likely model of sequence evolution. The application of both tests (hLRT and AIC) has been criticized since they usually select more complex models than necessary to reconstruct the correct phylogenetic topology, which increases the standard error (Nei & Kumar 2000). We tested the resulting models from both hLRT and AIC as well as a simpler model (JC) to assess the possible bias in phylogenetic reconstruction caused by the use of complex models.

Phylogenetic analyses

To root the *B. jararaca* sequences, we have used *B. erythromelas* and *B. atrox* as outgroup species, based on Wüster *et al.* (2002) where *B. erythromelas* is one of the more closely related species and *B. atrox* is a relatively more distant species. Phylogenetic inference was carried out using neighbor-joining (NJ), maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian Inference (BI) (see Holder & Lewis 2003 for a review). We inferred ML trees using PAUP* with the heuristic search (TBR) option and a NJ starting tree; confidence was estimated by 1000 bootstrap replicates using the NNI heuristic search option. Three putatively more efficient approaches for ML reconstruction were also used: genetic algorithms implemented in the programs MetaPIGA 1.02 (Lemmon & Milinkovitch 2002) and Treefinder (Jobb *et al.* 2004), quartet-puzzling implemented in Tree-Puzzle 5.0 software

(Strimmer & von Haeseler 1996) and a fast hill-climbing algorithm that adjusts tree topology and branch lengths simultaneously, implemented in the program phyML (Guindon & Gascuel 2003). For these three approaches statistical support was obtained by: 1,000 bootstrap replications (for Treefinder and phyML); percentage of cluster occurrence in 10,000 trees (for MetaPIGA); and percentage of how often the corresponding cluster was found among the intermediate trees in 100,000 puzzling steps (for Tree-puzzle).

BI was performed using Mr. Bayes v3.0b4 (Huelsenbeck & Ronquist 2001) software with 1,000,000 cycles for the MCMC algorithm using flat priors. MP was performed by heuristic search (TBR) with starting trees produced by 1,000 replications of random stepwise addition. To assess the MP statistical confidence, 1,000 bootstrap replicates were conduced using the same heuristic search, but with starting trees obtained from simple stepwise addition. NJ analysis, using the ML estimator of distance under the evolution models selected by MODELTEST, was evaluated with 1,000 bootstrap replications. Both MP and NJ analyses were performed in PAUP*.

Divergence time between clades was estimated using the linearized tree method as implemented in LINTREE (Takezaki *et al.* 1995). To calibrate the molecular clock we used three different substitution rates (μ) reported previously for pitvipers: 0.47% and 1.32% my^{-1} derived from the entire mtDNA reviewed by Zamundio & Greene (1997); 1.36% and 1.44% my^{-1} estimated with the *Lachesis* data from Zamundio & Greene (1997) by Pook *et al.* (2000); and 0.66% and 0.76% my^{-1} estimated from South American *Porthidium* data (Wüster *et al.* 2002).

Definition of the population genetic structure

Samples were grouped in populations according the municipal limits. As exact geographic location was not available for most of the samples, latitudinal and longitudinal

positioning was inferred using the geographical center of the town where the individuals were collected. The spatial analysis of molecular variance (SAMOVA, Dupanloup *et al.* 2002) was used to estimate the genetic structure of populations and possible barriers to gene flow to help to delimit groups of populations within the range of *B. jararaca* as needed for some analyses. This method is based on a simulated annealing approach to maximize the proportion of total genetic variance due to differences among groups of populations (F_{CT} index) and thus attempts to define the strongest structure of populations in genetic terms (Dupanloup *et al.* 2002). We analyzed an extensive range (one to eighty) of population partitions in the total distribution to find the largest F_{CT} value, which was suggested by Dupanloup (2002) to be associated with the correct number of groups in simulation analysis. The genetic structure observed using the grouping definitions from SAMOVA was then contrasted to that obtained by grouping our samples following approximately the three Atlantic forest subdivisions using an analysis of molecular variance (AMOVA, Excoffier *et al.* 1992), estimated for all geographical groups in ARLEQUIN 2.0 (Schneider *et al*. 2000).

Population parameters

DNASP 4.0 (Rozas *et al.* 2003) was used to estimate population diversity statistics such as nucleotide (π) and haplotype diversity (Hd), Tajima's (Tajima, 1983) and Fu & Li's (Fu & Li 1993) neutrality tests and their statistical significance, F-statistics (F_{ST}; Hudson, *et al.* 1992), number of migrants per generation (Nm, using $F_{ST} = 1/(1+2Nm)$, and mismatch distribution analyses (Rogers & Harpending, 1992). The spatial autocorrelation analysis was conduced with the AIDA software (Bertorelle & Barbujani, 1995) with 1000 replications and 15 distance classes, each class containing approximately equal numbers of comparisons. This autocorrelation statistics compares DNA sequences at several spatial scales, using the individual haplotype as the unit of analysis (Barbujani *et al.* 1995).

Network and Nested Clade Analyses

Two kinds of haplotype networks were constructed: the median-joining network (MJN) (Bandelt *et al.* 1999) estimated with the Network 3.1.1.1 software (www.fluxus-engineering.com), and the parsimony network (with 0.95 probability limits) using the program TCS v1.0 (Clement *et al.* 2000) with Crandall's assumptions (Crandall *et al.* 1994) to resolve ambiguities. Nested clade analysis (NCA) was conducted using the GeoDis 2.0 program (Posada *et al.* 2000) with 10000 (Monte Carlo) replications. The TCS haplotype network was edited and nested categories were assigned following the nested rules in Templeton *et al.* (1987), Templeton & Sing (1993) and Crandall (1996). Results were interpreted based on Templeton's key (Templeton 1998) using the key available at the website http://darwin.uvigo.es/software/geodis.html.

Coalescent analyses

Coalescent analyses were used for estimating divergence times and migration rates among groups of populations to evaluate the relative contributions of isolation and migration to the population structure in this species. We used the program MDIV (Nielsen & Wakeley 2001) which uses a Markov Chain Monte Carlo method within a likelihood framework to estimate the posterior distributions of: theta ($\theta = 2N_{ef}\mu$), the migration rate per generation ($M = N_{ef}m$, m = migration rate), and divergence time ($T = t/N_{ef}$, t = divergence time) (equations adjusted for mitochondrial data). The program also estimates the expected time to the most recent common ancestor (T_{MRCA}) for all sequences in the sample. Five runs were performed with 5,000,000 cycles each for the Markov Chain and the burn-in time of 10% as recommended by the program manual.

Female effective population size (N_{ef}) was estimated using $N_{ef} = \theta / 2\mu$. One generation time for *B. jararaca* was estimated using the data from Sazima (1992), that suggested jararacas attain adult size in their third or fourth year and they live approximately 10-12 years (Sazima 1992). We estimated generation time as an average of the youngest reported age at maturity and the shortest reported life span minus one year as a compensation for probability of survival until old ages. This procedure resulted in a generation time of 5.5 years for *B. jararaca*.

Results

mtDNA sequence variation

An alignment of 626 bp of cytochrome *b* was obtained for 171 individuals of the *B. jararaca* species complex and the two outgroups (*B. erythromelas* and *B. atrox*) (the sequences been submitted to GenBank, Accession AY865653 to AY865824). Sixty one variable sites (9.7%) were found among the ingroup taxa while 145 variable sites (23.2%) were found including the two outgroups. Forty-five haplotypes were found in the *B. jararaca* complex, 44 being present in *B. jararaca*, with a haplotype diversity (Hd) of 0.943 and a nucleotide diversity (π) of 0.0207 (Table 1). The five individuals of *B. alcatraz* share a common haplotype, identical to the most frequent and widespread *B. jararaca* haplotype (haplotype code N01). On the other hand, two haplotypes were found in the seven *B. insularis*: one exclusive of *B. insularis* and present in a single individual (N27), and another found in the other six individuals and identical to that of mainland *B. jararaca* from São Bernardo do Campo, SP (N26).

Phylogenetic analyses

Although there is a signal for substitution saturation evidenced by a deviation from the isometric line in plots of substitution for transitions at the third codon position, we used the full dataset to estimate the haplotype phylogenies below, as this signal is weak and only apparent in the comparisons involving the outgroups (Fig. 2) and the trees constructed without the third codon position or with transversions only resulted in a broadly unresolved ingroup topology (data not shown). Besides, the trees estimated with ingroup sequences only resulted in topologies very similar to those of found with the outgroups included (data not shown).

The HKY+Γ substitution model was selected by the hLRT in MODELTEST with a gamma shape parameter (α) of 0.1597. Alternatively, the TrN+I model was selected by AIC

with a proportion of invariable sites of 0.6937. The main difference observed in the respective tree topologies is the position of the root. In several analyses using the HKY+ Γ model (ML, Tree-puzzle, NJ, and BI, data not shown), the outgroup position is not consistent and each method places the root on a different branch. On the other hand, when the TrN+I model is used with all methods, phyML and Treefinder with the HKY+Γ model and all methods with the simpler JC model the root is placed within the same branch. The inconsistent rooting might be due to a large increase in branch lengths between the outgroup and the ingroup when the gamma correction was applied (JC mean distance 13.6%, HKY+Γ mean distance 31.2%; see Huelsenbeck *et al*. 2002 for problems of using distant outgroups for rooting trees). Therefore, we used TN+I for all analyses but those performed with MetaPiga, in which we used HKY because the TN+I model is not implemented.

All phylogenetic trees showed a very similar topology, the main difference being the relative positions of some subclades (see below) with a few differences in the statistical support values with MetaPiga, Tree-puzzle, BI and NJ presenting higher values, while ML, phyML, Treefinder and the majority rule MP consensus tree show lower values (Fig. 3). The main feature of these trees is the presence of two divergent (distance of 3.3% calculated using TrN) clades of haplotypes. One group, that we call the southern clade (Sc), includes 14 haplotypes (found in 76 individuals) mainly from the southern part of the jararaca distribution (PR, SC, and RS states) and the other, called the northern clade (Nc), includes 31 haplotypes (found in 95 individuals) mainly from the southeastern Brazilian region (SP, MG, RJ and ES states). Statistical support values for these clades vary: the Sc clade receives high support with all analyses, while the Nc presents values ranging from 50% to 85%. The two clades are also geographically independent; only two individuals don't occur in their respective geographic clade, bj164 from Telêmaco Borba, PR (N04) and bj144 from Juquitiba, SP (S12, Fig. 3).

Within these two major clades there are some subclades that are well supported by most of the analyses. In the southern clade two subclades (Sc1 and Sc2, Fig. 3) show high statistical support in all analyses, but without clear geographic structure. Within the Nc, six subclades could be recognized in all analyses (Nc1, Nc2, Nc3, Nc4a, Nc4b and Nc5; Fig. 3) although some received low support in a few analyses. Most of the subclades show limited geographic structure with some overlap. The haplotypes of Nc1 are all from the northeastern distribution of the area considered (ES state) while those of Nc2 are from the Northern coast of RJ state. In Nc3 individuals of Southern MG state occur close to samples from inland RJ state (see Fig. 3 for support values).

The seven individuals (haplotypes N26 and N27) of *B. insularis* from Queimada Grande Island and two mainland individuals from SP state build up the strongly supported Nc4a group. This subclade groups together with Nc4b, another strongly supported clade consisting of specimens from inland SP and samples of areas near the city of São Paulo. In the sixth northern subclade, individuals of the central and western areas of MG are placed in Nc5. Ncmc comprises a large clade of haplotypes with a lower support including the only haplotype found in *B. alcatraz* (haplotype N01, shared with individuals from SP and RJ states).

The relationship among the subclades within the Nc and the root position of this clade vary considerably across the trees obtained by different methods with very low statistical support in most of them (root positions were not statistically different using Shimodaira-Hasegawa test).

Grouping the populations

The SAMOVA applied to two groups of populations (as implied by the results above) suggests a geographic barrier approximately between the SP and PR states, in the vicinity of

the Paranapanema river (Fig. 1). This putative barrier divides the *B. jararaca* populations in a Southern (SG) and a Northern (NG) group, exactly as we found in our phylogenetic analyses. However, the maximum F_{CT} value was obtained when 80 populations were used (results not shown). This pattern could be due to a large genetic variation within several of the populations or may be caused by our sampling scheme of few individuals in most of the municipalities, here defined as equivalent to the "population" (see Material and Methods). With these features, when we increase the number of groups, the number of populations within the groups becomes smaller, the F_{SC} decreases while the F_{CT} increases due to the relationship $(1-F_{ST}) = (1-F_{SC})\ (1-F_{CT})$ if F_{ST} remains almost unaltered (see Dupanloup *et al.* 2002). As a result of this relationship, with present data set it is not possible to associate the largest F_{CT} to the best number of groups. Therefore, in an attempt to avoid an analytical bias in definition of populations and based on the concordance among our phylogenetic inference, all further analyses were performed considering only the above two groups of populations.

Statistical and demographic parameters

Several statistical parameters were estimated for the two groups of jararaca identified herein (table 1). The NG is more diverse than the SG defined by more haplotypes and higher nucleotide diversity. Two neutrality tests (Fu and Li's D and F) were significantly negative for the SG, suggesting a demographic process such as population contraction followed by expansion or some kind of selection process on mtDNA, although Tajima's D was negative but not significant. For the NG, the respective values were not significant and were all positive except for Tajima's D test. Considering *B. jararaca* as a whole, all statistics were positive but not significant.

The mismatch distribution considering all individuals or the two groups separately showed multimodal distributions (Fig. 4) that agree with the non-significant results of the

neutrality tests except for the SG group. Unimodal distributions for SG emerge only when the respective subclades are regarded separately (e.g. using Sc1 and Sc2, not shown).

We have found a large F_{ST} (0.711) between the two groups of populations (SG and NG) and consequently a low number of migrants per generation was estimated (0.2). Likewise, the AMOVA test detected significant structuring between SG and NG: 69.98% of the genetic variation is found between the two groups of populations while only 16.22% are found among populations within groups, and the remaining 13.80% was found within populations.

The correlograms (Fig. 5) produced by the spatial autocorrelation analysis considering all sequences and the population groups showed the same pattern, in which the *II* coefficient decreased from positively significant to negatively significant with the increase of geographical distance, describing a cline. Barbujani and Bertorelle (1996) listed three possible causes for a clinal pattern: a selective gradient, demic diffusion and range expansion accompanied by founder effects and followed by gene flow.

Networks and NCA

The median-joining (Fig. 6) and statistical parsimony networks (not shown) were very similar. Median-joining network showed 14 mutational steps between the two major clades (Sc and Nc) thus corroborating the strong differentiation between them found by phylogenetic methods. However, some subclades of each of these clades differ between the networks and the phylogenetic analyses. The most striking difference is the relationship of *B. insularis* and the São Bernardo do Campo (SP) haplotypes (N26, N27 and N31) , occurring closest to N07 and N01 (Ubatuba-SP, and the most widespread haplotype from Nc, respectively) in the networks, and not within the Nc4 subclade as suggested by all phylogenetic analyses. As previously indicated by the phylogenetic trees, individuals bj164 (N04) and bj144 (S12)

appear as possible migrants, because although belonging to SG and NG populations, their haplotypes belong to Nc and Sc clusters, respectively (Fig. 6).

In the NCA, most of the lower-level clades were not significant in the exact contingency tests ($P > 0.05$) that evaluate the geographical associations between haplotype distribution and geographic location. A few higher-level clades were significant ($P < 0.01$), and their genetic signature, as inferred from Templeton's inference key is shown in table 2. Considering the whole distribution of *B. jararaca* populations the contingency test showed a highly significant association ($P < 0.0001$) possibly produced by a past fragmentation event. Northern clade was inferred to have suffered a contiguous range expansion, however within this clade the results for Nc5 and Nc1+Nc2 suggest, that the null hypothesis of no geographical association cannot be rejected and that the sampling pattern did not allow to discriminate between isolation by distance and long distance dispersal. On the other hand, the diversity of Sc and Sc1 may be explained by restricted gene flow with isolation by distance.

Coalescent analyses and divergence times

The value for θ estimated by the likelihood coalescent approach was 9.416, similar to Waterson´s θ of 11.196 (estimated with DNASP). Using the extreme values reported for the mutation rate in pitviper species (0.47% and 1.44% my^{-1}) we get a historical N_{ef} of 290,948 to 94,962 individuals. The migration rate was estimated as M = 0.22, very similar to that calculated from F_{ST} (Nm = 0.21).

The divergence time between the two major groups estimated with MDIV was 1.9 units, which varies from about 3.04 to 0.99 Mya depending on which of the above mentioned substitution rate is used. Accordingly, the T_{MRCA} was estimated at 2.5 units corresponding to 4.00 to 1.30 Mya. The molecular clock test implemented in LINTREE proves three sequences to be significantly different from the rest, showing lower rates. Excluding these sequences the

1 divergence time of Nc and Sc is estimated about 1.8 Mya (fig 7). Therefore, both methods
2 suggest a separation between a southern and a northern population of *B. jararaca* by the end
3 of Pliocene or in the beginning of Pleistocene (3.6 - 1.0 Mya). The diversification of the
4 clades into subclades began more recently about 0.8 Mya. It is interesting to note that most of
5 the subclades of Nc seem to have diverged almost simultaneously, about 0.5 Mya and
6 diversification within subclades started between 0.3 to 0.13 Mya.
7

Discussion

The genetic diversity found in the *B. jararaca* complex is relatively high when compared to those observed in other *Bothrops* species. Puorto *et al.* (2001) working with cytochrome *b* and ND 4 sequences of *B. leucurus* (1401 bp) found a low genetic diversity both within this species (p-distances ranging between 0.07% and 0.087%) and between *B. leucurus* and *B. atrox* (p-distances ranging from 2.07% to 2.45%). Working with the *B. atrox* complex, Wüster *el al*. (1999) using cytochrome *b* sequences (580 bp) found p-distance ranging from 0.4% to 4.8% (average distances not reported). The *B. atrox* complex has a wider distribution than the *B. jararaca* complex occupies a broader range of habitats and includes seven species. Despite these facts *B. jararaca* appears to be more diverse than the *B. atrox* complex.

Phylogeographic patterns

The phylogenetic analyses and network methods using cytochrome *b* sequences identified two highly distinct phylogroups within *B. jararaca*, which diverged 3.6 - 1.0 Mya. These clades are also consistent in our population analyses which shows only two individuals positioned in different populations in relation to their clades. One of these populations is represented by individuals from the São Paulo state northward and the other by the individuals from Paraná state southward (Fig. 1). The coalescent analysis and the F_{ST} value suggest a scenario of high isolation between these two groups. These two groups of populations are parapatric in the vicinity of the Paranapanema River, a tributary of Paraná River. However it is not clear if this river could be the barrier that caused the differentiation of these two groups. Although the present drainage of the Paraná River system was probably established in the Mid-Tertiary (Potter 1998) and underwent several fluctuations in water level and flow which could be compatible with our estimated divergence times, rivers of this

extension do not seem to be an effective dispersal barrier for *B. jararaca*. (Other rivers of similar size exist along the distribution of the species that do not seem to have affected the dispersal of the species.)

Hoge *et al.* (1977) describe an interesting variation in the number of ventral scales in *B. jararaca*. They analyzed 522 specimens from most of the distribution of the species and distinguished two overlapping groups, one with fewer scales in the southern parts of the distribution (SC and PR), and the other with a larger number of scales in the northern parts of the range (RJ, MG and ES). Specimens from SP state were morphologically intermediate between the two patterns. Hoge *et al.* (1977) considered average annual temperature to be functionally linked to the observed variation and referred to Fox (1948) and Fox & Fox (1961) who correlated temperature variation and somite formation in *Thamnophis elegans* populations. Considering the results of the present study the morphological distinction between southern and northern populations found by Hoge *et al*. (1977) may be better explained by a past fragmentation event of the species range into two refugial populations. The deep mtDNA lineage divergence found in *B. jararaca* suggests that, for conservation purposes, this species should be provisionally divided into two separate geographical units because 70% of its variability is found between the two groups. However it has been presumed by Sazima (1992) that females of *B. jararaca* show a sedentary tendency while males have a wandering pattern, so this phylogeographic pattern may be mainly matrilineal. Further studies including bi-parental markers and additional traits are necessary to better understand which management and taxonomic status should be assigned to this two major population groups.

Phylogeographical patterns within SG are conflicting. The results of the neutrality test (Table 1) support the scenario of a relatively large expansion, but the multimodal mismatch distribution (Fig. 5) apparently conflicts with this model, since a population expansion is

usually associated to unimodal pattern. However, a model of moderate bottleneck followed by a large size expansion could accommodate these conflicting results, since if two genetically distinct founders remained in the population, a ragged distribution could arise (Rogers & Harpending 1992). As emphasized by Mahoney (2004), mismatch distribution graphics may be considered much more for hypothesis formulation than as a statistical test of demographic history. This scenario for SG is also supported by the results of the autocorrelation analyses, which showed a cline (Fig. 5), that could suggest the occurrence of founder effects accompanied by range expansion with gene flow.

On the other hand, NG shows a more complex pattern, including a signal of range expansion (Table 2, Fig. 5), absence of both strong bottleneck and size expansion (Table 2, Fig. 4), as well as some geographical structuring (Fig. 3). Therefore, a possible scenario for this group of populations involves the absence of any strong bottleneck in its inception but with range expansion followed by regional size expansion with moderate gene flow.

Diversification patterns and paleoecology in the Brazilian Atlantic forest

There are several major hypotheses trying to explain the diversification of the South American fauna (see Moritz *et al*. 2000 for a review). The refugia model is one of the most widely debated one and suggests a pivotal role for Pleistocene climatic changes in promoting isolation and speciation (Haffer 1969, Müller 1973). Some molecular studies have found a divergence time correspondent to climatic changes throughout the Pleistocene (e.g. Ribas & Miyaki 2004). However, contrarily to the classical late Pleistocene Refuges hypothesis, most molecular studies in different areas of South America estimated older times for species/lineage divergence predating the Pleistocene (e.g. Cortés-Ortiz *et al.* 2003, Lara & Patton 2000, see Moritz *et al*. 2000). Thus the Pliocene seems to have been a period of active

speciation for many organisms in South America, possibly due to changes in the humidity levels, which ultimately promoted changes in the phytophysiognomic domains.

For the Brazilian Atlantic Forest there are few studies to test this hypothesis using data on patterns of divergence. Nevertheless, Lara & Patton (2000) analyzing the results of Vasconcelos *et al*. (1992) tried to explain the divergence of three major clades of spiny rats of the genus *Triomys* dated between 1.6 and 7.4 million years ago (Mya) based on the uplift of the coastal mountains and the interruption of precipitation in Southeastern Brazil by the early Pliocene at about 5.6 Mya which coincides with the transition from tropical humid to semi-arid or arid conditions. This uniform stable dry climate persisted until the Middle to Upper Pliocene when a gradual increase in humidity happened. They concluded the deep divergence seen in *Triomys* to be older than the late Pleistocene and to be more likely caused by events that dating back to Miocene/Pliocene times (Lara & Patton 2000).

B. jararaca is a forest dweller (Sazima 1992) and is likely that such a fragmentation, in drier periods, could have divided a single widespread group into several isolated populations. This scenario with the assumption of restricted gene flow could have persisted until the establishment of more humid climatic conditions in the Early Pleistocene. The results of the present study suggest that the divergence of the two lineages of *B. jararaca* may be associated with an older fragmentation event in the Brazilian Atlantic Forest during the Pliocene similarly to the findings reported by Lara & Patton (2000). On the other hand, the initial diversification time estimated for the subclades ranges from 200,000 to 130,000 years ago suggesting that the genetic and geographic variability of subpopulations of *B. jararaca* may have been shaped by late Pleistocene climatic oscillations.

This model does not fit the clinal pattern detected by autocorrelation analyses with the whole dataset (Fig. 5). A cline is defined by relationships across geographical distances and genetic divergence in a gradient of correlation without fragmentation. . However, one of the

three principal causes promoting a clinal pattern comprises fragmentation with a weak and differential bottleneck on two populations followed by a moderated range expansion and subsequent restricted gene flow (Barbujani and Bertorelle 1996) thus supporting the hypothesis suggested herein.

The origin and status of the insular jararacas and the validity of *Bothrops jararaca*

According to Marques *et al.* (2002) the two insular species may have been originated from populations of a *B. jararaca*-like ancestor from the eastern coast of Brazil that may have been isolated on islands during one of the last sea level oscillations in the late Pleistocene. Our results corroborate the idea of a very recent isolation of *B. insularis* and *B. alcatraz* but, based on the haplotype network; it is evident that these two species derived from *B. jararaca* itself and not from another ancestral species. Although with the present data it is not possible to test whether both species originated from one or from two different source populations, the results of the present study suggest an independent origin. At first, the two haplotypes from *B. insularis* (N27 and N26, the latter shared with mainland jararaca) belong to a very divergent subclade (Nc4a, fig. 03) with a very limited distribution being restricted to a single sampling site (São Bernardo do Campo, SP). The single haplotype from *B. alcatraz* is located within a distant subclade (N31) although this one is identical with the most frequent and widely distributed mainland haplotype (N01). The shared haplotypes from both species occur in mainland sampling sites very near Queimada Grande and Alcatraz islands. The low genetic diversity found within each island suggests a possible founder effect for the origin of these insular species. Additional data are needed to test this hypothesis.

Does the phylogenetic position of *B. insularis* and *B. alcatraz* mtDNA haplotypes inside *B. jararaca* populations represent a problem for the delimitation or validity of these

species, as suggested by Salomão *et al.* (1997)? In fact, the gene genealogy as described herein is very likely when differentiation involves a small population isolated from a larger ancestral one. Actually a large number of such "paraphyletic" species phylogenies based on mtDNA are known (Avise 2000). Johnson *et al.* (2000) discussing island biogeography models demonstrated that phylogenies of island and mainland populations showing paraphyly of mainland alleles occur when the mainland population is very large and exhibits geographic structure and/or when a short time has passed since colonization. Rapid speciation events were also recognized by Moritz (1994) as a restriction to the definition of Evolutionary Significant Units based on the reciprocal monophyly concept, but his argument is that it does not affect conservation priorities because these taxa are frequently recognized as different species based on other biological criteria. Therefore, the paraphyly of the mtDNA alleles and the recent peripatric model of differentiation or speciation of the two island jararacas as suggested herein should not influence the conservation status of any such population. Many striking biological features observed in *B. insularis* and *B. alcatraz* (Marques *et al.* 2002) are not found in mainland jararacas, thus supporting their consideration as valid taxa and distinct evolutionary units for conservation.

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Figure Legends

Fig. 1 Geographical distribution of the *Bothrops jararaca* complex with sampling localities for this study. Light gray area represents the geographical distribution of *Bothrops jararaca*. Squares indicate individuals from the northern clade and crosses indicate individuals from the southern clade. Solid line indicates the main genetic barrier as defined by SAMOVA. Brazilian states: RS – Rio Grande do Sul, SC – Santa Catarina, PR – Paraná, SP – São Paulo, RJ – Rio de Janeiro, ES – Espírito Santo, MG – Minas Gerais, MS - Mato Grosso do Sul, and GO - Goiáis.

Fig. 2 Isometric plots of the number of pairwise transitions against Tamura-Nei distances showing levels of saturation at all codon positions (open squares) and 3rd codon position (black triangles) in mtDNA sequences of *B. jararaca* group. Circumference indicates only the ingroup.

Fig. 3 The phyML tree with TN+I model for cyt *b* mitochondrial gene in *B. jararaca* complex and outgroups. Letters and numbers in the terminals represents the haplotype number (S from southern clade and N from northern clade) and the Brazilian states (RS – Rio Grande do Sul, SC – Santa Catarina, PR – Paraná, SP – São Paulo, RJ – Rio de Janeiro, ES – Espírito Santo, and MG – Minas Gerais). Black bars indicate haplotypes from the southern group, open bars indicate haplotypes from northern group and crossed square indicate haplotypes shares by southern and northern groups. Numbers near internal branches are support values from phyML, Treefinder, Tree-puzzle, MetaPIGA, NJ, BI, MP and ML trees, respectively. (*) value lower than 50%.

Fig. 4 Mismatch distributions for the (A) Northern clade; (B) Southern clade; (C) Whole distribution.

Fig. 5 Correlogram showing the coefficient of spatial autocorrelation for whole *B. jararaca* distribution (circles), southern groups (triangles) and northern groups (squares). *, $P < 0.05$, solid symbol, P <0,001; open symbol, non significant.

Fig. 6 Median-joining network showing the two clades within *B. jararaca.* Letters and numbers represent the haplotype number (S from southern clade and N from northern clade).

Fig. 7 Linearized neighbor joining tree showing the divergence time among clades and subclades. Numbers in italic indicate divergence time calculated using the medium value for substitution rates; numbers in the gray bars represent divergence time calculated using our extreme substitution rates (0.47% and 1.44% my^{-1}).

Table 1 Summary statistics observed in southern group (SG), northern group (NG) and the whole distribution of *Bothrops jararaca* (SG/NG).

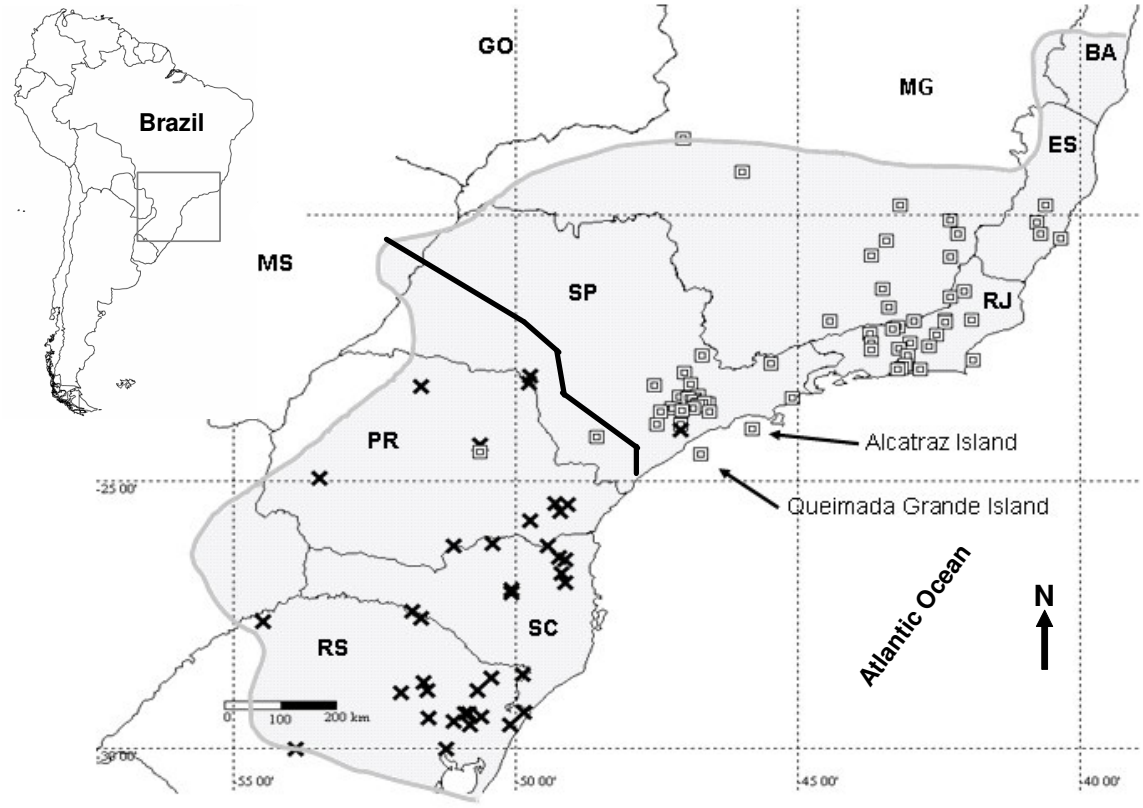
	N	S	h	Hd	π	D	F'	D'
SG	76	32	15	0.862 ± 0.022	0.00761 ± 0,00074	-0.99754	-2.71499*	-3.05309*
NG	95	37	31	0.904 ± 0,021	0.01081 ± 0.00065	-0.27207	0.16639	0.40752
whole	171	61	45	0.943 ±0.008	0.02068 ± 0.00047	0.47881	0.58120	0.48785

N, number of sequences; S, number of variable sites; h, number of haplotypes; Hd, haplotype diversity; **π**, nucleotide diversity; D, Tajimas' D; F', Fu and Li's F; D', Fu and Li's D. * P < 0.05.

Table 2 Demographic Inference chain results from the nested clade analysis.

Clade	Chain of inference	Inference
Total Cladogram	1-2-3-5-15-no	Past Fragmentation
Northern Clade	1-2-11-12-13-Yes	Contiguous Range Expansion
Nc5	1-No	The null hypothesis cannot be rejected
Nc1+Nc2	1-2-3-4-No	Sampling inadequate to discriminate between Isolation by distance X Long distance dispersion
Southern Clade	1-2-3-5-6-7-Yes	Restricted gene flow with isolation by distance
Sc1	1-2-11-17-4-No	Restricted gene flow with isolation by distance
Sc2	1-No	The null hypothesis cannot be rejected

Fig. 1


Brazil
GO
MG
BA
ES
MS
SP
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PR
Alcatraz Island
Queimada Grande Island
Atlantic Ocean
N
SC
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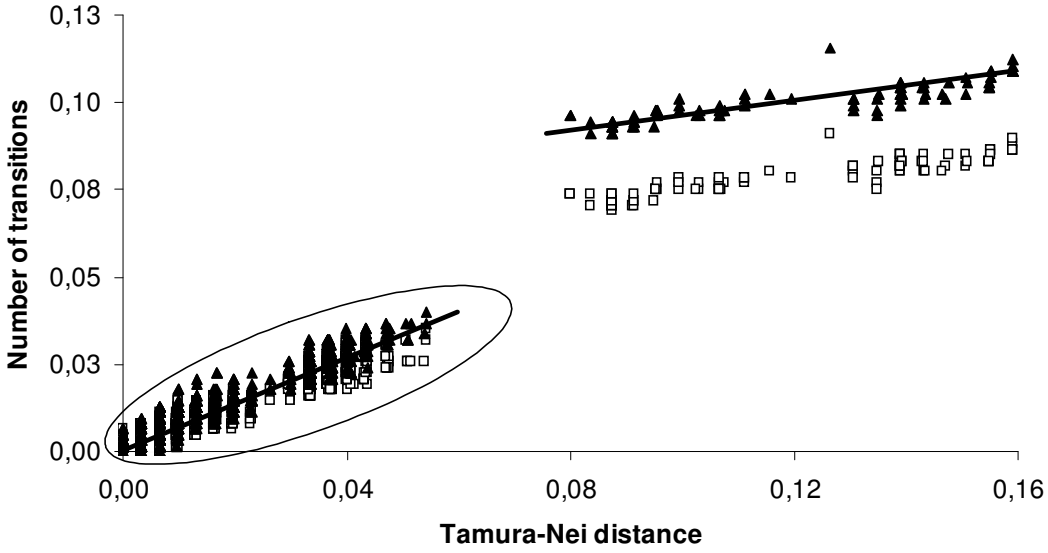
0
100
200 km
25 00'
30 00'
55 00'
50 00'
45 00'
40 00'

Fig. 2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0,10
0,08
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0,00
Number of transitions
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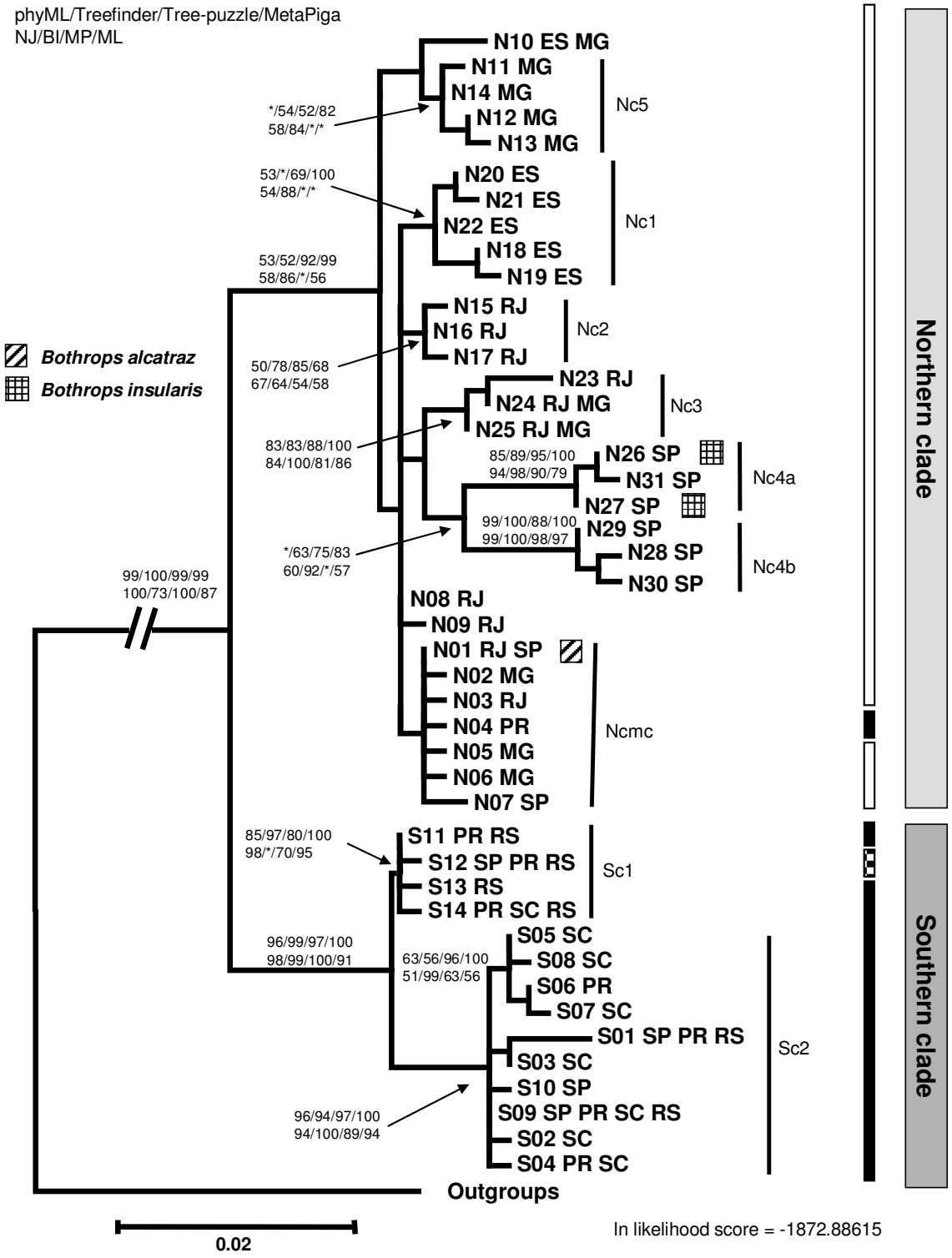
0,04
0,08
0,12
0,16
Tamura-Nei distance

Fig. 03


phyML/Treefinder/Tree-puzzle/MetaPiga
NJ/BI/MP/ML
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N11 MG
N14 MG
N12 MG
N13 MG
Nc5
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58/84/*/*
53/*/69/100
54/88/*/*
N20 ES
N21 ES
N22 ES
N18 ES
N19 ES
Nc1
53/52/92/99
58/86/*/56
N15 RJ
N16 RJ
N17 RJ
Nc2
50/78/85/68
67/64/54/58
Bothrops alcatraz
Bothrops insularis
N23 RJ
N24 RJ MG
N25 RJ MG
Nc3
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84/100/81/86
85/89/95/100
94/98/90/79
N26 SP
N31 SP
N27 SP
Nc4a
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99/100/98/97
N29 SP
N28 SP
N30 SP
Nc4b
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100/73/100/87
N08 RJ
N09 RJ
N01 RJ SP
N02 MG
N03 RJ
N04 PR
N05 MG
N06 MG
N07 SP
Ncmc
85/97/80/100
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S12 SP PR RS
S13 RS
S14 PR SC RS
Sc1
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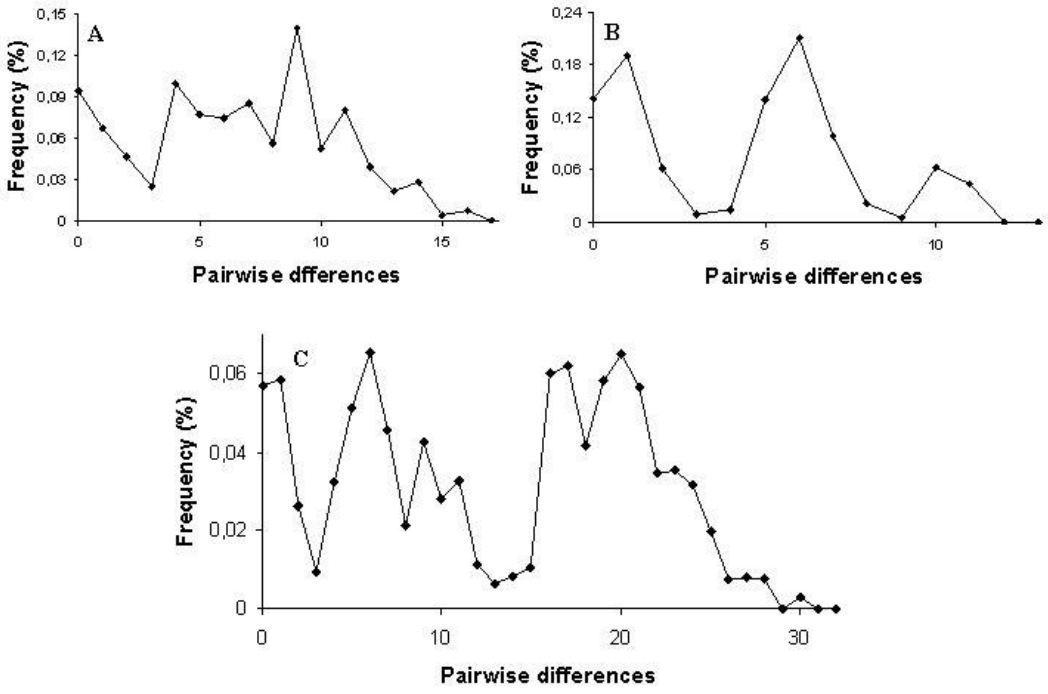
98/99/100/91
63/56/96/100
51/99/63/56
S05 SC
S08 SC
S06 PR
S07 SC
S01 SP PR RS
S03 SC
S10 SP
S09 SP PR SC RS
S02 SC
S04 PR SC
Sc2
96/94/97/100
94/100/89/94
Outgroups
0.02
ln likelihood score = -1872.88615
Northern clade
Southern clade

Fig. 4


A
Frequency (%)
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Pairwise dfferences
B
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0
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10
Pairwise differences
C
Frequency (%)
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0,02
0
0
10
20
30
Pairwise differences

Fig. 5

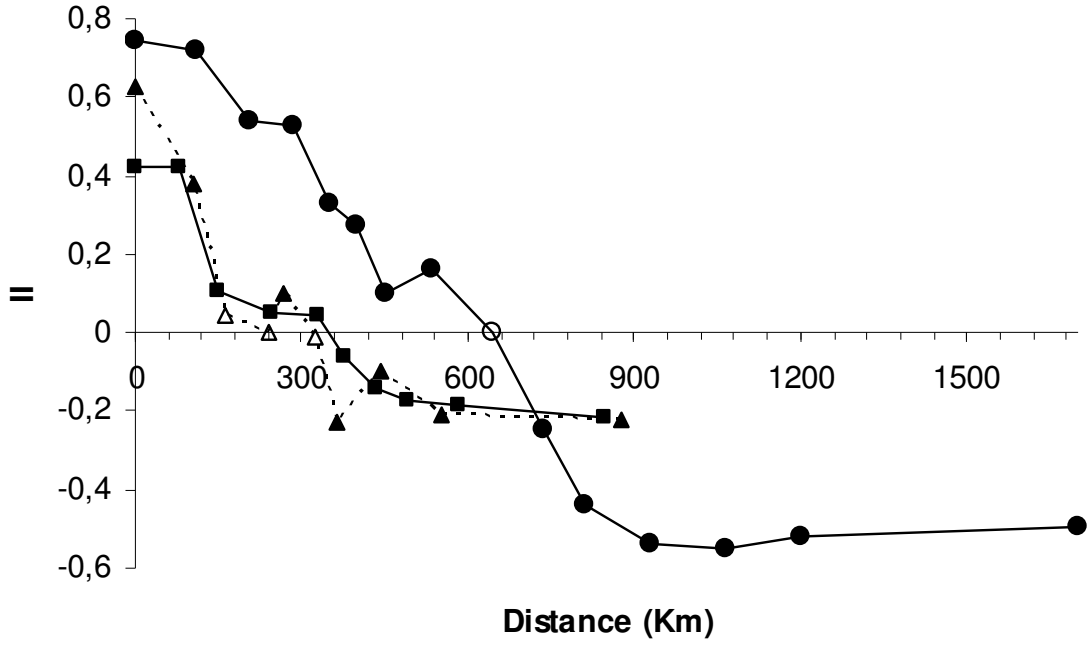
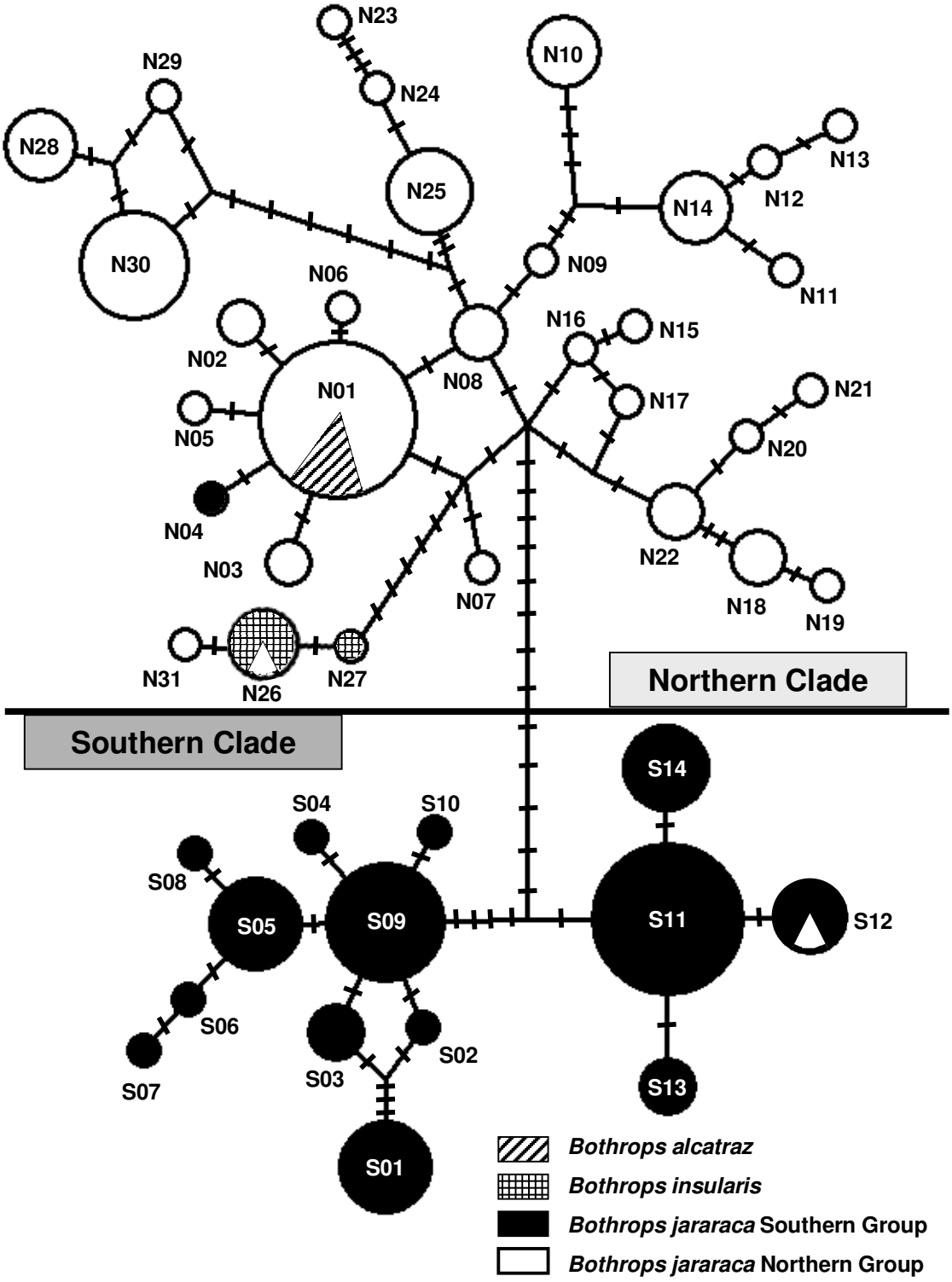


Fig. 06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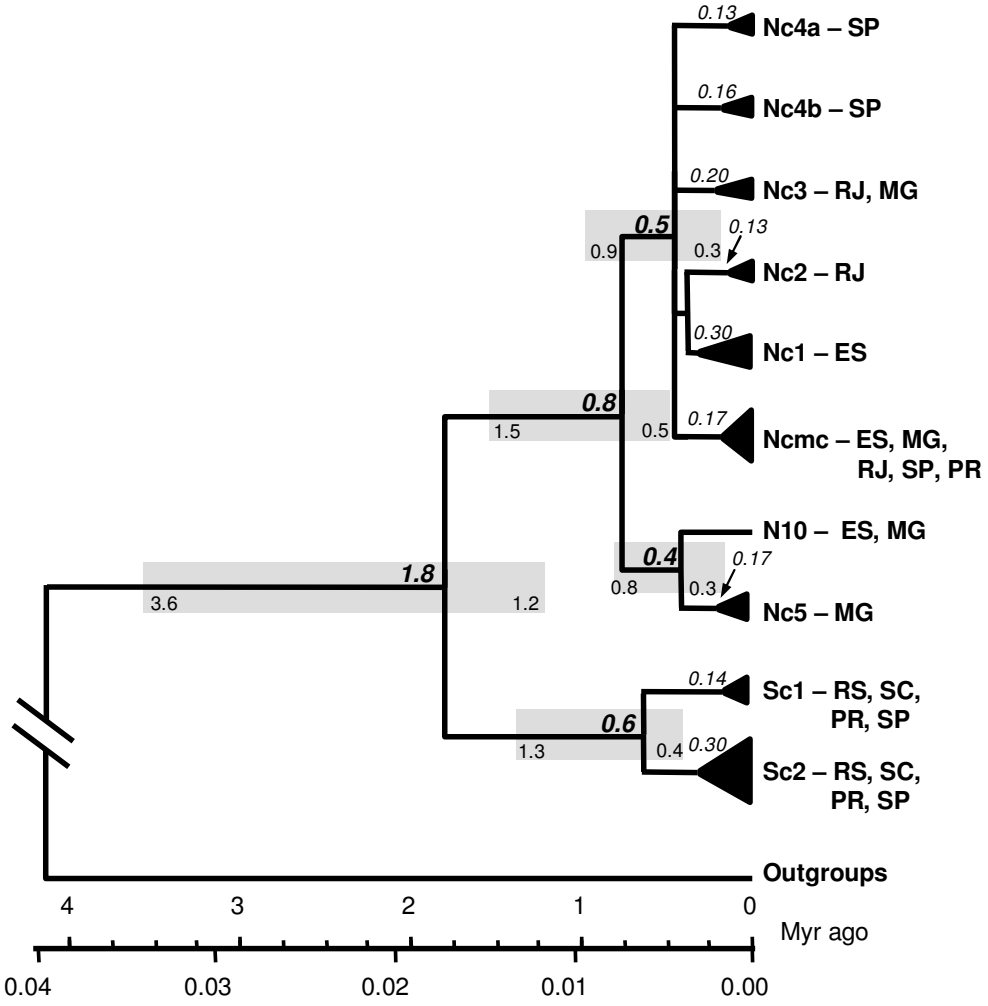
N04
N22
N03
N07
N18
N19
N31
N26
N27
Northern Clade
Southern Clade
S14
S04
S10
S08
S05
S09
S11
S12
S06
S02
S03
S13
S07
S01
Bothrops alcatraz
Bothrops insularis
Bothrops jararaca Southern Group
Bothrops jararaca Northern Group

Fig. 7


0.13
Nc4a – SP
0.16
Nc4b – SP
0.20
Nc3 – RJ, MG
0.5
0.9
0.3
0.13
Nc2 – RJ
0.30
Nc1 – ES
0.8
1.5
0.5
0.17
Ncmc – ES, MG,
RJ, SP, PR
N10 – ES, MG
0.4
0.8
0.3
0.17
Nc5 – MG
1.8
3.6
1.2
0.14
Sc1 – RS, SC,
PR, SP
0.6
1.3
0.4
0.30
Sc2 – RS, SC,
PR, SP
Outgroups
4
3
2
1
0
Myr ago
0.04
0.03
0.02
0.01
0.00

Appendix 1

Localities, haplotypes and vouchers for specimens used in this paper.

Nh	NºCBGM	Species	Nº voucher	Localities name	states	Longitude	Latitude
S01	bj160	*Bothrops jararaca*	CMM - 21	Telêmaco Borba	PR	50°37'02''W	24°20'15''S
S01	bj161	*Bothrops jararaca*	CMM - 22	Telêmaco Borba	PR	50°37'02"W	24°20'15"S
S01	bj162	*Bothrops jararaca*	CMM - 23	Telêmaco Borba	PR	50°37'02"W	24°20'15"S
S01	bj163	*Bothrops jararaca*	CMM - 24	Telêmaco Borba	PR	50°37'02"W	24°20'15"S
S01	bj166	*Bothrops jararaca*	CMM - 27	Astorga	PR	51°39'53"W	23°14'22"S
S01	bj195	*Bothrops jararaca*	CMM - 56	Cachoeira do Sul	RS	53°53'46"W	30°02'22"S
S01	bj225	*Bothrops jararaca*	CMM - 88	Machadinho	RS	51°40'00"W	27°34'25"S
S01	bj327	*Bothrops jararaca*	IB - *	Chavantes	SP	49°43'02"W	23°02'00"S
S02	bj230	*Bothrops jararaca*	IB - 67665	Blumenau	SC	49°05'48"W	26°55'05"S
S03	bj237	*Bothrops jararaca*	IB - *	Canoinhas	SC	50°23'38"W	26°10'52"S
S03	bj238	*Bothrops jararaca*	IB - 68554	Canoinhas	SC	50°23'38"W	26°10'52"S
S03	bj240	*Bothrops jararaca*	IB - 68559	Canoinhas	SC	50°23'38"W	26°10'52"S
S04	bj091	*Bothrops jararaca*	IB - 66557	Jaraguá do Sul	SC	49°05'50"W	26°29'01"S
S04	bj168	*Bothrops jararaca*	CMM - 29	Curitiba	PR	49°17'03"W	25°25'50"S
S05	bj090	*Bothrops jararaca*	IB - 66567	Jaraguá do Sul	SC	49°05'50"W	26°29'01"S
S05	bj092	*Bothrops jararaca*	IB - 66556	Jaraguá do Sul	SC	49°05'50"W	26°29'01"S
S05	bj093	*Bothrops jararaca*	IB - 65314	Corupa	SC	49°13'13"W	26°26'47"S
S05	bj095	*Bothrops jararaca*	IB - 65313	Corupa	SC	49°13'13"W	26°26'47"S
S05	bj148	*Bothrops jararaca*	CMM - 9	São Bento do Sul	SC	49°24'36"W	26°13'06"S
S05	bj150	*Bothrops jararaca*	CMM - 11	São Bento do Sul	SC	49°24'36"W	26°13'06"S
S05	bj153	*Bothrops jararaca*	CMM - 14	Pomerode	SC	49°10'26"W	26°45'16"S
S05	bj217	*Bothrops jararaca*	CMM - 80	Piratuba	SC	51°48'09"W	72°70'00"S
S06	bj175	*Bothrops jararaca*	CMM - 36	São José dos Pinhais	PR	49°10'44"W	25°34'39"S
S07	bj154	*Bothrops jararaca*	CMM - 15	Pomerode	SC	49°10'26"W	26°45'16"S
S08	bj094	*Bothrops jararaca*	IB - 65323	Corupa	SC	49°13'13"W	26°26'47"S
S09	bj087	*Bothrops jararaca*	IB - 66809	Guapiava	SP	48°32'16"W	24°11'12"S
S09	bj088	*Bothrops jararaca*	IB - 66794	Guapiava	SP	48°32'16"W	24°11'12"S
S09	bj149	*Bothrops jararaca*	CMM - 10	São Bento do Sul	SC	49°24'36"W	26°13'06"S
S09	bj152	*Bothrops jararaca*	CMM - 13	Pomerode	SC	49°10'26"W	26°45'16"S
S09	bj167	*Bothrops jararaca*	CMM - 28	Astorga	PR	51°39'53"W	23°14'22"S
S09	bj169	*Bothrops jararaca*	CMM - 30	União da Vitória	PR	51°04'03"W	26°14'00"S
S09	bj199	*Bothrops jararaca*	CMM - 60	Timbó do Sul	SC	49°51'45"W	28°49'17"S
S09	bj212	*Bothrops jararaca*	CMM - 75	Veranópolis	RS	51°32'55"W	28°56'19"S
S09	bj228	*Bothrops jararaca*	IB - 67666	Blumenau	SC	49°05'48"W	26°55'05"S
S09	bj229	*Bothrops jararaca*	IB - 67663	Blumenau	SC	49°05'48"W	26°55'05"S
S09	bj231	*Bothrops jararaca*	IB - 67662	Blumenau	SC	49°05'48"W	26°55'05"S
S09	bj235	*Bothrops jararaca*	IB - *	Canoinhas	SC	50°23'38"W	26°10'52"S
S09	bj328	*Bothrops jararaca*	IB -69878	Canoinhas	SC	50°23'38"W	26°10'52"S
S10	bj089	*Bothrops jararaca*	IB - 66792	Guapiava	SP	48°32'16"W	24°11'12"S
S11	bj146	*Bothrops jararaca*	CMM - 7	Ribeirão Claro	PR	49°44'00"W	23°10'04"S
S11	bj171	*Bothrops jararaca*	CMM - 32	Curitiba	PR	49°17'03"W	25°25'50"S
S11	bj177	*Bothrops jararaca*	CMM - 38	Nova Petrópolis	RS	51°37'01"W	27°07'00"S
S11	bj178	*Bothrops jararaca*	CMM - 39	Nova Petrópolis	RS	51°37'01"W	27°07'00"S
S11	bj180	*Bothrops jararaca*	CMM - 41	Nova Petrópolis	RS	51°37'01"W	27°07'00"S
S11	bj182	*Bothrops jararaca*	CMM - 43	Nova Petrópolis	RS	51°37'01"W	27°07'00"S
S11	bj186	*Bothrops jararaca*	CMM - 47	Nova Petrópolis	RS	51°37'01"W	27°07'00"S
S11	bj188	*Bothrops jararaca*	CMM - 49	São Francisco de Paula	RS	50°34'45"W	29°26'35"S
S11	bj189	*Bothrops jararaca*	CMM - 50	São Francisco de Paula	RS	50°34'45"W	29°26'35"S
S11	bj196	*Bothrops jararaca*	CMM - 57	Canela	RS	50°48'34"W	29°21'48"S
S11	bj197	*Bothrops jararaca*	CMM - 58	São Francisco de Paula	RS	50°34'45"W	29°26'35"S
S11	bj198	*Bothrops jararaca*	CMM - 59	Gramado	RS	50°52'38"W	29°22'33"S
S11	bj200	*Bothrops jararaca*	CMM - 61	Dom Pedro de Alcântara	RS	49°50'57"W	29°20'59"S
S11	bj203	*Bothrops jararaca*	CMM - 64	Canela	RS	50°48'34"W	29°21'48"S
S11	bj204	*Bothrops jararaca*	CMM - 65	Porto Alegre	RS	51°13'01"W	30°02'23"S
S11	bj205	*Bothrops jararaca*	CMM - 67	Terra de Areia	RS	50°04'06"W	29°35'00"S
S11	bj209	*Bothrops jararaca*	CMM - 72	Tucunduva	RS	54°26'59"W	27°39'02"S

S11	bj213	*Bothrops jararaca*	CMM - 76	Igrejinha	RS	50°47'38"W	29°34'45"S
S11	bj216	*Bothrops jararaca*	CMM - 79	Morro Reuter	RS	51°05'01"W	29°31'57"S
S11	bj219	*Bothrops jararaca*	CMM - 82	Salvador do Sul	RS	51°31'00"W	29°27'04"S
S11	bj220	*Bothrops jararaca*	CMM - 83	Salvador do Sul	RS	51°31'00"W	29°27'04"S
S12	bj144	*Bothrops jararaca*	CMM - 5	Juquitiba	SP	47°03'04"W	23°57'00"S
S12	bj174	*Bothrops jararaca*	CMM - 35	Piraquara	PR	49°03'53"W	25°27'09"S
S12	bj192	*Bothrops jararaca*	CMM - 53	Cazuza Ferreira	RS	50°39'17"W	28°55'59"S
S12	bj218	*Bothrops jararaca*	CMM - 81	Bom Jesus	RS	50°24'03"W	28°41'58"S
S12	bj221	*Bothrops jararaca*	CMM - 84	Anta Gorda	RS	52°01'06"W	28°59'02"S
S13	bj193	*Bothrops jararaca*	CMM - 54	Cazuza Ferreira	RS	50°39'17"W	28°55'59"S
S13	bj194	*Bothrops jararaca*	CMM - 55	Cazuza Ferreira	RS	50°39'17"W	28°55'59"S
S13	bj207	*Bothrops jararaca*	CMM - 70	Machadinho	RS	51°40'00"W	27°34'25"S
S14	bj170	*Bothrops jararaca*	CMM - 31	Lapa	PR	49°43'52"W	25°45'53"S
S14	bj172	*Bothrops jararaca*	CMM - 33	Cascavel	PR	53°27'08"W	24°58'02"S
S14	bj202	*Bothrops jararaca*	CMM - 63	Tucunduva	RS	54°26'59"W	27°39'02"S
S14	bj210	*Bothrops jararaca*	CMM - 73	Tuncunuva	RS	54°26'59"W	27°39'02"S
S14	bj211	*Bothrops jararaca*	CMM - 74	Nova Prata	RS	51°36'05"W	28°47'04"S
S14	bj232	*Bothrops jararaca*	IB - 68275	Fraiburgo	SC	50°03'34"W	27°03'20"S
S14	bj233	*Bothrops jararaca*	IB - 68276	Fraiburgo	SC	50°03'34"W	27032'00"S
S30	bj157	*Bothrops jararaca*	CMM - 18	Juquitiba	SP	47°03'04"W	23°57'00"S
N01	bz001	*Bothrops alcatraz*	IB - 00061C0EBA	Ilha de Alcatrazes	SP	45°46'35"W	24°01'50"S
N01	bz002	*Bothrops alcatraz*	IB - 0061C17E9	Ilha de Alcatrazes	SP	45°46'35"W	24°01'50"S
N01	bz003	*Bothrops alcatraz*	IB - 000618E21E	Ilha de Alcatrazes	SP	45°46'35"W	24°01'50"S
N01	bz004	*Bothrops alcatraz*	IB - 000610D8E2	Ilha de Alcatrazes	SP	45°46'35"W	24°01'50"S
N01	bz005	*Bothrops alcatraz*	IB - 00061C0D7D	Ilha de Alcatrazes	SP	45°46'35"W	24°01'50"S
N01	bj109	*Bothrops jararaca*	IVB - 060/02	Nova Friburgo	RJ	42°31'39"W	22°15'49"S
N01	bj110	*Bothrops jararaca*	IVB - 186/00	Niteroi	RJ	43°07'55"W	22°54'11"S
N01	bj111	*Bothrops jararaca*	IVB - 75/99	Niteroi	RJ	43°07'55"W	22°54'11"S
N01	bj114	*Bothrops jararaca*	IVB - 184/02	Teresópolis	RJ	42°58'30"W	22°24'57"S
N01	bj116	*Bothrops jararaca*	IVB - 115/02	Rio de Janeiro	RJ	43°11'54"W	22°54'34"S
N01	bj118	*Bothrops jararaca*	IVB - 129/99	Niteroi	RJ	43°07'55"W	22°54'11"S
N01	bj119	*Bothrops jararaca*	IVB - 31/01	Valença	RJ	43°42'16"W	22°14'44"S
N01	bj120	*Bothrops jararaca*	IVB - 16/01	Teresópolis	RJ	42°58'30"W	22°24'57"S
N01	bj130	*Bothrops jararaca*	IVB - 85/99	São Gonçalo	RJ	43°04'22"W	22°50'07"S
N01	bj138	*Bothrops jararaca*	IVB - 195/00	Magé	RJ	43°02'23"W	22°39'38"S
N01	bj139	*Bothrops jararaca*	IVB - 146/01	Santa Maria Madalena	RJ	42°01'38"W	21°57'29"S
N01	bj140	*Bothrops jararaca*	IVB - 61/01	Maricá	RJ	42°49'05"W	22°54'59"S
N01	bj227	*Bothrops jararaca*	IB - *	Monte Alegre do Sul	SP	46°41'03"W	22°39'55"S
N01	bj244	*Bothrops jararaca*	IBV - 183/01	Petrópolis	RJ	43°11'08"W	22°31'13"S
N01	bj257	*Bothrops jararaca*	IVB - 331/02	Niterói	RJ	43°07'55"W	22°54'11"S
N01	bj258	*Bothrops jararaca*	IVB - 210/02	Niterói	RJ	43°07'55"W	22°54'11"S
N01	bj288	*Bothrops jararaca*	IB - *	Jundiaí	SP	46°52'24"W	23°11'58"S
N01	bj292	*Bothrops jararaca*	IB - *	Atibaia	SP	46°33'25"W	23°07'26"S
N01	bj300	*Bothrops jararaca*	IB - 68793	Monte Alegre do Sul	SP	46°41'03"W	22°39'55"S
N01	bj301	*Bothrops jararaca*	IB - 68790	Valinhos	SP	46°59'28"W	22°58'27"S
N02	bj272	*Bothrops jararaca*	FUNED - 9902	Bocaina de Minas	MG	44°24'06"W	22°10'02"S
N02	bj274	*Bothrops jararaca*	FUNED - 9934	São Francisco da Gloria	MG	42°16'00"W	20°47'58"S
N03	bj137	*Bothrops jararaca*	IVB - 66/01	Eng. Paulo de Frontin	RJ	43°40'58"W	22°33'10"S
N03	bj249	*Bothrops jararaca*	IVB - 003/02	Santa Cruz da Serra	RJ	42°17'04"W	21°33'00"S
N04	bj164	*Bothrops jararaca*	CMM - 25	Telêmaco Borba	PR	50°37'02"W	24°20'15"S
N05	bj285	*Bothrops jararaca*	FUNED - *	Paraíba do Sul	MG	43°17'33"W	22°09'37"S
N06	bj126	*Bothrops jararaca*	IVB - 177/02	Juiz de Fora	MG	43°21'19"W	21°45'11"S
N07	bj294	*Bothrops jararaca*	IB - *	Ubatuba	SP	45°04'34"W	23°27'09"S
N08	bj117	*Bothrops jararaca*	IVB - 202/01	Vassouras	RJ	43°40'04"W	22°24'37"S
N08	bj133	*Bothrops jararaca*	IVB - 185/02	Petrópolis	RJ	43°11'08"W	22°31'13"S
N08	bj245	*Bothrops jararaca*	IBV - 194/00	Petrópolis	RJ	43°11'08"W	22°31'13"S
N09	bj135	*Bothrops jararaca*	IVB - 179/01	Areal - Três Rios	RJ	43°12'29"W	22°06'57"S
N10	bj100	*Bothrops jararaca*	IB - 66959	Santa Maria Jetiba	ES	40°44'00"W	20°02'00"S
N10	bj102	*Bothrops jararaca*	IB - 67045	Domingos Martins	ES	40°40'05"W	20°21'59"S
N10	bj103	*Bothrops jararaca*	IB - 67014	Domingos Martins	ES	40°40'05"W	20°21'59"S
N10	bj266	*Bothrops jararaca*	FUNED - 9415	Patos de Minas	MG	46°30'48"W	18°35'21"S
N10	bj269	*Bothrops jararaca*	FUNED - 9502	Santana do Monte	MG	43°41'03"W	20°46'59"S

N11	bj278	*Bothrops jararaca*	FUNED - 9925	Matutina	MG	45°57'59"W	19°13'02"S
N12	bj267	*Bothrops jararaca*	FUNED - 9704	João Monlevade	MG	43°09'24"W	19°50'09"S
N13	bj265	*Bothrops jararaca*	FUNED - 9901	Santa Bárbara	MG	43°24'39"W	19°58'22"S
N14	bj268	*Bothrops jararaca*	FUNED - 9909	Caputira	MG	42°16'04"W	20°10'02"S
N14	bj271	*Bothrops jararaca*	FUNED - 9919	Matutina	MG	45°57'59"W	19°13'02"S
N14	bj276	*Bothrops jararaca*	FUNED - 9924	Matutina	MG	45°57'59"W	19°13'02"S
N14	bj277	*Bothrops jararaca*	FUNED - 9922	Matutina	MG	45°57'59"W	19°13'02"S
N14	bj279	*Bothrops jararaca*	FUNED - 9927	Matutina	MG	45°57'59"W	19°13'02"S
N15	bj127	*Bothrops jararaca*	IVB - 190/02	Campos Novos - Búzios	RJ	41°53'00"W	22°45'04"S
N16	bj129	*Bothrops jararaca*	IVB - 86/99	Búzios	RJ	41°53'00"W	22°45'04"S
N17	bj108	*Bothrops jararaca*	IB – B1329	Barra do Pirai	RJ	43°49'31"W	22°28'16"S
N18	bj098	*Bothrops jararaca*	IB - 66970	Santa Maria Jetiba	ES	40°44'00"W	20°02'00"S
N18	bj099	*Bothrops jararaca*	IB - 66953	Santa Maria Jetiba	ES	40°44'00"W	20°02'00"S
N18	bj128	*Bothrops jararaca*	IVB - 53/99	Santa Tereza	ES	40°34'56"W	19°56'11"S
N19	bj123	*Bothrops jararaca*	IVB - 95/02	Vitória	ES	40°18'25"W	20°18'21"S
N20	bj250	*Bothrops jararaca*	IVB - 133/03	Fazenda Santa Maria	ES	40°40'05"W	20°21'59"S
N22	bj104	*Bothrops jararaca*	IB - 67053	Domingos Martins	ES	40°40'05"W	20°21'59"S
N22	bj115	*Bothrops jararaca*	IVB - 094/02	Vitória	ES	40°18'25"W	20°18'21"S
N22	bj262	*Bothrops jararaca*	IVB - *	Fazenda Santa Maria	ES	40°40'05"W	20°21'59"S
N22	bj263	*Bothrops jararaca*	IVB - *	Fazenda Santa Maria	ES	40°40'05"W	20°21'59"S
N23	bj113	*Bothrops jararaca*	IVB - 222/01	Niteroi	RJ	43°07'55"W	22°54'11"S
N24	bj121	*Bothrops jararaca*	IVB - 162/00	São João do Manhaçu	MG	42°08'57"W	20°23'03"S
N25	bj125	*Bothrops jararaca*	IVB - 268/02	Cachoeira de Macacu	RJ	42°39'09"W	22°29'14"S
N25	bj131	*Bothrops jararaca*	IVB - 117/99	Cantagalo	RJ	42°21'54"W	21°59'03"S
N25	bj132	*Bothrops jararaca*	IVB - 166/01	Sapucai	RJ	42°55'04"W	22°00'27"S
N25	bj261	*Bothrops jararaca*	IVB - 277/02	Cordeiro	RJ	42°21'49"W	22°02'06"S
N25	bj270	*Bothrops jararaca*	FUNED - 9916	Juíz de Fora	MG	43°21'19"W	21°45'11"S
N25	bj283	*Bothrops jararaca*	FUNED - *	Rio Espera	MG	43°29'05"W	20°50'57"S
N25	bj284	*Bothrops jararaca*	FUNED - *	Rio Espera	MG	43°29'05"W	20°50'57"S
N26	bis002	*Bothrops insularis*	IB - *	Ilha Queimada Grande	SP	46°42'00"W	24°30'00"S
N26	bis003	*Bothrops insularis*	IB – KZ952	Ilha Queimada Grande	SP	46°42'00"W	24°30'00"S
N26	bis004	*Bothrops insularis*	IB – KZ961	Ilha Queimada Grande	SP	46°42'00"W	24°30'00"S
N26	bis005	*Bothrops insularis*	IB – KZ950	Ilha Queimada Grande	SP	46°42'00"W	24°30'00"S
N26	bis006	*Bothrops insularis*	IB – KZ951	Ilha Queimada Grande	SP	46°42'00"W	24°30'00"S
N26	bis007	*Bothrops insularis*	IB – KZ959	Ilha Queimada Grande	SP	46°42'00"W	24°30'00"S
N26	bj010	*Bothrops jararaca*	IB - *	São Bernardo do Campo	SP	46°32'39"W	23°42'45"S
N27	bis001	*Bothrops insularis*	IB - *	Ilha Queimada Grande	SP	46°42'00"W	24°30'00"S
N28	bj009	*Bothrops jararaca*	IB - *	Araçariguama	SP	47°04'09"W	23°26'00"S
N28	bj239	*Bothrops jararaca*	IB - *	Ribeirão Grande	SP	45°27'03"W	22°47'58"S
N28	bj289	*Bothrops jararaca*	IB - *	Piedade	SP	47°24'41"W	23°42'44"S
N28	bj295	*Bothrops jararaca*	IB - *	Ibiuna	SP	47°13'06"W	23°39'02"S
N28	bj297	*Bothrops jararaca*	IB - Bjf001-15	Caucaia do Alto	SP	47°02'00"W	23°40'57"S
N29	bj287	*Bothrops jararaca*	IB - *	Tapiraí	SP	47°28'59"W	23°57'05"S
N30	bj032	*Bothrops jararaca*	IB - 67546	Porto Feliz	SP	47°31'14"W	23°12'43"S
N30	bj143	*Bothrops jararaca*	CMM - 4	Juquitiba	SP	47°03'04"W	23°57'00"S
N30	bj236	*Bothrops jararaca*	IB - CN102	Ribeirão Grande	SP	45°27'03"W	22°47'58"S
N30	bj286	*Bothrops jararaca*	IB - *	Taboão da Serra	SP	46°46'08"W	23°36'11"S
N30	bj290	*Bothrops jararaca*	IB - *	Santana do Parnaíba	SP	46°55'05"W	23°26'59"S
N30	bj291	*Bothrops jararaca*	IB - *	São Paulo	SP	46°37'42"W	23°32'01"S
N30	bj296	*Bothrops jararaca*	IB - *	Carapicuíba	SP	46°48'12"W	23°31'11"S
N30	bj298	*Bothrops jararaca*	IB - 9788	Embú	SP	46°50'26"W	23°38'56"S
N30	bj299	*Bothrops jararaca*	IB - *	Perus	SP	46°44'48"W	23°24'47"S
N31	bj329	*Bothrops jararaca*	IB - 69882	São Bernardo do Campo	SP	46°32'39"W	23°42'45"S
#	be001	*Bothrops erythromelas*	IB - *	#	#	#	#

Nh, number of haplotypes; S, southern haplotypes; N, northern haplotypes; NºCBGM, tissue collection number of Centro de Biologia Genômica e Molecular - PUCRS; IB, Instituto Butantan; CMM, Markus Monzel tissue collection; FUNED, Fundação Ezequiel Dias; IVB, Instituto Vital Brazil; *, no voucher number; #, not available. Brazilian states: RS – Rio Grande do Sul, SC – Santa Catarina, PR – Paraná, SP – São Paulo, RJ – Rio de Janeiro, ES – Espírito Santo, MG – Minas Gerais.